MacVector 10.6

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Introduction

The following chapters introduce you to the structure of this user guide and provide an overview of the MacVector application.

Chapter 1, “Introduction to the User Guide”, describes the conventions used in this user guide and outlines the content of each user guide part.

1 Introduction to the User Guide

Overview

This chapter describes the information provided in this user guide, where to find it, and the conventions used throughout.

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Introduction to the User Guide

The MacVector documentation set

Please refer to the release notes included with your copy of MacVector for new and updated information about a particular release. Also please check our website for news of updates, recent issues, and also for freely downloadable updaters for your version of MacVector.

http://www.macvector.com

About this user guide

This user guide describes the MacVector sequence analysis software program and how to use it. The user guide is divided into the following parts:

Introduction

An outline describing the MacVector documentation set and the major functional areas of MacVector.

Using MacVector

A description of using the features of MacVector on a task by task basis.

Understanding MacVector

A description of the theory behind the analyses performed by MacVector, with full references.

Appendices

Additional information, e.g. the file formats that can be used with MacVector, and reference tables of code formats.

Index

A comprehensive index to the MacVector User Guide.

Conventions in this user guide

This user guide assumes that you are familiar with the following:

- the graphical user interface of your operating system
- principles of basic file management.

Refer to Chapter 4, “Working with MacVector Files”, for instructions on file management and an explanation of how MacVector manages files.
Navigation aids

Interface conventions

The following styles are used in this user guide to describe actions and components of the interface:

- Choose File | Open means: choose the File menu, then choose Open from that menu
- Items on the interface that you need to select, such as buttons or check boxes, are shown in bold, for example:
  - the Offset check box
  - the and radio button
- Text that you need to type in, or text that is displayed in a window, is shown as follows:
  - 2.0
  - example1.seq

Navigation aids

There is a full table of contents for this user guide on page 1.

As explained earlier, this user guide is divided into five parts, where each part contains one or more chapters.

Each chapter has a short table of contents and an overview that explains exactly what information is contained within the chapter.

Where possible, information on any particular topic is grouped together, ensuring that you can easily locate the information that you need. There are also cross references to other relevant information about a topic.

There is a comprehensive index at the end of the user guide, so that you can easily locate all the information about a particular topic.
Overview

MacVector is a sequence analysis application for Macintosh computers. This chapter provides an overview of MacVector, introducing:

- MacVector sequence files
- the search methods and other analyses that can be applied to your sequence data
- the various data views that can be used to analyze sequence data.

Data displayed in the various views can be printed and saved in a quality suitable for publication.

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Introduction to MacVector

MacVector sequence files

MacVector uses its own binary file format to store sequences, along with features and annotations of the sequence. It can also read the GeneWorks binary file format.

All major ASCII sequence formats are supported. Refer to Appendix G, “Supported file formats and file extensions” for a summary.

The Sequence window

Any sequence file opened in MacVector is displayed in a Sequence window. This comprises a context dependent OS X style toolbar and four tabbed and linked views of the sequence: Editor, Map, Features and Annotations. Refer to “The Sequence window” on page 86, for further details.

Editing sequence data

The Sequence Editor view allows you to enter and edit sequence data. You can use the readback facility to check that the data has been entered correctly. See “The Editor view” on page 92.

![Sequence Editor View]

Standard sequence conversions allow you to reverse, complement, and reverse complement regions within a sequence and to create new sequences using complement, translate, and reverse translate functions.

Sequence maps

MacVector gives you complete control over the appearance of the Sequence Map view. You choose the way that the features, ruler, and sequence are displayed, and can choose to display some or all of the
sequence features. You can optimize the maps either for on-screen appearance or for high-quality printed output.

Features and annotations

Sequence features and other regions of interest can be marked in a number of ways to assist rapid identification.

Features can be displayed either as annotations or as a list in the Sequence Features view. See “The Features view” on page 99.

When a sequence is unlocked, features are edited easily from the table, using the toolbar at the top of the Sequence window (or double clicking). Clicking once on a feature automatically selects the residues corresponding to that feature in the Editor view.
The Annotated Sequence window

An Annotated Sequence window can be created and customized to show additional information including defined sequence features, and, in the case of nucleic acid sequences, the complement strand and translation of major features.

Site and motif searches

MacVector includes protein motif, nucleic acid motif, restriction site and proteolytic site searching. Motifs can consist of 1, 2 or 3 parts, and you can define the gap between each part. ORF detection can be used to assist gene detection. Search results can be displayed as annotated sequences, subsequence maps, fragment predictions and sizes, and tabular data summaries, allowing flexible processing of data.
All motif searches are based upon subsequence files, which contain a set of motifs. You can edit and create new files to contain your own search analyses.

Sequence comparison, alignment and phylogeny

MacVector uses Pustell matrix analysis to compare two sequences, generating a dot plot that provides an excellent overview of the sequence similarity. You can also compare a query sequence via the Internet to databases available on the NCBI server, using the BLAST algorithm.

You can compare and align a set of sequences that are stored in a folder, or use the ClustalW algorithm to align multiple sequences. The MSA Editor lets you enter, import and edit multiple sequences, and perform phylogenetic analyses, generating tree diagrams of their evolutionary relationships.
Protein and nucleic acid toolbox analysis

Property profiles for both nucleic acid and protein sequences can be plotted.

MacVector can be customized to display various combinations of properties. It is easy to magnify regions of interest.
This part describes how to use MacVector and includes the following chapters:

Chapter 3, “General Procedures”, describes how you can customize the appearance of the sequences and graphics displayed by MacVector.

Chapter 4, “Working with MacVector Files”, introduces all the file types that MacVector uses, and how to modify non-sequence files.

Chapter 5, “Working with Sequences”, describes how to create and modify sequences and their associated features and annotations.

Chapter 6, “Working with Multiple Sequences”, describes how to handle, display and edit multiple sequences.

Chapter 7, “Using the NCBI Entrez Database”, describes how to locate and extract complete sequences from the Entrez database released by NCBI.

Chapter 8, “Calculating Sequence Properties”, describes how to calculate property profiles and how to search a nucleic acid sequence for coding regions.

Chapter 9, “Searching for Sites and Motifs”, provides guidelines on working with the sequence
search methods in MacVector.

Chapter 10, “Primer and Probe Design”, describes the methods used to screen for PCR and sequencing primers.

Chapter 11, “Using Transcription and Translation Functions”, describes the facilities for controlling the transcription and translation of DNA to protein using any genetic code, and also reverse translation.

Chapter 12, “Comparing Sequences using Pustell Matrix Analysis”, describes the MacVector implementation of Pustell matrix analysis for sequence similarity.

Chapter 13, “Aligning Sequences”, describes the range of methods available in MacVector for sequence comparison and alignment.

Chapter 14, “Reconstructing Phylogeny”, describes methods for generating phylogenetic trees from multiple alignments of nucleic acid or protein sequences.

Chapter 15, “Aligning Sequences to a Reference”, describes the Sequence Confirmation interface for small scale resequencing projects.

Chapter 16, “Assembler”, describes the Assembler add-on module for Contig Assembly.
Overview

This chapter describes several generally useful functions available in MacVector for visualising and interpreting results. The results can be manipulated by:

- formatting the annotated sequence display
- formatting the aligned sequence display
- formatting the map display.

You can also use the numeric keypad to make it easier to enter nucleotide sequences.

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Viewing results
The results of a MacVector sequence analysis are displayed in one or more tabbed views. All graphic results are displayed as a map in a Map view, whose appearance is controlled by two dialog boxes:
- the Symbol Editor, see “Editing the global symbol set” on page 43
- the Graphics Palette, see “Editing the general map appearance” on page 56.
Non-graphical results appear in views as text listings.

Magnifying a graphic area
You can enlarge a region of any displayed graphic result.

To magnify an area of the graphic
1. Move the mouse cursor into the graphic area so that the mouse cursor changes to a magnifying glass.
2. Hold the mouse button down at one side of the area you want to enlarge and drag the cursor to the other side of the required area.
3. When you have selected the required area for enlargement, release the mouse button.

The selected area of the graphic is enlarged and redisplayed.

To return a magnified area to the original size
1. Double-click the mouse button in the graphic area.

The graphic is redisplayed with the entire residue range.

Outputting results
You can output the results of an analysis in two ways:
- Print the results
- Save the results to a file.

To print results
1. Make the plot or list you want to print the active tab, by clicking on it.
2. Choose File | Print.

A dialog box is displayed, enabling you to choose your printer and its settings.
3. Select Print to print the results.
Outputting results

A dialog box is displayed, informing you of the progress of the printing.

Tip. All or part of a list result can be copied to the clipboard by highlighting the required text.

To save results to a file
1. Make the plot or list the you want to save the active tab by clicking on it.
2. Choose File | Save As.

Saving graphics

In line with most modern OS X applications, MacVector uses the PDF format for exporting most graphics. Some parts of MacVector still use the older PICT format, for example, Phylogenetic trees and ClustalW guide trees still use the PICT format.

Graphics copied from Map views are placed on the clipboard in PDF format which retains high resolution and can be pasted into many applications (Adobe Illustrator, TextEdit, Pages, KeyNote etc) with no loss of resolution.

However, many older applications, particularly Microsoft Office 2004 programs such as PowerPoint or Word, cannot import clipboard information in this format. To get around this limitation, MacVector also places graphical information on the clipboard in bitmap format. You can control the resolution used by MacVector for bitmap copies using the Map View preferences dialog accessible using MacVector | Preferences from the menu, then clicking the Map View icon on the preferences dialog, or by clicking the Prefs icon on the toolbar when a Map view is selected.

PDF files

Most graphics can be exported as PDF files. This format is best as long as the intended application supports PDF. PDF is a vector format, and if your intended application supports both PDF file input and vector image editing (e.g. Adobe Illustrator CS3, or Adobe Photoshop CS3), then the MacVector graphic can be edited further in that application.

To Save a PDF image of a graphic
1. Select the graphics tab and select File | Print.
   The Print dialog box is displayed.
2. Click the PDF button and choose Save as PDF from the menu.
3. Navigate to the folder where you want to save the graphic data and choose **Save**.

**To Copy a PDF image of a graphic to another application**
1. Select the graphics tab and select **File | Copy**
2. Navigate to the application you want to export the graphic to, and select **Edit | Paste** in that application.

**Bitmap files**

To increase the quality of the exported bitmap image you can use oversampling to increase the resolution of the image placed in the clipboard. By default, MacVector has bitmapped copy turned on with 1x oversampling. This copies an image with the same resolution as you can see on the screen.

When you choose **Edit | Copy**, the entire graphical image is copied and placed on the clipboard, not just the visible region. For large sequences this can be a tremendous amount of information, particularly if you use 2x or 4x oversampling to increase the bitmap resolution. You can limit the amount of memory MacVector allocates for the bitmap copy by changing the appropriate value in the **Map View** preferences dialog. By default this is set to 16 MB. If the required memory would exceed this value, the bitmap copy is simply skipped.

**PICT files**

PICT files are specific to Macintosh computers. They include extra high-resolution information, which is not visible on the screen, but increases the printed quality. If you are always working and printing on a Mac, PICT files are standard and convenient.
Formatting the annotated sequence display

Note. This feature is included for legacy purposes only. Most graphics in MacVector are now exported in bitmap or PDF format.

Formatting the annotated sequence display

You can customize the appearance of the annotated sequence display, both as it appears on the screen and as it will be printed. The formatting enables you to control:

- the number of residues that occur per sequence block, between sequence numbers, and between sequence marks
- how the sequence itself is displayed: uppercase or lowercase letters, single- or double-stranded format, and the fixed width font that is used.
- which feature types will be included among the annotations to the sequence
- which translated feature types will have their amino acid sequences printed beneath the nucleic acid sequence.

Annotated sequence formatting also affects some of the format properties of the aligned sequence display. Examples of formatting can be found in Appendix D, “Formatting Examples”.

This functionality may be accessed at any time, whether an annotated sequence is present or not. If an annotated sequence is present, it will be updated immediately to reflect changes.
Formatting residue sequences

The sequence layout can affect the clarity of the displayed information, particularly if a lot of feature information is also visible.

To format a residue sequence

1. Choose MacVector | Preferences from the menu, then click the Text Display icon on the preferences dialog, or click the Prefs icon on the toolbar when the Features tab is selected.

The Text Display preferences dialog box is displayed.

Note. You can also access the Text Display preferences dialog using Options | Format Annotated Display from the menu.

2. Type a number in the Line Length text box to specify the number of residues per line.

3. Type a number in the Blocking text box to draw residues in groups of that number.

4. Type a number in the Numbering text box to number the sequence residues at that interval.

5. Type a number in the Marking text box to place an asterisk (*) at that interval along the sequence.

6. This is often set at half the interval of the Numbering value.

7. Select the Letter case as upper or lower as required.
8. Select the display of amino acid codes as one or three letters when a nucleic acid sequence translation is displayed.

9. Select either single or double to display a nucleic acid sequence as either a single- or double-stranded molecule.

10. Select OK to apply the formatting.

Formatting features

When the sequence is displayed, you can control the type of features that are shown beneath the sequence. The features must be present in the features table of the sequence.

**To display features as sequence annotations**

1. Choose MacVector | Preferences from the menu, then click the Text Display icon on the preferences dialog, or click the Prefs icon on the toolbar when the Features tab is selected.

   The Text Display preferences dialog box is displayed.

   **Note.** You can also access the Text Display preferences dialog using Options | Format Annotated Display from the menu.

2. In the Displayed Feature Types panel, select and deselect check boxes as required.

   Selected features that occur in the sequence features table will appear as annotations to the sequence in the display.

3. Select OK to apply the formatting.

   The features are displayed below the appropriate residues.

   **Note.** If two or more features overlap, one appears below the other.

   You can also view the translation of certain features, if they occur in the sequence’s features table. These features are:

   - mature protein product (mat_peptide)
   - protein coding region (CDS)
   - signal peptide (sig_peptide)
   - exon regions (exon)

**To display feature translations as sequence annotations**

1. Choose MacVector | Preferences from the menu, then click the Text Display icon on the preferences dialog, or click the Prefs icon on the toolbar when the Features tab is selected.

   The Text Display preferences dialog box is displayed.
Note. You can also access the Text Display preferences dialog using Options | Format Annotated Display from the menu.

2. In the Translate panel, select and deselect check boxes as required.
3. To display the translated region in codon groups, select block to phase.

This display style overrides the Blocking value set for the remainder of the sequence.

4. Select OK to apply the formatting.

Translatable features of the selected types are displayed in translated form beneath the main sequence.

To change the font used

1. Choose MacVector | Preferences from the menu, then click the Fonts icon on the preferences dialog.

The Fonts preferences dialog box is displayed.

2. In the Result window font panel, use the drop down menu to change the font.

3. Check the Enable anti-aliasing option to enable anti-aliasing for displaying the font. This makes fonts appear smoother at the expense of reduced sharpness. High resolution monitors may display better without this option.

4. Select OK to apply the formatting.

Note. Certain printer drivers do not correctly align Monaco text when printed out, although the alignments look perfect on the screen. Andale Mono is a good substitute that is correctly aligned on all printers we have tested to date.

Formatting the aligned sequence display

You can customize the appearance of the alignment portion of the aligned sequence display, both as it appears on the screen and as it will be printed. This formatting does not apply to multiple sequence alignments, which have their own formatting options.

The aligned sequence formatting enables you to control:

- whether or not a query sequence line will appear
- the style and display of a score line
- how often the aligned sequences will be numbered and how often vertical alignment characters will appear
Formatting the aligned sequence display

- the display of vertical and horizontal alignment characters
- the representation of matching and mismatching residues.

Characteristics of the query sequence are customized using the **Text Display** options. See “Formatting the annotated sequence display” on page 35. Examples of formatting can be found in Appendix D, “Formatting Examples”.

This functionality can be accessed at any time, whether an aligned sequence is present or not. If an aligned sequence is present, it will be immediately updated to reflect changes.

There are three possible components to an aligned sequence display:
- the aligned sequence
- the score line
- the query sequence.

If all three are present, they appear beneath each other in the order shown above.

**Formatting the aligned sequence**

The format of an aligned sequence affects the numbering of residues, representation of matched and mismatched residues, and visual alignment aids.

**To format an aligned sequence**
General Procedures

1. Choose MacVector | Preferences from the menu, then click the Aligned Display icon on the preferences dialog.

The Aligned Display preferences dialog box is displayed.

**Note.** You can also access the Aligned Display preferences dialog using Options | Format Aligned Display from the menu.

2. Type a number in the Numbering text box to number the sequence residues at that interval.

3. Type a number in the Vertical Alignment text box to insert vertical alignment characters at that interval through the display.

This is an aid to keeping your place as you scan down through the aligned sequences. If you enter a zero in this box, no vertical alignment characters will appear.

4. Type a character in the Vertical Alignment char. text box to use as a vertical alignment character.

If you do not want any vertical alignment characters to appear, type a space in the box or leave it empty.

5. Type a character in the Horizontal Alignment char. text box to use as a horizontal alignment character.

This is an aid to keeping your place on the line as you scan left to see the sequence name. If you do not want any horizontal alignment characters to appear, type a space in the box or leave it empty.

**Note.** Do not use a hyphen, because MacVector uses hyphens to represent deletions and gaps in the aligned sequence.

6. From the Match Character panel, select the radio button corresponding to how you want to represent residues in the aligned sequences that match residues in the query sequence.

The character radio button enables you to enter a character of your choice in the text box.

7. From the Mismatch Character panel, select the radio button corresponding to how you want to represent residues in the aligned sequences that do not match residues in the query sequence.

The character radio button enables you to enter a character of your choice in the text box.

8. Select OK to apply the formatting.

Each aligned sequence display is updated with the formatting changes.
Formatting the aligned sequence display

Displaying a score line

Each character in the score line represents the score assigned to the comparison between the residue in the aligned sequence and the corresponding residue in the query sequence. This gives you a semi-graphical indication of which regions along the length of the sequence match the query sequence well and which match poorly.

To display a score line

1. Choose MacVector | Preferences from the menu, then click the Aligned Display icon on the preferences dialog.

The Aligned Display preferences dialog box is displayed.

Note. You can also access the Aligned Display preferences dialog using Options | Format Aligned Display from the menu.

2. Select the Score Line check box to display a score line beneath each of the sequences in the alignment.

3. Type a character of your choice in each of the boxes labeled +1 or greater, between -1 and +1, and -1 or less.

These characters can identify matching residues along the aligned sequence. Most scoring matrices give a positive score to an exact or equivalent match, and a negative score to a mismatch. Any or all of these boxes may contain a space character or be left blank.

4. Select OK to apply the score line.

Each aligned sequence display tab is updated with the formatting changes.

Displaying a query line

The query sequence can be displayed beneath each of the sequences in the alignment. The format of the query sequence is controlled using the Text Display options. See “Formatting the annotated sequence display” on page 35.

To display a query line

1. Choose MacVector | Preferences from the menu, then click the Aligned Display icon on the preferences dialog.

The Aligned Display preferences dialog box is displayed.

Note. You can also access the Aligned Display preferences dialog using Options | Format Aligned Display from the menu.
2. Select the **Query Line** check box to display a query line beneath each of the sequences in the alignment.

3. Select **OK** to apply the query line.

Each aligned sequence display is updated with the formatting changes.

**Formatting the map display**

You can customize the graphical results tabs by manipulating the appearance of the following elements:

- title
- sequence line, either showing residue letters or a single line
- ruler indicating the scale of the sequence line
- feature sites
- result sites, if there are any
- segment map

This is done by editing symbol sets, of which there are three types:

- sequence default symbol set, which can be specified and saved for each sequence
- global default symbol set, which can be specified and saved as the default set used when a new sequence is created
- MacVector default symbol set, which cannot be edited, only reapplied to the editable symbol sets
Formatting the map display

There are also a number of options that can be used to control the overall appearance of the map and to optimize the map for printing.

Editing the global symbol set

The global default symbol set is edited using the Symbol Editor dialog box. Editing the global default symbol set affects only new sequences or features created after modifications are made; existing sequences or features are not affected.

When no windows are present, the dialog box is displayed by choosing Options | Default Symbols. If a sequence tab, map tab or results tab is active, then the menu says Options | Symbols for name, where name is a description of the active window. In this case, you need to hold down the option key before making the menu selection, and the item Default Symbols will be present again. If any other tab type is active, the item will be grayed out.

The dialog box consists of six panels, which are used to modify the appearance of the following elements of the graphical displays:

- feature types
- result types
General Procedures

- title
- ruler
- sequence
- segment map.

The use of each panel is described in the following sections.

Features panel

Using this panel of the Symbol Editor dialog box, you can select each feature type and alter its settings.

To modify the global feature symbols

1. Choose Options | Default Symbols from the menu.

Note. If a sequence tab, map tab or results tab is active, you must hold down the option key before making this menu selection.

The Symbol Editor dialog box is displayed.

2. Select Features from the drop-down menu at the top left of the dialog box.

A list of all available features is displayed in the scrolling panel. The symbols to the left of the feature name identify the feature as either a protein or nucleic acid feature.
3. From the scrolling list, select a feature name whose appearance you want to modify.

**Note.** You can select more than one feature type to modify by holding down the `<shift>` or `<command>` key while selecting features. Each selected feature will be given the modified settings when **OK** is selected.

4. Choose a style for the feature symbol from the **Style** drop-down menu.

5. If you want the symbol to be filled, select the **FILL** check box, then choose the fill color and pattern from the associated drop-down menus.

6. If you want a border around the symbol, select the **PEN** check box, then choose the color and border thickness from the associated drop-down menus.

7. If you want to label the symbol, select the **Show label** check box, then do the following in the Label panel:
   - enter the text for the label in the text box. The following special symbols are available for the label text:
     - `<Description>` inserts the feature comment text
     - `<Type>` inserts the feature type name, e.g., CDS
     - `<Start>` and `<Stop>` insert the start and stop residue numbers.
     For example, “`<Start>:<Stop> <Type>`” would display “123:456 CDS” for a CDS feature that spanned residues 123 to 456.
   - choose the label color from the **Color** drop-down menu
   - use the **Set** button to choose an alternative font style, size and weight to the one displayed in the **Font** box.
   - choose the position and orientation of the label with respect to the feature symbol from the **Orientation** drop-down menus.

**Note.** When plotting circular maps, vertical label text runs along a radius of the circle, and horizontal label text runs parallel to the circumference. Align to the left means that the text begins at the left edge of the symbol.

8. If you want the symbol visible by default, select the **Initially visible** check box.

9. Choose the position of the symbol relative to the sequence line from the **Level** drop-down menu.

There are six levels above or below the sequence line. They can be thought of as lines on which the feature symbol is displayed. You would
General Procedures

normally leave this as “above or below”, in which case MacVector assigns a level automatically.

10. To make changes permanent, do one of the following:
   - select OK to save the changes you have made and to close the dialog box
   - select Cancel to discard all changes made
   - select Shipping Defaults to revert to the MacVector default symbols.

Results panel

Using this panel of the Symbol Editor dialog box, you can select each result type and alter its settings.

To modify the global results symbols

1. Choose Options | Default Symbols from the menu.

Note. If a sequence tab, map tab or results tab is active, you must hold down the option key before making this menu selection.

The Symbol Editor dialog box is displayed.

2. Select Results from the drop-down menu at the top left of the dialog box.
A list of all available result types is displayed in the scrolling panel. The symbols to the left of the result type identify the result as either a protein or nucleic acid result.

3. From the scrolling list, select a result type whose appearance you want to modify.

4. Choose a style for the symbol from the **Style** drop-down menu.

5. If you want the symbol to be filled, select the **FILL** check box, then choose the fill color and pattern from the associated drop-down menus.

6. If you want a border around the symbol, select the **PEN** check box, then choose the color and border thickness from the associated drop-down menus.

7. If you want to label the result symbol, select the **Show label** check box, then do the following in the Label panel:
   - enter the text for the label in the text box. The following special symbols are available for the label text:
     - `<Description>`, `<Desc>` or `<Type>` inserts the result type name
     - `<Start>` and `<Stop>` insert the start and stop residue numbers. For example, `<Start> <Type>` would display 123 BamHI on a restriction enzyme map that had a BamHI cut site at residue 123.
     - choose the label color from the **Color** drop-down menu
     - use the **Set** button to choose an alternative font style, size and weight to the one displayed in the **Font** box.
     - choose the position and orientation of the label with respect to the result symbol from the **Orientation** drop-down menus.

**Note.** Align to the left means that the text begins at the left edge of the symbol.

8. If you want the result type visible by default, select the **Initially visible** check box.

9. Choose the position of the result symbol relative to the sequence line from the **Level** drop-down menu.

There are six levels above or below the sequence line. They can be thought of as lines on which the result symbol is displayed. You would normally leave this as “above or below”, in which case MacVector assigns a level automatically.

10. To make changes permanent, do one of the following:
General Procedures

- select **OK** to save the changes you have made and to close the dialog box
- select **Cancel** to discard all changes made
- select **Shipping Defaults** to revert to the MacVector default symbols.

**Title panel**

The Title panel is used to define the title text.

**To modify the global map tab title**

1. Choose **Options | Default Symbols** from the menu.

**Note.** If a sequence tab, map tab or results tab is active, you must hold down the option key before making this menu selection.

The Symbol Editor dialog box is displayed.

2. Select **Title** from the drop-down menu at the top left of the dialog box.
3. If you want the title visible by default, select the **Initially visible** check box.
4. Enter a title in the **Title** text box.

The text `<Sequence Name>` is special text that is replaced by the name of the sequence whose map is displayed.

5. Use the **Set** button to choose an alternative font style, size and weight to the one displayed in the **Font** box.
Formatting the map display

6. Choose the title position from the **Position** drop-down menu.
7. Choose the text color from the **Color** drop-down menu.
8. To make changes permanent, do one of the following:
   - select **OK** to save the changes you have made and to close the dialog box
   - select **Cancel** to discard all changes made
   - select **Shipping Defaults** to revert to the MacVector default symbols.

Ruler panel

The ruler panel controls the appearance of the sequence numbering.

![Ruler panel screenshot](image)

To modify the global ruler markings

1. Choose **Options | Default Symbols** from the menu.

**Note.** If a sequence tab, map tab or results tab is active, you must hold down the option key before making this menu selection.
   - The Symbol Editor dialog box is displayed.

2. Select **Ruler** from the drop-down menu at the top left of the dialog box.
3. If you want the ruler visible by default, select the Initially visible check box.

4. If you want tick marks, select the Tick marks check box, then do the following:
   - enter the tick interval in the text box
   - choose the tick color from the Color drop-down menu
   - choose the tick thickness from the Thickness drop-down menu.

5. If you want to label the ruler ticks, select the Show residue numbers check box, then do the following in the Label panel:
   - choose the label color from the Color drop-down menu
   - use the Set button to choose an alternative font style, size and weight to the one displayed in the Font box.
   - choose the orientation of the label with respect to the ruler from the Orientation drop-down menus

6. Choose the level of the ruler from the Level drop-down menu.

The levels above or below the sequence line can be thought of as lines on which the graphic features are displayed.

7. To make changes permanent, do one of the following:
   - select OK to save the changes you have made and dismiss the dialog box
   - select Cancel to discard all changes made
   - select Shipping Defaults to revert to the MacVector default symbols.
Formatting the map display

Sequence panel

The sequence panel defines the default appearance of any new sequence maps that are created.

To modify the global sequence appearance

1. Choose Options | Default Symbols from the menu.

   Note. If a sequence tab, map tab or results tab is active, you must hold down the option key before making this menu selection.

   The Symbol Editor dialog box is displayed.

2. Select Sequence from the drop-down menu at the top left of the dialog box.

3. If you want the ruler visible by default, select the Initially visible check box.

4. If you want to view the sequence only as a line, select the Show as a line radio button.

5. If you want to display the sequence letters, select the Show residue letters if room radio button.

   The sequence letters will be displayed if there is room. This will depend on the settings on the Graphic Palette dialog box, see “Editing the general map appearance” on page 56.

6. Choose the line color and thickness by selecting from the Color and Thickness drop-down menus.
General Procedures

7. Choose the residue color by selecting from the **Color** drop-down menu.

8. Choose an alternative font style, size and weight to the one displayed in the **Font** box for the residue using the **Set** button.

9. To make changes permanent, do one of the following:
   - select **OK** to save the changes you have made and to close the dialog box
   - select **Apply** to save the changes you have made, leaving the dialog box displayed
   - select **Cancel** to discard all changes made
   - select **Shipping Defaults** to revert to the MacVector default symbols.

**Segment Map panel**

The segment map panel controls the appearance of the segment map. It is only available when MacVector is displaying a linear map showing the results of an enzyme digest or subsequence search.

![Segment Map dialog box](image)

**To modify the global segment map appearance**

1. Choose **Options | Default Symbols** from the menu.

   **Note.** If a sequence tab, map tab or results tab is active, you must hold down the **option** key before making this menu selection.

   The Symbol Editor dialog box is displayed.
Formatting the map display

2. Select Segment Map from the drop-down menu at the top left of the dialog box.

3. If you want the segment map visible by default, select the Initially visible check box.

4. Choose the color and thickness of the horizontal lines, by selecting from the Color and Thickness drop-down menus in the Horizontal Line Style panel.

5. Choose the color and thickness of the vertical cut lines, by selecting from the Color and Thickness drop-down menus in the Cut Line Style panel.

6. To alter the appearance and position of the map labels, do the following in the Label panel:
   • choose the label color from the Color drop-down menu
   • choose the label position from the At End drop-down menu
   • use the Set button to choose an alternative font style, size and weight to the one displayed in the Font box.

7. Choose the position of the segment map (above or below the cut map) from the Level drop-down menu.

8. To make changes permanent, do one of the following:
   • select OK to save the changes you have made and to close the dialog box
   • select Apply to save the changes you have made, leaving the dialog box displayed
   • select Cancel to discard all changes made
   • Select Shipping Defaults to revert to the MacVector default settings.

Editing the sequence symbol set

When a new sequence is created, its symbol set is defined to be the same as the global symbol set, see “Editing the global symbol set” on page 43. The symbol set for each sequence can be individually edited and saved.

When a change is made to the sequence symbol set, the active maps for the sequence are updated immediately. If you are refining the appearance of a large map, you can interrupt redrawing at any time by one of the following methods:
General Procedures

- pressing the **command** and **period** (.) keys together
- pressing the **esc** key
- changing any setting in the Graphics Palette dialog box.

To edit the sequence symbol set

1. Do one of the following:
   - if a sequence, map or results tab is active, choose **Options | Symbols for name**, where *name* is a description of the active window.
   - select the item that you want to edit from the scrolling list of the Graphics Palette dialog box, then select **Edit**.

**Note.** The Graphics Palette dialog box is displayed by selecting **Window | Show Graphics Palette**. This menu item is a toggle, and once set, the Graphics Palette dialog box will be displayed whenever a map tab is selected, until you choose **Window | Hide Graphics Palette**.

The Symbol Editor dialog box is displayed. If you used the Edit button on the Graphics Palette dialog box, the appropriate Symbol Editor panel is displayed.

**Note.** The scrolling list shows a tree view of the map items on the Features and Results panels. These can be expanded or contracted by clicking on the triangles to the left of the items.
Formatting the map display

2. For details of how to edit the symbols, see the corresponding sections in “Editing the global symbol set” on page 43, (omit step 1 in each case):
   - “Features panel” on page 44
   - “Results panel” on page 46
   - “Title panel” on page 48
   - “Ruler panel” on page 49
   - “Sequence panel” on page 51
   - “Segment Map panel” on page 52

Note. When you access the dialog boxes in this way, they each have one additional button, next to the Cancel button. It is the Apply button. This lets you update the open maps with the changes you make, without closing the dialog box. This means that you can make a number of edits to the map display conveniently.

   The tree hierarchy of map items for the Features and Results panels enables you to modify the symbol for each item individually, for a complete group of features, or any combination of features.

Note. Modifying the symbol of an item on the Results or Features panel affects all other items subordinate to it in the tree hierarchy.

3. The edited symbol set is saved with the sequence, so you can save different map views of the same sequence.
Editing the general map appearance

The Graphics Palette dialog box is used to control the overall appearance of the graphic map. Any changes made directly from this dialog box are applied immediately to the map tab.

To modify the general map appearance

1. When a map tab is active, choose Windows | Show Graphics Palette if the Graphics Palette dialog box is not visible.

   Note. This menu item is a toggle. Once set, the Graphics Palette dialog box will be displayed each time a map tab is active, until you choose Windows | Hide Graphics Palette.

   The Graphics Palette dialog box is displayed. The scrolling list shows a tree directory of all the map elements whose symbols can be edited. The list can be expanded or contracted by clicking on the triangles to the left of the items. The items on the dialog box differ, depending on whether the sequence is displayed as a linear or circular map.

2. If the graphic map is for a DNA sequence, you can toggle between linear and circular display by selecting the linear/circular button.

3. To see the map at a scale where you can read the individual residue letters, press the magnifying-glass button.

   Note. This only applies to linear maps.

4. Otherwise, to adjust the display density of residues, do the following:
Formatting the map display

- select inch, cm or line from the Residues/ drop-down menu
- enter the number of residues to appear in that interval in the associated text box.

**Note.** This only applies to linear maps.

5. If your map is circular, you can control the size by doing one of the following:
   - select a value for the radius units from the Radius in drop-down menu, then enter a value in the text box
   - to scale the map to the current page size automatically, select the button to the right of the Radius in text box

**Note.** The page size is determined by the current settings of the page setup dialog.

- to scale the map to the current window size automatically, select the Fit to Window button.

**Note.** The Fit to Window button re-sizes a circular map to the longest dimension of your window. It will not shrink the map below a minimum legible size, so some scrolling will be needed to view the entire map if your window is small.

6. If your map is linear, you can fit it to your window width automatically, by selecting the Fit to Window button. The map is fitted by adjusting the line length and residue density.

7. Otherwise, you can adjust the line length by doing one of the following:
   - enter a value in the Line wrap text box. This is in inches by default, but you can type in a value immediately followed by cm or mm to enter a value in those units, e.g., 15cm. This sets the width at which the sequence continues on a new line
   - choose whether to make one long unbroken line of residues, or use the printer-configured paper width, by selecting from the Line wrap drop-down menu.

**Note.** The line wrap length, coupled with the residues-per-inch setting, governs how many residues are plotted in each panel of a linear map plot.

8. If your map is circular, you can rotate the map by a number of residues by entering a value in the Rotation text box.

The number entered is the number of residues by which the sequence is moved clockwise around the map circle, with residue number 1 initially at the top of the map. For example, for a sequence of 1500 residues, a
value of 100 in the Rotation text box would place residue 1401 at the top of the map.

9. If you want to modify the map by refiltering your results, select Filter. This displays the results dialog box appropriate to the origin of your map data.

10. You can change the height of the graphic items by selecting a value in the Graphics Height popup menu. This affects all of the graphic objects in the display and is designed to let you adjust the proportions of the graphics for a more aesthetically pleasing effect.

11. There is a checkbox to enable a drop shadow effect so that the graphics appear to be floating slightly above the background. If this is selected you can click on the Options... button to set the amount of blur and the X and Y offset you would like applied to the shadow.

12. MacVector draws all graphics using antialiasing which improves the appearance of text and circular graphics tremendously. However, depending on the monitor you are using, you may find that linear graphics look a little fuzzy, especially if you have a lot of narrow lines on the display. Select the Suppress Antialiasing checkbox to turn off antialiasing in linear views only.

To display a region of a sequence
You can restrict the display to a certain region of the sequence, using either the mouse or the Graphics Palette dialog box:

- Using the mouse, click and drag to select the region on the map that you want to display. If your map is linear, the selection will be fitted to the window automatically.

- Using the Graphics Palette: type, in the Range panel, the sequence numbers of the first and last residues of the selected region, separated by a colon (:). Alternatively, you can select a region from the features table drop-down menu at the right of the text box. When you make a selection from the Graphics Palette, your current settings for residue density and line wrap are retained.
Note. For results maps, the range cannot be set outside the range that was specified when the results calculation was performed. For example, if restriction enzyme cut sites were only computed between bases 1000 and 2000, the range cannot be set to show bases 1 to 4000, because result data is not available for the wider range. If the user types in range values outside the permitted limit, they will be changed to be within the limit (i.e. no less than 1000, no more than 2000 in the example just given.)

Printing maps

After you have drawn a map to your requirements, you can print it. MacVector generates high-quality print output. The map tab can show you how the map will be printed if it is large enough to cover several pages, or alternatively you can use Page Setup... to scale a map to fit onto a single page.

Maps are always printed at 100% view; the view scale controls on the map tab have no effect on the printed output and are there only to enable you to see the split of the map over several pages.

When choosing a font for labels and titles, be aware of the differences between fonts that look good on the screen and fonts that look good on the printer. Geneva, Monaco and Chicago fonts are designed to look clear and legible on the screen. However, when you print them to a standard PostScript laser printer, their letter spacing can look a little uneven. (In fact, when you try to print Geneva you actually get Helvetica letters, and when you print Monaco you may get Courier letters, but with letter spacing appropriate to their original screen font. The mismatch between which font's letters are being drawn and which font is being used for the inter-letter spacing accounts for the uneven appearance.)

Geneva is the default label font supplied with MacVector, to give best legibility on screen. If your primary concern is to get the best quality printed map, edit the symbols to set them to use a true PostScript printer font like Helvetica. You will notice the difference, particularly in the quality of rotated text on circular maps.

To print a map view

1. If you want to preview how the map will split across printed pages, click the page mode button in the lower left corner of the view.

This toggles the page mode. In page mode, the tab has dotted lines that represent page boundaries, so you can see where your map will split.
2. If necessary, adjust the view so that the whole map appears in the window, by selecting **Fit In Window** from the **View scale** drop-down menu next to the **page mode** button.

**Note.** This does not affect the printed output size.

3. If you want to scale the map, either to fit on a single sheet of paper, or to control where the map splits across a page, do the following:
   - choose **File | Page Setup**. The Page Setup dialog box appears
   - select **Page Attributes** from the drop-down menu at the top left of the dialog box
   - enter a value in the **Scale** text box, then select **OK**.

The map tab is redrawn. If you need to adjust the size further, repeat this step as necessary.

4. Choose **File | Print**.

5. Adjust the print options as required.

6. Select **Print**.

The map is printed to the chosen device.

**Configuring the numeric keypad**

This option enables you to assign the one-letter codes for nucleotides to keys of the numeric keypad (if your Macintosh has one) to make it easier to enter sequence data with one hand.

Assignments can be made whenever the menu bar is accessible, but they will be in effect only when a nucleic acid sequence tab is active. At other times, the keypad keys will retain their normal assignments as numbers or symbols. The assignment is not retroactive; if you type in
some bases from the keypad, then change the keypad assignment in mid-sequence, the bases you previously typed in will not be changed.

To configure the numeric keypad

1. Choose Options | Set Keypad.

The Keypad Assignments dialog box appears.

2. To cancel all keypad assignments that are currently in effect, select the Clear Keys button.

3. Click on the box corresponding to the key that you want to assign.

4. Type the letter to assign to that key.

Only valid IUPAC one-letter codes will be accepted.

Note. When assigning IUPAC codes to the keypad, the letter U is not used. Instead, the T key enters T into a DNA sequence and U into an RNA sequence.

5. To make changes permanent, do one of the following:
   - select the Cancel button to discard all changes made. MacVector ignores any changes you may have made to the keypad assignments and retains existing ones.
   - Select OK to accept the keypad assignments that are currently displayed.

The assignments are saved when you exit MacVector.
Overview

This chapter introduces all the file types that are used by MacVector. It also describes how the following file types can be modified:

- enzyme files
- subsequence files
- scoring matrix files

Chapter 5, “Working with Sequences” describes how to manage and edit sequence files.

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MacVector file types

Sequence files

These two icons are used for nucleic acid and protein sequence files. They are in a binary file format and can only be opened and edited within MacVector.

MacVector can also open nucleic acid and protein sequences in all major ASCII formats. See Appendix G, “Supported file formats and file extensions” for a complete list of the sequence formats MacVector supports.

For more information about sequence files, see “Sequence files” on page 82, and Chapter 5, “Working with Sequences”.

Enzyme files

These icons are used to represent restriction enzyme and proteolytic enzyme files. They are in a binary file format and can only be opened and edited within MacVector.

Subsequence files

These file icons are used for nucleic acid and protein subsequence files. They are in a binary file format and can only be opened and edited within MacVector.
MacVector file types

Scoring matrix files

These icons are used to represent the nucleic acid and protein scoring matrix files that are used in the matrix comparisons and database searches. They are in a binary file format and can only be opened and edited within MacVector.

Codon bias files

This icon is used for files that contain codon bias tables. You cannot open and edit a codon bias file directly.

Multiple alignment files

These icons are used for files generated by MacVector that contain multiple alignments of DNA or protein sequences. They can be opened and edited within MacVector.
Managing files

This section describes some general procedures for managing files:

• creating a file
• opening a file
• saving a file
• closing a file

Creating new files

You can create new files of the following MacVector file types:

• restriction enzyme or proteolytic enzyme files
• nucleic acid or protein sequence files
• nucleic acid or protein subsequence files
• nucleic acid or protein multiple alignment files
• nucleic acid or protein scoring matrix files
• primer databases

Codon bias files are created by a different process. See Chapter 10, “Primer and Probe Design” for further details.

To create a new file

1. Choose File | New from the menu.
2. Choose a file type from the submenu.

An untitled window of the appropriate type is displayed.

Alternatively, press <command> + N on the keyboard to create a new nucleic acid sequence file.

Opening files

You can open any of the following types of files for editing:

• restriction enzyme or proteolytic enzyme files
• nucleic acid or protein sequence files
• nucleic acid or protein subsequence files
• nucleic acid or protein multiple alignment files
• nucleic acid or protein scoring matrix files

Codon bias files can only be accessed during codon preference analysis. They cannot be opened for editing.
Managing files

You can open any file that MacVector recognises using the Open dialog box.

To open a file
1. Choose File | Open.
   The Open dialog box is displayed.
2. To restrict the types of files displayed, click the Enable arrow button and choose the required file type from the drop-down list.
3. Browse to the appropriate directory and choose the required file from the list. You can also use the search box at the top right of the dialog box to help you locate your files.
4. To open more than one file, hold down the <shift> key and continue to choose files from the list. (A second <shift>-click on the same file will deselect it.)
5. If you have selected a file containing multiple sequences, the Open Multiple Sequence Files As: menu enables you to choose whether to open each sequence in a separate window, or all the sequences
aligned, in a single window. See “Opening multiple sequences” on page 118 for details.

6. Select Open to open the file(s) and close the dialog box.
7. The files are displayed in windows of the appropriate type.

Saving files

When files are modified, the entries take immediate effect on the copy in memory. The changes are only made permanent when you use either File | Save or File | Save As.

To save a file

1. Ensure the required window is active.
2. Do one of the following:
   • choose File | Save to save changes to the file.
   • choose File | Save As to save the changes as a new file. If you do this, you must provide a new name for the file in the Save As edit box, before saving the file using the Save button.

Closing files

To close a file

   The file and its associated window are closed.

Enzyme files

Enzyme files contain a list of enzymes and details of the recognition site they require for cleaving a sequence.

Restriction enzymes (for cleaving nucleic acid sequences) and proteolytic enzymes (for cleaving amino acid sequences) are stored in separate files. MacVector is supplied with several enzyme files. The restriction enzyme files are a mirror of the REBASE database of restriction enzymes.

You can edit restriction enzyme and proteolytic enzyme files. MacVector enables you to:
   • select a subset of enzyme entries and save the selection for use with the restriction enzyme or proteolytic enzyme analyses
   • delete existing enzyme entries from the file
   • change the data for existing enzyme entries
Enzyme files

- add new enzyme entries to the file

Selecting enzymes for site analysis

You can choose a set of enzymes to use for a site analysis. This may be useful, for example, when you want to restrict your analysis to those enzymes readily available in your laboratory.

To select a subset of enzymes in an enzyme file

1. Open the required enzyme file, and ensure that the file window is active.

   A selection of enzyme files is available in the Restriction Enzymes folder.

2. If the file is locked, unlock it by clicking the Locked icon in the toolbar.

3. Scroll through the enzyme list to find the name of each enzyme you want to select.

   Tip. You can navigate through the list by typing the first character of the restriction enzyme, or by using the up and down arrows on the keyboard.
4. Click on each required enzyme. A check mark appears, and a line at the bottom of the window indicates how many enzymes have been selected.

**Tip.** Use the **clear selection** button to deselect all entries.

5. Save the file to disk, using **File | Save**. Your selections are saved and you will not have to repeat the selection process every time you open the file.

**Note.** When you perform a restriction or proteolytic site search, you may use either all entries in the enzyme file, or only those enzymes selected.

**Tip.** If you save the changes using **File | Save As**, you can save different selection subsets.

### Adding entries to an enzyme file

Entries can be added to an enzyme file at any time that the window for that file is active.

![Enzyme Editor](image)

**To add a new entry to an enzyme file**

1. Open the required enzyme file and ensure that the file window is active.
2. If the file is locked, unlock it by clicking the **Locked** icon in the toolbar.
3. Click the **Add** button on the toolbar. The **Enzyme Editor** dialog box is displayed.
4. Type a name for the enzyme in the **Name** text box.
Enzyme files

5. Click the sequence representation in the center of the dialog box to position the insertion point, then type the recognition site using the IUPAC one-letter codes.

For proteins, enter the recognition sequence in the amino to carboxy direction; for nucleic acids, in the 5' to 3' direction.

Note. Use parentheses to enclose a combination of amino acids for which there is no one-letter assignment. For example, if the agent cleaves after an aspartic (D) or glutamic (E) acid residue, denote it as (DE).

6. Drag the arrow-shaped control to the cut position.

For restriction enzymes, there are two arrows: the upper one to indicate the cut position on the plus strand, the lower one to indicate the cut position on the minus strand.

7. If required, you can enter a comment up to 254 characters long.

8. Select OK to add the enzyme to the file.

9. Choose File | Save to make the changes permanent.

Copying entries between enzyme files

Copying existing entries between files can be useful, for example to create a customized enzyme subset from more than one file. This requires that one or more enzyme files are open at the same time, and that the file receiving the enzyme entries is unlocked.

To copy entries between files

1. Make the file from which you are copying entries the active window.

2. Select the enzymes that you want to copy using one of the following methods:
   • click to select a single entry
   • hold down the mouse button and drag to select a continuous block of entries
   • hold the <shift> key in combination with either of the above to retain already selected entries.

3. Choose Edit | Copy.

4. Make the file that is receiving the entries the active window.

5. If the file is locked, unlock it by clicking the Locked icon in the toolbar.

The entries are added to the file in alphabetical order.

7. Choose **File | Save** to make the changes permanent.

**Note.** If any of the entries were marked as selected, the selection will be retained.

### Editing entries in an enzyme file

Entries in an enzyme file can be modified. This is generally useful only when a mistake has been noticed in the existing entry.

**To edit an enzyme entry**

1. Open the required enzyme file and ensure that the file window is active.
2. If the file is locked, unlock it by clicking the **Locked** icon in the toolbar.
3. Select the enzyme that you want to edit.
4. Click the **Edit** button on the toolbar.

The **Enzyme Editor** dialog box is displayed.
5. Modify the entry as required.

See “**To add a new entry to an enzyme file**” on page 70, for further details.
6. Select **OK** to add the changes to the enzyme file.
7. Choose **File | Save** to make the changes permanent.

### Deleting entries from an enzyme file

Entries in an enzyme file can be deleted when required.

**To delete an enzyme entry**

1. Open the required enzyme file, and ensure that the file window is active.
2. If the file is locked, unlock it by clicking the **Locked** icon in the toolbar.
3. Select the enzymes that you want to delete using one of the following methods:
   - click to select a single entry
   - hold down the mouse button and drag to select a continuous block of entries
   - hold the `<shift>` key in combination with either of the above to retain already selected entries
Subsequence files

4. Click the **Delete** button on the toolbar.
   The selected entries are deleted from the file.

5. Choose **File | Save** to make the changes permanent.

**Note.** This is equivalent to using **Edit | Clear**. If you want to paste the deleted entries into another file, use **Edit | Cut** instead of the **Delete** button.

Subsequence files

Subsequence files contain a list of motifs that can be used to look for structural and functional patterns within sequences. A subsequence may consist of up to three parts, with the permitted gap between each part defined, as well as the allowed mismatch for each part in a search. The logic operators OR and AND can be used to specify whether any or all of the parts need to be found in a sequence for it to be accepted.

MacVector is supplied with several subsequence files for both proteins and nucleic acids. You can edit these to:

- select a subset of entries and save the selection for use with the subsequence analyses
- delete existing entries from the file
- change the data for existing entries
- add new entries to the file
Selecting subsequences for searches

You can choose a set of subsequences to use for a search. This may be useful, for example, when you want to restrict your search to particular functional patterns.

To select a subset of subsequences

1. Open the required subsequence file and ensure that the file window is active.

2. If the file is locked, unlock it by clicking the Locked icon in the toolbar.

3. Scroll through the subsequence list to find the name of each subsequence you want to select.

Tip. You can navigate through the list by typing the first character of the subsequence, or by using the up and down arrows on the keyboard.

4. Click on each required subsequence.

A check mark appears and a line at the bottom of the window indicates how many subsequences have been selected.

Tip. Use the clear selection button to deselect all selected entries.

5. Save the file to disk, using File | Save.

Your selections are saved and you will not have to repeat the selection process every time you open this file.

Note. When you perform a search, you may use either all entries in the subsequence file, or only those subsequences selected.

Tip. If you save the changes using File | Save As, you can save different selection subsets.
Subsequence files

Adding entries to a subsequence file

Entries can be added to a subsequence file at any time that the window for the file is active.

To add a new entry to a subsequence file

1. Open the required subsequence file and ensure that the file window is active.
2. If the file is locked, unlock it by clicking the Locked icon in the toolbar.
3. Click the Add button on the toolbar.
   The Subsequence Editor dialog box is displayed.
4. Type a name for the subsequence in the Name text box.
5. Select the No. of parts radio button that corresponds to how many parts the subsequence will have.
6. In the Part # text boxes, type the sequence for each part of the subsequence, using standard IUPAC one-letter codes.

Note. Use parentheses to enclose a combination of amino acids for an ambiguous residue. For example, if either an aspartic (D) or glutamic (E) acid residue can
occur at a given position, denote it as (DE). However, for an ambiguous nucleic acid residue, use standard IUPAC symbols, not parentheses.

7. Use the **Perfect Match** and **Allowed mismatch** text boxes to adjust the tolerance of the match as follows:
   - to specify an exact match, type 0 in **Allowed mismatch** and leave **Perfect match** empty
   - to allow some degree of mismatching, type the maximum number of mismatching residues that you will accept in the **Allowed mismatch** text box
   - to require that specific residues match exactly, type X beneath those residues in the **Perfect match** box, using spaces to position the Xs. For amino acid motifs, only one X can be placed beneath each group of amino acids enclosed in parentheses. The allowed mismatch cannot exceed the length of the sequence for that part of the subsequence.

8. If you want to add an offset to the reported position of the subsequence when a search is performed, type a value in the **Offset** text box.
   Usually, the number of the first residue in the subsequence is reported.

9. If required, you can enter a comment up to 254 characters long.

10. For subsequences with more than one part, you can treat each part of the subsequence as if it were a separate motif:
    - select the **Logic: or** radio button to report a subsequence found if any combination of the subsequence parts is found
    - select the **Logic: and** radio button to report a subsequence found only if all subsequence parts are found.

11. For subsequences with more than one part, select one of the **Offset part** radio buttons to report the position of the subsequence relative to any of the parts.
    This option appears only if you are using AND logic. It is used in conjunction with the **Offset** value. If you select 1 as the offset part and type 0 as the offset, MacVector will report the position of the first residue of the first part of the subsequence. If you select 3 as the offset part and type 4 as the offset, MacVector will report the location of the subsequence to be the fifth residue of the third part of the subsequence.
Subsequence files

12. For subsequences with more than one part, use the Gap text boxes to specify the limits on the number of residues that may occur between each part of the subsequence.

This option appears only if you are using AND logic. For example, if your subsequence consists of two parts which may be separated by 10 to 20 residues, you would type 10 and 20 in the #1 - #2 Gap text boxes. The gap values can be any number from zero upwards, and the second number must be equal to or larger than the first.

13. Select OK to add the subsequence to the file.

14. Choose File | Save to make the changes permanent.

Copying entries between subsequence files

Copying existing entries between files can be useful, for example to create a customized subsequence subset from more than one file. One or more subsequence files must be open at the same time, and the file receiving the subsequence entries must be unlocked.

To copy entries between files

1. Make the file from which you are copying entries the active window.
2. Select the subsequences that you want to copy:
   • click to select a single entry
   • hold down the mouse button and drag to select a continuous block of entries
   • hold the <shift> key in combination with either of the above to retain already selected entries.
3. Choose Edit | Copy.
4. Make the file that is receiving the entries the active window.
5. If the file is locked, unlock it by clicking the Locked icon in the toolbar.

The entries are added to the file in alphabetical order.

7. Choose File | Save to make the changes permanent.

Note. If any of the entries were marked as selected, the selection will be retained.

Editing entries in a subsequence file

Entries in a subsequence file can be modified. This is generally useful only when a mistake has been noticed in the existing entry.
To edit a subsequence entry
1. If the file is locked, unlock it by clicking the **Locked** icon in the toolbar.
2. Click the **Edit** button on the toolbar.
   The **Subsequence Editor** dialog box is displayed.
3. Modify the entry as required.
   See “To add a new entry to a subsequence file” on page 75, for further details.
4. Select **OK** to add the changes to the subsequence file.
5. Choose **File | Save** to make the changes permanent.

Deleting entries from a subsequence file
Entries in a subsequence file can be deleted when required.

To delete a subsequence entry
1. If the file is locked, unlock it by clicking the **Locked** icon in the toolbar.
2. Select the subsequences that you want to delete:
   - click to select a single entry
   - hold down the mouse button and drag to select a continuous block of entries
   - hold the `<shift>` key in combination with either of the above to retain already selected entries.
3. Click the **Delete** button on the toolbar.
   The selected entries are deleted from the file.
4. Choose **File | Save** to make the changes permanent.

**Note.** This is equivalent to using **Edit | Clear**. If you want to paste the deleted entries into another file, use **Edit | Cut** instead of the **Delete** button.

Scoring matrix files
You can make the following modifications to the scoring matrix files to customize both the matrix comparisons and database searches:
- change the match/mismatch scores in the scoring matrix
- change the hash codes used during the hashing step
- change the deletion penalty
Scoring matrix files

- change the gap penalty
- change the parameters used to calculate the cut-off score for performing an optimized alignment

The default values for the above parameters in the scoring matrix files supplied with MacVector are listed in Appendix C, "Reference Tables". Modifications should be made with caution.

Editing match and mismatch scores

The scoring matrix is displayed as a lower-triangular matrix. The number located at coordinate \((H,V)\) in the matrix is the score that is assigned for an alignment between the two residues \(H\) and \(V\).

From left to right across the horizontal axis, and from top to bottom down the vertical axis, the order for nucleotide codes is:
- A C M G R S V T W Y H K D B N

and for amino acid codes is:
- A C D E F G H I K L M N P Q R S T V W Y B Z X *

The match and mismatch scoring is symmetric, so any change to the score for a pair \((H,V)\) is automatically applied to \((V,H)\) in the matrix.

To edit the match/mismatch scores
1. Open the scoring matrix that you want to edit.
A window opens, displaying the lower-triangular scoring matrix.

2. Select the value to be edited using one of the following methods:
   - click directly on a value
   - type the first letter of a pair, then hold down the `<shift>` key and type the second letter.

3. Type the new score (it may be positive or negative) and press the `Enter` key.

The scores are symmetrical, so if you change the score for the aligned pair A by C, the score for the pair C by A is changed simultaneously. Values assigned to the scoring matrix must lie between -99 and 99.

4. Select OK to save the changes.

5. Choose File | Save to make the changes permanent.

Editing hash codes

All residues that have the same hash code are treated as identical residues by the program. As an example, look at the hash codes for the nucleic acid scoring matrix DNA database matrix. The ambiguous bases W (which stands for A or T) and D (not C) are arbitrarily assigned the same hash code as T. There are 21 possible hash codes for amino acids (0 to 20) and four possible for nucleic acids (0 to 3).

To edit the hash codes

1. Open the scoring matrix that you want to edit.
   A window opens, displaying the lower-triangular scoring matrix.

2. Click the Hash button on the toolbar.
   The Hash Code Editor dialog box is displayed.
Scoring matrix files

3. Type values in the text boxes as required to make the residue types equivalent.
4. Select OK to save the changes.
5. Choose File | Save to make the changes permanent.

Editing tweak values

The tweak values are used to evaluate alignment scores, and can be edited if required.

The cut-off score parameters are substituted into an equation that is used to calculate the minimum score that an initial alignment must have before an optimized alignment is performed. Ordinarily, these parameters should be left at their default values. Accepted values are from 1 to 200.

The deletion penalty (single residue indel) is the value subtracted from the score of an aligned region for every one-residue insertion or deletion that was introduced in order to improve the alignment.

If an insertion or deletion longer than one residue was introduced into an alignment, the score is reduced by the gap penalty (continuing indel) times the number of bases in the insertion or deletion after the first one. Accepted values for the deletion and gap penalties are from 0 to 100. By altering the values for these penalties, you can control the number and size of the insertions or deletions that you will permit when aligning two sequences.

A large deletion penalty coupled with a small gap penalty will make it difficult to introduce an insertion or deletion into an alignment, but will make it fairly easy to extend one when it is introduced. Alignments performed using this combination will not contain many insertions or deletions, but those that do occur may be fairly long. This type of setting should be used if you think that sequences in the database may contain insertions or deletions of blocks of residues when compared to your query sequence.

A small deletion penalty coupled with a large gap penalty will tend to produce alignments containing many short insertions or deletions. You
would use this type of setting if you expect that differences between the sequences will involve single-residue insertions or deletions.

![Tweak Editor dialog box]

**To edit tweak values**

1. Open the scoring matrix that you want to edit. A window opens, displaying the lower-triangular scoring matrix.
2. Click the **Tweak** button on the toolbar. The **Tweak Editor** dialog box is displayed.
3. Type values in the text boxes as required to modify the tweak values.
4. Select **OK** to save the changes.
5. Choose **File | Save** to make the changes permanent.

**Sequence files**

MacVector sequence files are binary files, rather than ASCII text files. This means that you cannot read them using a word processor or text editor. MacVector stores sequence file annotation and features table data, as well as the sequence data itself. By using binary files that can be edited only with MacVector, we can ensure that the files will be properly formatted.

MacVector provides features table support for files in GenBank format (or GenBank variants). As new features are added to the GenBank format over time, support for them is included in upgrades of MacVector. See Appendix E, “*GenBank Feature Tables*” for details of the feature keywords supported in this version of MacVector.
When non-GenBank files are imported, MacVector creates a GenBank-style annotation section in memory for these files. Any nonsequence information in the original files is placed in the annotation section under Comment, so no information is lost.

When directed to open a file of type TEXT, MacVector parses it to see if it matches one of the supported file formats. If there is no match, it assumes that the file is a line file (sequence only).

MacVector reads the molecule type (nucleic acid or protein) directly from the file, if possible. When it encounters a file format that does not contain this information (such as line files, Staden files, and FastA files) MacVector displays an alert box for you to indicate whether the file contains a nucleic acid or a protein sequence.

See Appendix G, “Supported file formats and file extensions” for a complete list of the sequence formats MacVector supports.

For details of how to manage and edit sequence files, see Chapter 5, “Working with Sequences”.
Overview

This chapter describes the Sequence window and how to work with sequence files, including:

- opening, creating, saving and printing sequence files
- editing sequences
- editing features
- editing annotations
- using the proofreader
- searching sequences, features and results

Multiple sequences are described in Chapter 6, “Working with Multiple Sequences”

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The Sequence window

The Sequence window is displayed whenever a sequence is opened or created. It comprises a context dependent toolbar and four tabbed and linked views of the sequence: Editor, Map, Features and Annotations. The functionality available in each view is described in the sections below.

Managing sequences

This section describes some general procedures for managing sequences:

- opening a sequence
- creating a sequence
- saving a sequence
- printing a sequence
- closing a sequence
Managing sequences

Opening a sequence

You can use this procedure to open any sequence file that MacVector recognizes.

To open a sequence
1. Choose File | Open from the menu. The Open dialog box is displayed.
2. To choose a file type to view, select the Show arrow button and choose the required file type from the drop-down list.
3. Choose the required file from the list. Alternatively, you can also use any of the three buttons at the top right of the dialog box to help locate your files. These give you access to shortcuts, favorites, and recently used files.
4. To open more than one file, hold down the <shift> key and continue to choose files from the list. (A second <shift> click on the same file will deselect it.)
5. If you have selected a file containing multiple sequences, the **Open Multiple Sequence Files As:** menu lets you choose whether to open each sequence in a separate Sequence window, or all the sequences aligned in a single Multiple Sequence Alignment (MSA) window. See “Opening multiple sequences” on page 118 for details.

6. Select **Open** to open the file(s) and close the dialog box.

**Note.** Regardless of the file type opened, it is converted internally into MacVector binary format. This is the default format for saving the file.

### Creating a new sequence

You can create a new sequence at any time.

**To create a new sequence**

1. Choose **File | New** from the menu.
2. Select the required sequence type from the submenu.

A new empty Sequence window of the selected type is displayed.

### Saving a sequence

You can save a sequence using its existing file name or a new file name. You can save using any of the supported sequence file formats. See Appendix G, “Supported file formats and file extensions” for a complete list of the sequence formats MacVector supports.

**Save**

When a new sequence file is created or an existing file is opened, **File | Save** is disabled until the first change is made.

If you choose **File | Save** for a sequence that does not exist as a MacVector file, the Save As dialog box is displayed. This makes it unlikely that you will accidentally overwrite the original version of a file in another format.

**To save an existing MacVector sequence**

1. Ensure the required Sequence window is active.
2. Choose **File | Save** from the menu.

The file is saved as a MacVector binary file, overwriting the previous version of the file, regardless of the previous format.

**Note.** If the file is newly created, **File | Save** acts like **File | Save As** and a dialog box is displayed, asking for a filename.
Managing sequences

Save As

Use the **Save As** command when you want to:

- change the file name
- save the file to a different folder or to a different disk
- save the file in a format other than MacVector binary format
- change the molecule type or strand type of the sequence if the file is a nucleic acid sequence file

The **Save As** command is enabled whenever a Sequence window is active. If a file with the same name already exists on disk, it will be overwritten.

To save a sequence

1. Ensure the required Sequence window is active.
2. Choose **File | Save As** from the menu.
3. If required, navigate to the required folder, or create a **New** folder.
4. If required, change the file name by typing in the Save As text box.
5. Choose a format from the Format drop-down menu.
6. If necessary, select the required Sequence Type.
The Sequence Type option is only applicable to the IG_Suite and Gen-Bank file formats.
7. Select Save to save the file.

See also “Saving multiple sequence alignments” on page 121

The GCG RSF file format allows you to save several sequences to a single file. The MacVector interface allows you to write the contents of all currently open sequence windows to a single file in GCG RSF format. (Sequences in MSA windows cannot be saved in this way.)

To save several sequences to a single file
1. Ensure that the only open sequence windows are the ones to be saved, and that a sequence window is active.
2. Choose File | Save As from the menu.
The Save As dialog box is displayed.
3. If required, navigate to a new folder.
4. If required, change the file name by typing in the Save As text box.
5. Choose the GCG(RSF) format from the File Format drop-down menu.
6. Choose All open sequences from the Window drop-down menu.
7. Select Save to save the file.
All currently open sequences are saved to the file.

File extensions for sequence files
When you save a sequence using the Save As dialog box, MacVector will by default, add a filename extension appropriate to the format you select. See Appendix G, “Supported file formats and file extensions” for a complete list of the file extensions used by MacVector.
These file extensions may be useful when files are used with other operating systems which use extensions to identify the file type.

Printing a sequence
The information in sequence files can be printed as follows:
• as an annotated sequence file in a user-customizable format
Managing sequences

- in unannotated form, containing one or more of the three parts of the sequence file (annotations, features table, and sequence).

If you print an annotated sequence, you must format the appearance first. See Chapter 3, “General Procedures”, for details of formatting the annotated sequence display.

To print a sequence

1. Choose File | Page Setup from the menu.
   The Page Setup dialog box is displayed
2. Adjust the paper size and orientation as required.
3. Select OK.
4. Choose File | Print from the menu.
5. Adjust the print options as required.
6. Select Print.
   The sequence information is printed to the chosen device.

Closing a sequence

When you close a sequence that has not been saved, a message dialog box is displayed, asking whether you want to save the sequence before closing it.

To close a sequence

1. Choose File | Close from the menu.
   The sequence closes, and the Sequence window is closed.
The Editor view

The Editor view is displayed by default whenever a sequence is opened or created. To display it at any other time, click on the Editor tab in the Sequence window.

It enables you to perform the following operations on sequence data that appears in the Sequence window:

- insert or delete individual residues or groups of residues using the keyboard
- transfer blocks of residues within a sequence or between sequences of the same type using cut, copy, and paste operations
- reverse, complement, or reverse complement a sequence or a portion of a sequence
- verify your entries by having MacVector say the name of each residue as you enter it
- proofread the sequence by directing MacVector to read it back to you after you have finished entering it

Note. Sequences can be modified only if they are unlocked. This is done by clicking on the Locked indicator icon in the Sequence Editor toolbar.

If the sequence file has entries in the features table, any features that are affected by changes made to the sequence are updated automatically. In addition, cut, copy, and paste operations are automatically documented in the features table by the addition of a frag feature, which describes the operation performed.

The Editor has a toolbar containing icons that are used to perform the following functions:

Note. The Sequence Editor toolbar, like all toolbars in MacVector, can be customized. Right-click on the toolbar to access this functionality. The tools described below are those that appear in the default Sequence Editor toolbar. Some of these tools may be absent and other tools may be present depending on your settings.

For proteins, the sequence type indicator icon is Protein. For nucleic acids, the sequence type indicator icon is either DNA or RNA. Clicking
The Editor view

the icon changes how the sequences are interpreted from DNA to RNA and back again.

The Locked/Unlocked indicator icon displays the current lock status of the Sequence file. Clicking the icon changes the lock status from locked to unlocked and back again. Sequences can only be altered when the sequence file is unlocked.

The Text View icon provides access to the Annotated Sequence window, which contains the sequence text with features marked along its length. The format of this window controlled by the Text Display preferences dialog box, accessible via MacVector | Preferences on the main menu. See “Formatting the annotated sequence display” on page 35 for more information about this dialog box. The information in the window cannot be changed, but it can be highlighted and copied to the clipboard.

The Prefs icon provides access to the preferences dialog box, which enables you to specify general MacVector preferences, including the font used in the Sequence Editor.

The Replica icon is used to create a copy of the current sequence file in a new Sequence window.

The Blocking icon is a horizontal slider used to control the number of residues in a block. Drag the control with the mouse to change the number of residues in a block from 1 to 10.

The Voice Verify indicator icon displays the current voice verification status of the Sequence file. Clicking the icon changes the voice verification status from off to on and back again. When voice verification is on, each residue you enter is spoken by a computer voice. Refer to “The Map view” on page 98, for further information.

The Topology indicator icon shows which topological representation is currently being used in analyses of the sequence. Clicking the icon changes the topological representation from linear to circular and back again. This icon is displayed only if the Sequence window contains a nucleic acid sequence.

The Strands indicator icon shows whether a single or a double-stranded molecule is displayed in the Sequence window. Clicking the icon changes the view from single-stranded to double-stranded and back again. This icon is displayed only if the Sequence window contains a nucleic acid sequence.
The Add Feature icon provides access to the Feature Editor dialog box which you can use to add new features to the sequence. Refer to “Adding a feature” on page 100, for further information.

The Range text box displays the number or range of the current selection within the displayed sequence. You can edit the values in the box to change the selection. Refer to “Selection range” on page 94, for further information.

You can also alter the selected residues by using the Features drop-down menu. Choose from the list of sequence features or select the entire sequence.

To change the font used by the Sequence editor

1. Choose MacVector | Preferences from the menu, then click the Fonts icon on the preferences dialog, or click the Prefs icon on the toolbar when the Editor tab is selected.

The Fonts preferences dialog box is displayed.

2. In the Editor window font panel, use the drop down menus to change the font.

3. Select OK to apply the formatting.

Selection range

The Range text box shows one of the following:

- where the insertion point is located in the sequence
- the selected residue range

When you click within the content portion of the sequence window, a blinking vertical bar will appear within the sequence to mark the insertion point. The number of the residue immediately after the insertion point is displayed in the selection state window.

When one or more residues in the sequence are highlighted, the numbers of the beginning and ending residues of the block are displayed.

Clicking inside the Range box at any time enables you to alter the residue selection, by typing a new residue range or number.

The plus origin of the sequence is indicated by a small red plus sign above the sequence in the content part of the window, the minus origin by a small red minus sign. MacVector allows you to set the origin of the sequence to be a location other than the beginning or end of the sequence, so you can conform to the negative numbering system often used when referring to sequence data upstream from some significant
feature. To set the plus origin, place the pointer over the plus sign, hold down the mouse button, and drag the plus sign to the residue that you want to be number 1. The sequence numbering is adjusted accordingly, and the numbers of features in the features table are also adjusted to reflect the new origin.

Selecting residues

For many editing operations, you need to select a region of the sequence first. This may be done by using the mouse, the selection range tools, the Map view or the features table. The features table must contain entries for selection.

Mouse selections

To select a small block of residues using the mouse
1. Position the cursor to the left of the first residue in the block.
2. Hold down the mouse button and move the cursor until all the required residues are highlighted.

The residue block is highlighted.

To select a large block of residues using the mouse
1. Position the cursor to the left of the first residue in the block, then click the mouse button.
2. Scroll through the sequence to the required end point of the block of residues.
3. Hold down the <shift> key and click the mouse button after the last required residue.

The residue block is highlighted.

Selection range selections

The Range box and Features drop-down menu provide a convenient method of selecting residues when either the residue number range or feature name are known.

To select residues using the selection range box
1. Click inside the Range text box.
2. Type a residue number or range of numbers. Use a space or a colon (:) to separate the range values.

Either the residue range is highlighted, or the cursor is positioned immediately before the residue number typed.
Working with Sequences

To select residues using the features drop-down menu
1. Select a feature from the Features drop-down menu.
   The list contains the defined features of the sequence, plus All 1 to n for selecting the entire sequence.

Map view selections

To select a block of residues from the Map view
1. Click on the Map tab.
   The Sequence Map view is displayed.
2. Click on a feature in this view to select it.
   The residue block is highlighted.

Features table selections

To select a block of residues using the features table
1. Click on the Features tab.
   The Sequence Features view is displayed.
2. Scroll to the feature that you want to highlight.
3. Click on the feature.
   The residue block is highlighted.

Tip. To select the entire sequence, choose Edit | Select All.

Adding residues

You can add residues manually to a sequence displayed in the Sequence Editor view. You must use uppercase letters and the standard IUPAC code for either nucleic acids or amino acids.

Refer to Appendix C, “Reference Tables”, for details of these codes.

Tip. If you are unfamiliar with the one-letter codes for the nucleotides or amino acids, you can display a small movable window that contains the information by choosing Window | IUPAC Key from the main menu.

When you add a residue, the residues that follow it in the sequence are moved along one position.

In addition to entering bases using the alphanumeric portion of the keyboard, you can assign nucleotide codes to keys of the numeric keypad (if your Macintosh has one) to make it easier to enter nucleic acid sequence data with one hand. Choose Options | Set Keypad to use this facility.
The Editor view

To add residues to a sequence
1. Use the mouse cursor to select an insertion point in the sequence. Residues will be added immediately after this insertion point.
2. Enter the residues that you want to add.

Alternatively, you can choose Edit | Paste to add residues that have been copied to the clipboard.

Overwriting residues
You can overwrite residues by selecting a region of the sequence before adding the new residues. When you enter new residues, the selected region is overwritten.

Deleting residues
You can delete residues manually from a sequence displayed in the Sequence Editor view. When you delete residues, the residues that follow the deletion in the sequence are moved back the appropriate number of positions.

Any deletions made from a double-stranded nucleic acid sequence result in residues being deleted from both strands.

Deleting a single residue
You can delete single residues from a sequence.

To delete single residues from a sequence
1. Use the mouse cursor to select an insertion point in the sequence. Residues will be deleted immediately before this insertion point.
2. Use the <backspace> key to delete the unwanted residues.

Deleting a range of residues
You can delete a range of residues from a sequence.

To delete a range of residues from a sequence
1. Use the mouse cursor to select a region of the sequence that you want to delete.
2. Press the <delete> key to delete the selected residues.

Alternatively, choose Edit | Cut to remove the residues from the sequence and copy them to the clipboard for later use.
Working with Sequences

**Note.** Adding or deleting residues that are part of a feature will corrupt that feature. MacVector automatically adds a *frag* feature to the features table whenever a cut, edit or paste operation is performed, so you can keep track of changes.

**Reversing a sequence**

You can reverse the order of the residues in part or all of a sequence. This option applies to both protein and nucleic acid sequences in the sequence window.

**To reverse a region or the entire sequence**
1. Select either a region of the sequence, or the entire sequence.
2. Choose **Edit | Reverse**.

The region is reversed and updated in the sequence window.

**Complementing a sequence**

You can complement the residues in an entire sequence, or in a selected region of it. This option is available only if a nucleic acid sequence is displayed in the Sequence window.

**To complement a region or the entire sequence**
1. Select either a region of the sequence, or the entire sequence.
2. Choose **Edit | Complement**.

The region is complemented and updated in the sequence window.

**Reversing and complementing a sequence**

You can reverse then complement the order of the residues in an entire sequence, or in a selected region of it. This option is available only if a nucleic acid sequence is displayed in the Sequence window.

**To reverse and complement a region or the entire sequence**
1. Select either a region of the sequence, or the entire sequence.
2. Choose **Edit | Reverse & Complement**.

The region is reversed and complemented, and updated in the sequence window.

**The Map view**

The Map view displays an annotated graphic showing the features of the sequence. For DNA sequences, either a linear or circular map can be displayed.
The Features view

The map can be edited in many ways to give a tailored map for on-screen or printed presentation. See “Formatting the map display” on page 42, for further details.

To display a sequence map
1. Make the sequence window active by clicking inside it.
2. Select either a range of residues or the whole sequence.
   See “Selecting residues” on page 95.
3. Click on the Map tab.
The Map view is displayed, showing only the selected residues.
The appearance of the map can be controlled in a variety of ways, and can also be optimized for printing. See “Formatting the map display” on page 42.

The Features view

The Features view displays the features table associated with the sequence. A feature is an item of information about a sequence that is associated with a specific position within that sequence. The position may be a single residue, the gap between two residues, or a contiguous series of residues.

To display sequence features
1. Make the Sequence window active by clicking inside it.
2. Click on the Features tab.
The Features view is displayed.
You can modify sequence features as follows:
• add a new feature
• edit an existing feature
• delete an existing feature.
Features can be modified only when the sequence is unlocked. This is done by clicking on the Locked indicator icon in the Sequence window toolbar.

Feature keywords

The MacVector feature keyword sets for proteins and nucleic acids match the SwissProt and GenBank standard sets, respectively, see Appendix E, “GenBank Feature Tables” for further details. Any stan-
standard feature can be selected from the keyword list when editing the features table of protein or nucleic acid sequences.

Adding a feature

You can create a feature directly from a selected region in either the Editor view or the Map view. Or, you can create a feature from scratch using the Features view.

To add a protein feature

1. Click the Add Feature icon on the toolbar.

The Feature Editor dialog box is displayed.

By default, any region selected in the Sequence Editor or Sequence Map view is used to populate the Start Location and Stop Location boxes.

2. If necessary, supply or modify residue range of the feature using the Start Location and Stop Location text boxes and the associated At and Before (<) or After (> ) drop-down menus.

3. Scroll through the Feature Keyword list and select the feature type that you want to add.
A complete list of the protein feature keywords supported in MacVector can be found in Appendix E, “GenBank Feature Tables”.

4. Optionally, type a comment in the Comments text box.
5. Select OK to add the new feature.

To add a nucleic acid feature
1. Click the Add Feature icon on the toolbar. The Feature Editor dialog box is displayed.

By default, any region selected in the Sequence Editor or Sequence Map view appears as the first entry in the Location list.

2. Click on the + button to add additional segments to the feature.

A new sheet is displayed in the dialog box, which you can use to define additional segments.
3. Select a segment in the **Location** list and click **Edit** to modify it.

4. Select a segment in the **Location** list and click - to remove it from the feature.

5. Scroll through the **Feature Keyword** list and select the feature type that you want to add, or type the name of the feature type you want to add in the text box.

**Note.** If you type an invalid keyword into the **Feature Keyword** text box, then the **OK** button on the **Feature Editor** dialog box will be disabled. Select or type a valid feature keyword to re-enable the **OK** button.

A complete list of the nucleic acid feature keywords supported in MacVector can be found in Appendix E, “GenBank Feature Tables”.

6. Specify how the segments in the **Location** list should be combined to construct the feature using the **Operation** drop-down menu.

Select **Join** to indicate that the segments should be placed end-to-end to form one contiguous sequence. Alternatively, select **Order** to indicate that the segments can be found in the specified order (5’ to 3’ direction) but are not necessarily joined.

7. If the feature you are creating is located on the minus strand (the strand complementary to the one that is actually present in the sequence file), check the **Complementary** box. If the feature is located on the plus strand, ensure the this box is not checked.

8. To add GenBank qualifiers to the feature:

   - Ensure the **Qualifiers** tab is selected.
   - Click on the + button.

A new sheet is displayed in the dialog box, which you can use to add qualifiers.

   - Select the **Qualifier** you want to add from the drop-down list.

**Note.** Only qualifiers that are allowed by the GenBank specification for the selected feature type are available in the list.

   - Optionally, type **Comments** in the text box.
   - Click **OK** to add the qualifier to the feature or **Cancel** to dismiss the sheet without adding the qualifier.
   - Repeat these steps to add as many qualifiers as you want.
The Features view

Note. Some feature types have mandatory qualifiers. If one of these is selected, then MacVector will automatically add the appropriate qualifiers to the Qualifier list.

9. Alternatively, if you are familiar with GenBank format, you can type qualifiers in by hand using the Free-Form tab.

10. Select OK to add the new feature.

Editing a feature

To edit a feature

1. Select the feature that you want to edit by clicking on it with the mouse button.

2. Click the Edit icon on the toolbar to display the current feature information in full.

3. Edit the information as required.

Deleting a feature

To delete a feature

1. Select the feature that you want to delete by clicking on it with the mouse.

2. Click the Delete button on the toolbar to delete the selected feature.

Sorting features

The list of features displayed in the Features view can be sorted in several ways:

- in alphabetical order by feature keyword
- in ascending order by starting position on the 5' strand
- in ascending order by stopping position on the 5' strand
- in reverse-alphabetical order by feature keyword
- in descending order by starting position on the 5' strand
- in descending order by stopping position on the 5' strand
- by strand

To sort features

1. On the Features table, click the column heading label of the column you want to sort.

The feature list will be sorted by that column.
The Annotations view

The Annotations view displays text annotations associated with the sequence. Annotations provide extra information about the sequence, such as a description, academic references, or identifying numbers assigned by the databases from which the sequences are taken.

To display sequence annotations

1. Make the Sequence window active by clicking inside it.
2. Click on the Annotations tab.

The Annotation view is displayed.

MEDLINE abstracts can be retrieved directly from the Annotation view, by double-clicking on any reference that contain a MEDLINE ID.

You can also modify sequence annotations as follows:

- add a new annotation
- edit an existing annotation
- delete an existing annotation.

Annotations can only be modified when the sequence is unlocked. This is done by clicking on the Locked indicator icon in the Sequence window toolbar.

Types of annotation

There is one GenBank annotation field that you cannot edit directly in MacVector: the LOCUS line. MacVector fills in the LOCUS line information automatically whenever you save a file in GenBank or IG-SUITE format. The first ten characters of the name that you gave to the sequence file are used as the locus name. The current number of bases in the sequence file is computed by MacVector. The molecule representations (DNA or RNA, circular or linear) are taken from the current settings of the molecule and linear / circular icons unless you override the settings in the Save As dialog box. The entry date is the date the modifications were made.

You can edit all other GenBank annotation types directly.
### The Annotations view

<table>
<thead>
<tr>
<th>Field</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>definition</td>
<td>This is a short description of the sequence entry. It starts with the common name of the source organism, followed by the criteria that distinguish this sequence from the other parts of the source genome (gene name and what the gene codes for, the protein name and mRNA, or a description of the sequence’s function if it is a noncoding region). The definition line of coding regions can end with a completeness qualifier such as complete sequence, complete genome, or cds (complete coding sequence). MacVector limits this field to 254 characters.</td>
</tr>
<tr>
<td>accession</td>
<td>This contains one or more accession numbers that apply to the sequence entry. Accession numbers are assigned by GenBank and you would usually change them only if you knew there was an error in an existing number or if it was a new sequence and you had just received the accession number assignment from GenBank. Each accession number consists of an alphabetic character, followed by five digits. A space character is used to separate multiple accession numbers. The first accession number listed is unique to this particular sequence entry. MacVector limits this field to 254 characters.</td>
</tr>
<tr>
<td>version</td>
<td>This field contains a compound identifier, consisting of the primary accession number and a numeric version number associated with the current version of the sequence data in the record. This is followed by an integer key (a &quot;GI&quot;) assigned to the sequence by NCBI. Mandatory keyword/exactly one record.</td>
</tr>
<tr>
<td>NID/PID</td>
<td>An alternative method of presenting the NCBI GI identifier (described above) for nucleic acids and proteins. NID and PID are now obsolete, but are included for backwards compatibility.</td>
</tr>
<tr>
<td>DBSource</td>
<td>The DBSource line (DR line in SWISSPROT) is used as a pointer to information related to SWISSPROT and GENBANK entries and found in other data collections.</td>
</tr>
<tr>
<td>keywords</td>
<td>This field consists of short phrases that provide information about the sequence entry. Use semicolons to separate the keyword phrases and a period after the last keyword phrase. MacVector limits this field to 254 characters.</td>
</tr>
</tbody>
</table>
segment

This field is found only in segmented entries. It is used if two or more sequence entries of known relative orientation are separated by a short (less than 10 kb) segment of DNA. The format for the annotation is: n of total, where n is the segment number of the current entry and total is the total number of segments. Usually, you would not use this annotation.

source

This field has up to three subfields:

**Source** may contain an abbreviated form of the organism name and a molecule type.

**Organism** contains the scientific name for the source organism (genus and species, where applicable). Sequence files also contain in the Organism subfield a list of all the taxonomic classification levels for the organism, separated by semicolons and ending with a period.

If the sequence is from a parasitic organism, you can enter the name of the parasite’s host organism in the optional **Host** subfield. MacVector limits each subfield to 254 characters.

reference

This field has six subfields:

The optional **Title** subfield contains the title of the cited reference.

Type a number in the **Reference** subfield (references are numbered sequentially in a GenBank file, starting with 1).

The **Author** subfield is a list of authors in the order that they appear in the cited reference. Each name is listed in the form “lastname, A.A.”. The names are separated by a comma followed by a space. There is no comma after the penultimate name and the final name is preceded by the word “and”.

The **Journal** subfield contains the name of the journal, book, or thesis where the citation was published, or unpublished if the sequence has not been published. MacVector limits each subfield to 254 characters.

The **MEDLINE ID** subfield contains the MEDLINE references.

The **REMARK** subfield contains accredited comments that have been added by the database managers.

comment

This field is an optional, free-form text section. MacVector limits this field to 32,767 characters.
Using the proofreader

base count  The contents of this section cannot be changed by the user - the base count line can only be added to or removed from the file. If the base count field is present, MacVector automatically updates the base count whenever you edit a sequence.

origin  This field specifies how the first base is located within the genome. The Origin field for pBR322, for example, reads EcoRI site. This field may be left blank or the word Unreported may be entered. MacVector limits this field to 254 characters.

Adding an annotation

To add an annotation
1. Click the Add Annotation icon on the toolbar and select the type of annotation that you want to add from the submenu that appears.
2. Type the information as required.

Editing an annotation

To edit an annotation
1. Select the annotation that you want to edit by clicking on it with the mouse.
2. Click the Edit icon on the toolbar to display an editable version of the annotation.
3. Edit the information as required.

Deleting an annotation

To delete an annotation
1. Select the annotation that you want to delete by clicking on it with the mouse.
2. Click the Delete icon on the toolbar to delete the selected annotation.

Using the proofreader

MacVector provides a proofreading tool that enables you to have the residue sequence read back to you. This facility is available both as you type a sequence in, and as a proofreading tool on a selected sequence.
Reading a typed sequence

You can have a sequence repeated to you as you type.

To read back a sequence as it is typed
1. Choose **Windows | Show Proofreader** to display the proofreader.
2. Click on **MENU** to select a male or female voice and the volume level as required.
3. Position the cursor in the Sequence window at the insertion point where you want to enter the new or additional residue sequence.
4. Type in the sequence.
   As you type the sequence, it is read back to you.

Proofreading a sequence

After you have entered a sequence, you can proofread it.

To proofread a sequence
1. Select the required portion of the sequence to be proofread.
2. Choose **Windows | Show Proofreader** to display the proofreader.
3. Select a male or female voice and the volume level as required.
4. Select the **start** button on the proofreader to begin.
   Each residue is unhighlighted in turn as the sequence is read.
5. If required, you can pause the playback at any time with the **pause** button on the proofreader.
6. If required, you can move backwards or forwards along the sequence by using the **fast forward** and **rewind** buttons of the proofreader.

Selecting the sequence using the proofreader
The sequence for proofreading can be selected by using the fast forward and rewind buttons of the proofreader.

To select the sequence using the proofreader
1. Position the insertion point at the beginning or end of the required selection.
2. Use either the fast forward button or the rewind button to highlight residues from the insertion point.
3. Use the stop button when the required residue block has been highlighted.

The sequence can now be proofread as described previously.

Searching

The Find tool in MacVector provides powerful and flexible search and replace capabilities for finding specific subsequences in proteins and DNA in the current sequence. It also enables you to search features associated with a sequence and analysis results, such as BLAST search results.

Choose Edit | Find to display the Find dialog box. The dialog box has three tabs for the three different types of search: Feature, Sequence and Results.

Searching sequences

The sequence search enables you to find specific subsequences in proteins and DNA in the current sequence.

Tip. If you only need to find a specific residue number, use Edit | Jump to Location. The number you specify can be relative to the 5' end of the sequence, the 3' end of the sequence, the current position of the insertion point, or the plus or minus origin.
To perform a subsequence search on a sequence

1. Choose Edit | Find from the main menu. The Find dialog box is displayed.

2. Ensure that the Sequence tab is selected. This tab is selected by default if the Find dialog box is opened when a Sequence Editor view is active.

3. Type the subsequence that you want to find in the Find text box.

4. Choose the direction of search. Select the Forward checkbox to search in the 5' to 3' (amino to carboxy) direction. If not checked, the search will proceed in the 3' to 5' (carboxy to amino) direction.

5. Choose where to start the search from using the Search from drop-down menu.

6. Choose which strand to search from using the Strand drop-down menu.

7. Choose which phase to search from the Phase drop-down menu.

8. By default, the Find tool uses IUPAC codes to search for matching residues, so that the search string AGY will also locate the ambiguous residues AGT and AGC. If you just want to locate the sequence AGY, then check the Literal box.

9. If you want to search for the reverse of the typed subsequence, check the Reverse box.

10. If you want to search for the complement of the typed subsequence, check the Complement box. This can be used in conjunction with the Reverse checkbox to search for the reverse complement.

11. Do one of the following:
   - to find the first occurrence of the subsequence, select Find
   - to find the next occurrence of the subsequence, select Find Next

   If the subsequence is present in the sequence, it will be highlighted, if not, the alert will sound.

   You can also use the Find tool to search a protein sequence using a DNA subsequence and vice versa. This is useful if, for example, you want to find all subsequences in a DNA sequence that could code for the tripeptide leucine-serine-arginine.
Searching

MacVector automatically performs the translation (or reverse-translation) using the current genetic code.

**To search a DNA sequence using a protein subsequence**

1. Choose **Edit | Find** from the main menu.

The **Find** dialog box is displayed.

2. Ensure that the **Sequence** tab is selected.

This tab is selected by default if the **Find** dialog box is opened when a Sequence Editor view is active.

3. Click the **molecule** icon to toggle from DNA to protein.

4. Type the protein subsequence in the **Find** text box.

5. Select other options if required, as described in steps 2 through 8 in the procedure “To perform a subsequence search on a sequence” on page 110.

6. Select **Find** to perform the search.

**Tip.** If you select the **molecule** button again after entering the subsequence, you can see the translated DNA subsequence.

**To search a protein sequence using a DNA subsequence**

1. Choose **Edit | Find** from the main menu.

The **Find** dialog box is displayed.

2. Ensure that the **Sequence** tab is selected.

This tab is selected by default if the **Find** dialog box is opened when a Sequence Editor view is active.

3. Click the **molecule** icon to toggle from protein to DNA.

4. Type the DNA subsequence in the **Find** text box.

5. Select other options if required, as described in steps 2 through 8 in the procedure “To perform a subsequence search on a sequence” on page 110.

6. Select **Find** to perform the search.

The **Find** tool also enables you to specify a replacement string.

**To replace one subsequence with another in a sequence**

1. Choose **Edit | Find** from the main menu.

The **Find** dialog box is displayed.

2. Ensure that the **Sequence** tab is selected.
This tab is selected by default if the **Find** dialog box is opened when a Sequence Editor view is active.

3. Type the subsequence you want to find in the **Find** text box.

4. Type a replacement subsequence in the **Replace** text box.

5. Select other options if required, as described in the procedure “**To perform a subsequence search on a sequence**” on page 110.

6. Do one of the following:
   - to substitute the replacement string for the first occurrence of the subsequence, select **Find** then **Replace**. After replacement, the **Replace** button is unavailable.
   - to substitute the replacement string for the selected occurrence of the subsequence and find the next occurrence of the subsequence, select **Replace & Find**
   - to substitute the replacement string for all occurrences of the subsequence, select **Replace All**

**Searching features**

The feature search enables you to find specified text within the features associated with a sequence.

Search results are selected automatically in the Sequence Map and Sequence Features views, either all at once using the **All** button or one at a time using the **Next** and **Previous** buttons.

You can limit your search to a subset of the features by including a **Feature** type or a **Qualifier** type in your search.

You can also build more complex queries, by searching only those features that are already selected.

**To perform a feature search**

1. Choose **Edit | Find** from the main menu.
   
The **Find** dialog box is displayed.

2. Ensure that the **Feature** tab is selected.
   
   This tab is selected by default if the **Find** dialog box is opened when a Sequence Feature view or a Sequence Map view is active.

3. Type the term that you want to find in the **Find** text box.

4. Optionally, restrict your search to a particular **Feature** type by selecting it from the list.
Searching

5. Optionally, restrict your search to a particular **Qualifier** type by selecting it from the list.

6. Do one of the following:
   - to select all features that match the search parameters, use **All**
   - to scroll through the features that match the search parameters one at a time, use **Next** and **Previous**

Searching results

The results search enables you to find specified text within the results windows from analyses such as Blast and Align to Folder.

**To perform a results search**

1. Choose **Edit | Find** from the main menu.

The **Find** dialog box is displayed.

2. Ensure that the **Results** tab is selected.

This tab is selected by default if the **Find** dialog box is opened when Results window is active.

3. Type the term that you want to find in the **Find** text box.

4. Check **Ignore Case** to make your search insensitive to the case of any matching text.

5. Check **Wrap Around** to make your search locate matching text which straddles more than one line.

6. Scroll through the search results one at a time, using **Next** and **Previous**
Overview

This chapter describes the features of the Multiple Sequence Alignment (MSA) window, and how to use it with nucleic acid and protein sequence alignments. It describes how to import and export multiple sequence files, how to create an empty MSA document and insert sequences into it, and how to edit and display the alignment.

For information on ClustalW alignment, refer to Chapter 13, “Aligning Sequences”. For information on phylogenetic analysis, refer to “Phylogenetic reconstruction” on page 286.

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<td>The consensus sequence window</td>
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</tbody>
</table>
Multiple Sequence Alignments

Multiple sequence alignments may come from one of several sources. MacVector can generate a multiple sequence alignment automatically, by means of a ClustalW analysis. Alternatively, you can open a pre-calculated alignment which has been generated by another program such as the GCG Wisconsin Package PileUp command. You can also create a completely new alignment, by importing sequence data into a new MSA document window, or typing in the residues manually.

There are very few differences between an alignment which has been generated automatically by ClustalW, and one that has been imported or created manually. In all cases, the consensus line will be shown correctly and a phylogenetic tree can be generated. The only real difference is that the ClustalW guide tree cannot be displayed unless a ClustalW analysis has been performed (see “Displaying a guide tree” on page 284).

The Multiple Sequence Alignment window

The Multiple Sequence Alignment (MSA) window is displayed whenever a multiple sequence alignment document is opened or created. It comprises a context dependent toolbar and six tabbed and linked views of the multiple sequence alignment: Editor, Text, Pairwise, Matrix, Picture and Guide Tree. The functionality available in each view is described in the sections below.

All of the MSA tabs are visible in all MSA windows, irrespective of whether the corresponding views have been calculated. However, if a view has not been calculated, then the tab is greyed out and is non-clickable.

The Alignment Views dialog box enables you to generate any missing views, post-alignment. You can choose from the following output options:

- multiple alignment text display
- pairwise alignment text display
- multiple alignment picture display
- guide tree (for ClustalW alignments; see “Displaying ClustalW alignment results” on page 282)
- a consensus sequence
In addition, this dialog box includes an information panel. If you have performed a ClustalW multiple alignment, a summary of the results and parameters is listed here. The **Recalculate** button lets you perform (or repeat) a ClustalW alignment (see “Aligning multiple sequences using ClustalW” on page 277).

**Note.** The settings and parameters reported in the information panel are only valid following a ClustalW alignment.

To display the **Alignment Views** dialog box, click the **Views** icon on the MSA window toolbar. This dialog box is also displayed whenever a ClustalW alignment is performed.

The data in the different views is updated in real time but it is refreshed only when a new view is displayed after the underlying alignment has been edited. To see this, open an MSA document and click the **Replica** icon on the MSA window toolbar to create a duplicate MSA window. Display the MSA Editor view in one window and the MSA Text view in the other. If you edit the alignment in the Editor view, then nothing will happen to the Text view in the other window. However, if you click on the second window, to make it active the Text view is refreshed to reflect the changes you made it the Editor view. The same will happen with the other views.

This is different to the way the views in the Sequence window are updated. The reason for the difference is that some of Text views can take a considerable time to be refreshed. So if they were updated every
time you typed a character, updating the views could be very slow, pre-
venting you from being able to edit the alignment easily. This approach
ensures the views are only updated when you are ready to look at them.

Managing multiple sequence alignments

Opening multiple sequences

You can open multiple sequences from various sources:

- Several single-sequence files
- One multiple-sequence file
- Several multiple-sequence files, or a combination of single- and
  multiple-sequence files.

When you open multiple sequences from any of these sources, you may
want to open them in individual sequence windows, or in a single MSA
window.

The default behavior of MacVector is to use the following guidelines
when opening one or more sequence files:

<table>
<thead>
<tr>
<th>File type</th>
<th>How the files are opened</th>
</tr>
</thead>
<tbody>
<tr>
<td>MacVector, GCG RSF, ASCII/Plain</td>
<td>Individual sequence windows</td>
</tr>
<tr>
<td>GenBank, EMBL/Swiss-Prot or NBRF files, containing single or multiple sequences</td>
<td>Individual sequence windows</td>
</tr>
<tr>
<td>NEXUS, GCG MSF, PHYLIP or FastA (MacVector multiple sequence) files, containing single or multiple sequences</td>
<td>MSA windows, one for each input file</td>
</tr>
</tbody>
</table>

You can override the defaults and require either that all sequences are
opened in individual windows, or that all sequences are opened in a sin-
gle MSA window. In the File | Open dialog box, the Open Multiple Sequence Files As drop-down menu allows you to make this choice.

When Multiple Alignment is chosen, all sequences are opened in a single
MSA window. If the sequences are unaligned and are of unequal length,
gap symbols are appended to the end of the shorter sequences to make
the lengths equal.
Managing multiple sequence alignments

When Single Sequences is chosen, all sequences are opened in individual sequence windows.

To open multiple sequences from one or more files
1. Choose File | Open.
   The Open dialog box is displayed.
2. Select the sequence files you want to open.
3. Select the Open Multiple Sequences As arrow button and choose an option from the drop-down menu:
   • Auto: MacVector will use the default guidelines to open the files
   • Multiple Alignment: All sequences will be opened in a single MSA window
   • Single Sequences: All sequences will be opened in individual sequence windows.
4. Select Open to open the sequence files.

Creating new multiple sequence alignments

You can create a new MSA document in MacVector. This is particularly useful if you want to import aligned sequences from databases or web pages, by cutting and pasting from other applications. You may also want to type sequences into an empty MSA document.

Creating an empty MSA document

To create an empty MSA document
1. From the File menu, choose either New | Nucleic Acid Alignment or New | Protein Alignment.
   An MSA window of the appropriate type is displayed.
Inserting sequences into an empty MSA document

You can either type a sequence into the new window, or paste residues or full sequences from the clipboard. You can also add sequences from files.

**To type a sequence in an empty MSA document:**

1. Ensure that the Editor tab is selected and type in the required sequence, using the spacebar to indicate any gaps.

When you are working with nucleic acid sequences, the DNA/RNA icon on the toolbar indicates whether you should type in DNA or RNA codes,

**Note.** It is not possible to type in both DNA and RNA codes in the same MSA window.

As you type the sequence, the sequence numbering will appear over the line at the set intervals.

**Note.** You can program the numeric keypad for entering sequences. See “Configuring the numeric keypad” on page 60.

**To paste a sequence into an empty MSA document**

1. Ensure that the Editor tab is selected and choose Edit | Paste.

The clipboard contents will be inserted at the cursor position, as follows:

- If one or more full sequences have been selected for copying (see “Selecting sequences in the MSA Editor” on page 137), they will be inserted as new sequences
Managing multiple sequence alignments

- If, in a single sequence, only residues have been selected (i.e. not the sequence name), they will be pasted into the untitled sequence at the insertion point
- If the selection is a block extending over several sequences, it will be inserted as new sequences

Saving multiple sequence alignments

You can save multiple sequence alignments in the MSA window in any of the supported multiple sequence formats. See Appendix G, “Supported file formats and file extensions” for a complete list of the multiple sequence formats MacVector supports.

For all formats except PHYLIP, you save MSA documents in exactly the same way as single sequences. The File | Save As dialog box for MSA documents looks like this:

![Save As dialog box](image)

The Options button is only enabled when Format is set to PHYLIP.

Saving sequences as PHYLIP files

When saving aligned sequences as a PHYLIP file, you need to set the name length (the length of the field containing the sequence names). If the value you enter is less than the length of any of the current sequence names, they will be truncated. In cases where truncation would result in two or more sequences being given the same name, MacVector will
append numbers to the truncated name to distinguish the different sequences.

Before saving as a PHYLIP file, you also need to indicate whether the sequences should be interleaved or non-interleaved.

**To save a multiple aligned sequence as a PHYLIP file**
1. Ensure the required MSA window is active.
2. Choose **File | Save As** to display the **Save As** dialog box.
3. If required, navigate to the required folder, or create a **New** folder.
4. If required, change the file name by typing in the **Name** text box.
5. Choose **PHYLIP** from the **Format** drop-down menu. The **Options** button becomes active.
6. Select **Options**. The **File Format Options** dialog box is displayed.
7. Set the interleaving control:
   - If you want the sequences to be interleaved, ensure that the check box is selected.
   - If you want the sequences to be non-interleaved, ensure that the check box is not selected.
8. Type the required name length in the **PHYLIP name length** text box.
9. Select **OK** to apply the settings and return to the Save As dialog box.
10. Select **Save** to save the file.

**File extensions for aligned sequence files**

When you save an aligned sequence file using the Save As dialog box, MacVector will by default add a filename extension appropriate to the format you select. See Appendix G, “Supported file formats and file extensions” for a complete list of the file extensions used by MacVector.
The MSA Editor view

These file extensions will be particularly useful when files are used with other operating systems which use extensions to identify the file type.

The MSA Editor view

The MSA Editor view is displayed by default whenever a multiple sequence alignment is opened or created. To display it at any other time, click on the Editor tab in the MSA window.

It is used for modifying and comparing the alignment of several selected sequences. It can be used to edit individual sequences in multiple alignments, and to delete parts of sequences and import other parts.

The MSA Editor has a toolbar containing icons that are used to perform the following functions:

**Note.** The MSA Editor toolbar, like all toolbars in MacVector, can be customized. Right-click on the toolbar to access this functionality. The tools described below are those that appear in the default MSA Editor toolbar. Some of these tools may be absent and other tools may be present depending on your settings.

For protein alignments, the sequence type indicator icon is Protein. For nucleic acid alignments, the sequence type indicator icon is either DNA or RNA. Clicking the icon changes how the sequences are interpreted from DNA to RNA and back again.

The Locked/Unlocked indicator icon displays the current lock status of the MSA document. Clicking the icon changes the lock status from locked to unlocked and back again. Sequences can only be altered when the MSA document is unlocked.

The Align icon provides access to the ClustalW Alignment dialog box which enables you to align multiple sequences using the ClustalW algorithm. See “Aligning multiple sequences using ClustalW” on page 277.

The Phylogeny icon provides access to the Phylogenetic Reconstruction dialog box. This button becomes enabled when the MSA editor contains at least four sequences. See “Phylogenetic reconstruction” on page 286.

The Views icon provides access to the Alignment Views dialog box. See “The Multiple Sequence Alignment window” on page 116 for more information about this dialog box.
The **Prefs** icon provides access to the **Multiple Alignment Options** dialog box. See “Setting the MSA Editor display options” on page 125 for more information about this dialog box.

The **Replica** icon is used to create a copy of the current MSA document in a new MSA window.

The **Blocking** icon is a horizontal slider used to control the number of residues in a block. Drag the control with the mouse to change the number of residues in a block from 1 to 10.

The **Line Wrap** indicator icon displays the current line wrap display style of the MSA document. Clicking the icon changes the display style from linear mode to page mode and back again.

In linear mode, each sequence is displayed on a single horizontal line, and sequences are aligned on top of each other. You use the horizontal scroll bar to scroll through the entire sequence.

In page mode, the linear display is wrapped to fit into the window, as in a word processor. You use the vertical scroll bar to scroll through the sequence.

The **Voice Verify** indicator icon displays the current voice verification status of the MSA document. Clicking the icon changes the voice verification status from off to on and back again. When voice verification is on, each residue you enter is spoken by a computer voice. Refer to “The **Map view**” on page 98, for further information.

The **Color** indicator icon displays the current color display style of the MSA document. Clicking the icon changes the display style from color to black and white and back again.

In black and white mode, sequences are displayed as black text in a monospaced font on a white background. In color mode, sequences are displayed as black text with residue-specific color backgrounds. To help visualize similarities during manual alignments, each amino acid or nucleotide can be assigned a unique color. Furthermore, groups of amino acids can be given colors to permit visual alignments based on properties. The color groups can be edited using the **Color Group Editor** dialog box.

The **Groups** icon provides access to the **Color Group Editor** dialog box. This allows you to edit the colors and similarity groups used to calculate the consensus.

The **Width** icon is a horizontal slider used to control the width of the displayed residues when **Color** mode is enabled. Drag the slider to the left
The MSA Editor view

to reduce the width of the residues, and to the right to increase the width. If the width you set is less than the width of the default font, no characters are displayed and the residues are represented as color bars only.

Position mask

A position-masking bar can be displayed across the top of the aligned sequences. It is used in phylogenetic analysis, to exclude positions in the sequence from the analyses. For instructions, refer to “Position masking” on page 286.

Setting the MSA Editor display options

You can modify the appearance of the MSA window. Controls for all multiple alignment preferences are grouped together in the Multiple Alignment Options dialog box, which you can access using the Prefs icon on the MSA Editor toolbar.

In the Multiple Alignment Options dialog box, settings are organized into categories, accessed by selecting tabs at the top of the dialog box. General MSA Editor display options are set using the Editor tab. For information about setting the options on the Consensus tab, see “Calculating the consensus line” on page 126. For information about setting the options on the Text Display, Picture Fonts and Picture Shading tabs, see “The Multiple Sequence Alignment window” on page 116.
To change the Editor settings in the MSA window

1. Click the Prefs icon on the toolbar.

The Multiple Alignment Options dialog box is displayed, with the Editor tab selected.

2. To control sequence numbering, do one of the following:
   • select the None radio button if you do not want residue numbers to be displayed.
   • select the Top radio button to display residue numbers across the top of the alignment; then use the text box to set the interval at which numbers will be displayed (default is 10).

3. Select the Outline colors check box to display each residue with a dark outline, for greater contrast.

Note. The color display style must be enabled for this option to work. To toggle the color display style, click on the Color icon on the toolbar.

4. To allow regions of the aligned sequences to be excluded from phylogenetic reconstruction analyses, select the Show position mask check box.

5. Select Apply to see the effects of your changes on any open MSA windows.

6. Select OK to save the changes and close the dialog box.

7. Alternatively, select Defaults to reset the default settings, or Cancel to close the dialog box without saving.

Note. The font used in the MSA Editor view can be controlled using the MacVector preferences dialog box which is accessed by choosing MacVector | Preferences from the main menu, then clicking the Fonts icon on the preferences dialog.

Calculating the consensus line

Underneath an aligned sequence, you can display a line of residue codes to show the nature and extent of their consensus. To set the consensus calculation options, use the Consensus tab on the Multiple Alignment Options dialog box.
MacVector offers three basic modes for calculating the consensus line:

- Consensus Identity
- % Identity
- Property-based color groups, of which ClustalW default groups is a special case.

These modes are discussed in “Working with color groups” on page 130.

For protein sequence alignments, the default options for consensus calculations are:

- ClustalW default groups
- Consensus Identity
- % Identity

and the property-based color groups:

- Functionality
- Alpha-helix
- Alpha-helix + P450
- Hydrophobicity + Charge
- Acidity + Basicity
Working with Multiple Sequences

- Smooth Scaling
- Structural Position
- Steric Bulk
- Gascuel & Golmard
- Chou-Fasman Alpha Helix
- Chou-Fasman Beta Sheet
- Levitt Alpha Helix Forming
- Levitt Beta Sheet Forming
- Levitt Turn Forming
- Dayhoff Matrix
- Helix Termintors
- Chemical Type

For nucleotide alignments, the corresponding default options are:

- % Identity
- Consensus Identity
- DNA Residues

User-defined color groups will also appear in these menus.

Use threshold mode

MacVector offers a choice of two methods to deal with conflicting nucleic acids in the consensus calculation: Use threshold and Ignore threshold. The Use threshold option generates a consensus residue code for each position based on the highest percentage agreement that can be found between the aligned sequences. For example, if in 10 aligned sequences there are 9 Gs and one C, there is 90% agreement with a consensus code of G. The percentage agreement must exceed a minimum threshold value. Use the Threshold text box to set this value, within the range 51-100%.

If the consensus threshold is not met by any single-base code, MacVector then tests all 2-base ambiguities against the criterion (see “IUPAC-IUB codes for nucleotides and amino acids” on page 430). For example, if the separate percentages of G and C fail the criterion, but their combined percentage meets it, the consensus line will indicate S at that position. If no 2-base ambiguity meets the criterion, MacVector then
The MSA Editor view

tests all 3-base ambiguities. If all these fail, the consensus residue at that position will be N.

In Threshold mode, ambiguous base codes in sequences are treated as equal probabilities of the alternative bases (e.g. an S residue contributes 0.5 C and 0.5 G to the consensus base for its position).

Ignore threshold mode

When the Ignore threshold option is selected, the residue code on the consensus line must match every residue in that column, so that ambiguous residue codes may be required. For example, if in 10 aligned sequences there are 9 Gs and one C, the consensus code is S. The Threshold text box is disabled when you select this option, since the effective threshold is fixed at 100%.

For aligned protein sequences, the threshold method is always used to generate the consensus code.

Treatment of gaps in sequences

The Treat gaps as valid characters check box lets you choose whether to treat gaps as valid characters in the consensus calculation, or to ignore them. If gaps are valid characters, then the consensus line will show a gap wherever the percentage of gaps exceeds the threshold. For example, if the threshold is set at 51% and there are 11 aligned sequences, with 6 gaps, 3 Gs and 2 Cs, then the consensus code for that position will be “-” (gap), with 55% agreement.

If gaps are ignored, agreement is calculated as a percentage of the remaining total. In the example above, the consensus code will be G, with 60% agreement.

Displaying the consensus line

You can display the consensus line in the Editor, Text and Picture views in the MSA window. To set the consensus display options, use the Consensus tab on the Multiple Alignment Options dialog box.

Appearance of the consensus line

The Show consensus line check box lets you display the consensus line. It will appear in the MSA window in the Editor, Text and Picture views.

The No spaces in consensus check box lets you replace undefined characters in the consensus by N (in nucleotide windows) or X (in protein windows).
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The **No gaps in consensus** check box allows you to replace the gap character “-” in the consensus line with a space.

The **No ambiguous in consensus** check box is used only with nucleotide alignments. It allows you to display only unambiguous residue codes (A, G, C, T or U) on the consensus line.

**To change the Consensus settings in the MSA window**

1. Click the **Prefs** icon on the toolbar.
2. Choose the **Consensus** tab.
3. To display a consensus line in the MSA window, select the **Show consensus line** checkbox.
4. Set the required calculation mode for generating the consensus, by choosing from the **Calculate using** drop-down menu.
5. Set the threshold criterion, if required, by typing a value in the **Threshold** text box.
6. For nucleotide alignments, choose a **DNA Consensus** method for dealing with conflicts in the calculation:
   - select the **Ignore threshold** radio button if you want the consensus residue code to match all residues in the column
   - select the **Use threshold** radio button to calculate the consensus using percentage agreement and the **Threshold** criterion.
7. If required, select the **Treat gaps as valid characters** check box.
8. If required, select the **No spaces in consensus** check box.

**Note.** This option is not applicable in **Ignore threshold** mode, because spaces are never present.

9. If required, select the **No gaps in consensus** check box.
10. Select **Apply** to see the effects of your changes on any open MSA windows.
11. Select **OK** to save the changes and close the dialog box.
12. Alternatively, select **Defaults** to reset the default settings, or **Cancel** to close the dialog box without saving.

**Working with color groups**

For the color display, you can specify the color for each nucleic acid base or amino acid residue. You can assign amino acid residues to color
The MSA Editor view

groups according to their chemical properties. Alternatively, residues can be colored according to degree of consensus among the sequences at each position. In addition, you can create and apply your own color schemes.

To choose or edit a color group use the Color Group Editor dialog box, which you can access using the Groups icon on the MSA Editor toolbar.

The Color Group Editor dialog box differs slightly between protein and nucleotide sequence alignments, with some different Color By options available in each case.

Consensus Identity and % Identity color groups

These groupings are based on the degree of consensus among the aligned sequences at each position.

The Consensus Identity option uses three colors:

- **100% Identity** - where all the sequence residues match the consensus, then the 100% Identity color is used for the entire column
- **Consensus Match** - where one or more residues in a column do not match the consensus, those that do match will be displayed in this color
- **Mismatch** - this color is used for all the residues that do not match the consensus.

The % Identity option colors residues according to the percentage of residues at a given position that share the same symbol. The color displayed depends on the residues in the other sequences, not on the calculated consensus.
Property-based color groups

When there is a protein alignment in the MSA Editor view, you can select a range of preset color groups that are based on chemical or physical properties of the residues. The Color By drop-down menu includes the following property options:

- Chemical Type
- Functionality
- Alpha-helix
- Alpha-helix + P450
- Hydrophobicity + Charge
- Acidity + Basicity
- Smooth Scaling
- Structural Position
- Steric Bulk
- Gascuel & Golmard
- Chou-Fasman Alpha Helix
- Chou-Fasman Beta Sheet
- Levitt Alpha Helix Forming
- Levitt Beta Sheet Forming
The MSA Editor view

- Levitt Turn Forming
- Dayhoff Matrix
- Helix Termintors

The default option for protein alignments is Chemical Type. When you choose a color group from the menu, the corresponding groups are displayed in the Color Group Editor dialog box.

ClustalW Default Groups

The default property groups used by ClustalW alignment are included as a Color By option for protein alignments. These groups are unusual in that a given amino acid can appear in more than one group, and therefore could be represented by more than one color. To simplify the MSA editor view, these groups are always shown in white.

To specify individual residue colors
1. With nucleotide or protein sequences displayed in the MSA window and the Editor tab selected, click the Groups icon.

The Color Group Editor dialog box is displayed.
2. If you are using DNA sequences, ensure that DNA Residues is selected in the Color By drop-down menu.
3. If you are using protein sequences, select Smooth Scaling from the Color By drop-down menu.
4. To alter the color scheme, choose a color for each residue from the drop-down color palette to the right of each residue symbol. Alternatively, use the system Color Picker to select a color, by clicking on the current color swatch.
5. Select Apply to preview the changes. If they are not satisfactory, select Revert to restore the previous color set.
6. Select OK to save the changes.

Applying color groups

To apply an existing residue color scheme
1. With nucleotide or protein sequences displayed in the MSA window and the Editor tab selected, click the Groups icon.

The Color Group Editor dialog box is displayed.
2. Choose a preset color scheme from the Color By drop-down menu.
3. Select Apply to preview the changes. If they are not satisfactory, select Revert to restore the previous color set.

4. Select OK to save the change to the display.

Creating, renaming and deleting color groups

To create a new residue color scheme
1. With nucleotide or protein sequences displayed in the MSA window and the Editor tab selected, click the Groups icon.

The Color Group Editor dialog box is displayed.

You can now choose a preset color scheme to use as a template for your own scheme, as follows:

2. Choose a scheme from the Color By drop-down menu.

3. Choose Copy This Group from the Color By drop-down menu. A New Color Group Name prompt is displayed. The default name will be Copy of Name, where Name is the scheme you selected.

Note. You can modify a preset scheme, but we recommend that you always make a copy of the scheme to modify, because you can delete it later if necessary.

4. Type a name for the new scheme, and select OK to return to the Color Group Editor dialog box.

5. Edit the residue groups as required. For each group you create, do the following:
   • type a name for the group
   • type the IUPAC one-letter code for each residue in the group
   • choose a color for the group from the drop-down palette.

Note. Each residue type can only appear in one group. If you enter a residue that already appears in another group, it is automatically removed from the previous group.

6. Select Apply to preview the changes. If they are not satisfactory, select Revert to restore the previous color set.

7. Select OK to save the changes.

To rename or delete a color scheme:
1. With nucleotide or protein sequences displayed in the MSA window and the Editor tab selected, click the Groups icon.

The Color Group Editor dialog box is displayed.
2. Choose the user-defined color group that you want to rename or delete from the Color By drop-down menu.

**Note.** You cannot use the Color Group Editor to rename or delete the pre-defined color groups.

3. Do one of the following:
   - To rename the color group, choose Rename This Group from the Color By drop-down menu. A prompt to enter a New Color Group Name is displayed. Type the new name in the text box, and select OK to return to the Color Group Editor.
   - To delete the color group, choose Delete This Group from the Color By drop-down menu.

4. Select OK to close the Color Group Editor dialog box.

**Editing aligned sequences**

The MSA Editor allows you to copy, move, insert, rename and delete sequences. For your source material, you can select and use single or multiple sequences, or parts of sequences, from sequence windows, MSA windows, or other applications.

The MSA Editor incorporates familiar text-editor features, such as double-clicking to select a contiguous sequence of residues and moving sequences by selecting the sequence label and dragging. This section describes the available editing actions in the MSA window. Selection in single-sequence windows is described in “Selecting residues” on page 95.

**Adding sequences to an existing alignment**

You can add a new blank sequence to the MSA window, insert one or more existing sequences by pasting from the clipboard, or import sequences from files.

Before adding a sequence, ensure that the MSA document is unlocked. If necessary, click on the Locked icon on the toolbar to unlock the MSA document.

**To create a new sequence in an existing alignment**

1. Ensure that the MSA document you want to edit is active and the Editor tab is selected.
2. Choose Edit | Add New Sequence from the menu.
A newly created sequence is displayed below any existing sequences. Its length is set to the alignment length, and it consists entirely of gap characters. The cursor is positioned at the first character.

3. To input the sequence, do either of the following, as appropriate:
   - Paste in the required residues (see “Inserting residues” on page 138)
   - Type the required residues, using the cursor arrows to move along the sequence if necessary.

When you type, residues are inserted into the sequence. At the end of the sequence, gaps are truncated to keep the length constant.

**Tip.** You can program the numeric keypad for entering sequences. See “Configuring the numeric keypad” on page 60.

**To paste sequences into an existing alignment**

1. Ensure that the MSA document you want to edit is active and the **Editor** tab is selected.

2. Choose where you want the sequences to be inserted, and position the cursor somewhere in the sequence line immediately above this point.

**Note.** When there are multiple sequences on the Clipboard, the **Paste** command will not work if any sequences or residues are selected.

3. Choose **Edit | Paste** from the menu.

The pasted sequences will be inserted below the cursor.

Another way to add sequences is to import them from single or multiple-sequence files. You can import several files in one operation.

**To add sequences from a file**

1. Ensure that the MSA document you want to edit is active and the **Editor** tab is selected.

2. Choose where you want the sequences to be inserted, and position the cursor somewhere in the sequence line immediately above this point.

3. Choose **Edit | Add Sequences from File** from the menu.

The **Open** dialog box is displayed.

4. Select the sequence file(s) you want to open.

5. Select **Open** to open the sequence files.

The sequences are inserted at the cursor.
Selecting sequences in the MSA Editor

MacVector supports a full range of options to select residues and sequences.

To select all the contents of the MSA editor

1. Ensure that the MSA document you want to edit is active and the Editor tab is selected.
2. Choose Edit | Select All from the menu.

All the residues are highlighted. If you want to cancel the selection, click on white space anywhere in the sequence alignment display.

Tip. If you need to insert gaps at the start of an alignment, choose Edit | Select All. Then move the entire alignment to the right, by dragging with the mouse.

You can select one or more full sequences for copying or deletion. (Full sequences include both the sequence name and all its residues.)

To select full sequences in the MSA editor

1. Ensure that the MSA document you want to edit is active and the Editor tab is selected.
2. Select a sequence name by clicking on it.
3. To select multiple sequences, hold down the Shift key while clicking on the required sequence names.

If you need to de-select a sequence, hold down the Shift key and click on its name again. To de-select all sequences, click anywhere in the sequence alignment display.

You can also select all or part of a residue sequence without selecting its name.

To make selections in sequences

1. Do one of the following:
   - click and drag on one or more residues. You can select a block of residues from several sequences in this way. However, discontinuous blocks cannot be selected
   - double-click a residue to select that residue and all residues contiguous with it. The selection is made in both directions, until an inserted gap or the end of the sequence is encountered
Working with Multiple Sequences

- click the cursor in front of the first residue in the range you want to select. Holding down the Shift key, scroll the window until you can see the last residue in the required range, and click after it.

The selected residues are highlighted. You can copy them to the clipboard using Edit | Copy, or move them as described in the next section.

Changing and deleting sequences

Before changing or deleting a sequence, ensure that the MSA document is unlocked. If necessary, click on the Locked icon on the toolbar to unlock the MSA document.

To move a selection within a sequence
1. Do one of the following:
   - place the cursor over the selection until the cursor symbol changes to a hand, then drag the selection to a new location
   - use the cursor keys to move the selection to the left or right.

Note. A selection can only be moved within the gaps between it and the rest of the sequence. If there are no gaps around the selection, it cannot be moved, and the cursor symbol will not change to a hand.

Inserting residues

When you type or paste residues, they are inserted into the sequence. At the end of the sequence, gaps are truncated to keep the alignment length constant. If there are no gaps at the end of the sequence, gaps are appended to all the other sequences in the alignment to keep them the same length.

To insert and remove gaps in a sequence
1. Position the cursor where you want to add or remove a gap.
2. Do one of the following:
   - to insert gaps, use the spacebar
   - to remove gaps, use the backspace key

To insert residues by typing
1. Position the cursor where you want to add the residues.
2. Type the residues, using the keyboard or programmed numeric keypad.

To paste residues into a sequence
1. Position the cursor where you want to add the residues.
The MSA Editor view

2. Use **Edit | Paste** to insert the residues from the clipboard.

**Note.** If you paste a block of residues from more than one sequence into the alignment, they are always inserted as new sequences, with names based on their parent sequences. You cannot insert residues into more than one sequence at a time.

During alignment, it may be necessary to insert one or more gaps in an entire block of sequences:

**To insert gaps in multiple sequences**

1. Select a block of residues immediately after the position where the gap should be.
2. Press any “gap” key (space, hyphen, or period). A gap will be inserted in front of the selected block, in all the selected sequences. Repeat as required.

**Deleting and replacing residues**

**To delete residues in a sequence**

1. Select the residues you want to delete. You can select a block of residues from more than one sequence.
2. Choose **Edit | Cut** to delete the residues.

To keep all sequences the same length, gaps are appended to the affected sequences as required.

You can also use the delete key to delete residues.

**To replace or clear residues in a sequence**

1. Select the residues you want to replace.
2. Do one of the following:
   - Choose **Edit | Clear** to replace all the residues by gaps
   - Type or paste in the new residues.

**Note.** You cannot use a block of residues from more than one sequence to replace another selected block. You must insert residues into one sequence at a time.

**To delete sequences**

1. Select the name of a sequence you want to delete. To select more than one sequence, hold down the **Shift** key and click on the required names.
2. Choose **Edit | Delete Sequence** to delete the sequences. Alternatively, use the **Cut** function; the sequences will be placed on the Clipboard.
Ordering and naming sequences

To control the ordering and naming of sequences, use the sequence name buttons in the panel to the left of the displayed residues in the MSA editor.

Changing the sequence display order

You can change the order in which the sequences are displayed.

To change the order in which sequences are displayed
1. Select a sequence label by clicking on it.
2. Hold down the mouse button and drag the sequence to a new position

Note. Altering the sequence order does not affect the order of sequences in the results windows, until you regenerate the results.

To view the new sequence order in the results windows
1. Click the Views icon on the toolbar.

The Alignment Views dialog box is displayed.
2. Do one of the following:
   - If you have a ClustalW alignment, select Recalculate and use the Alignment Views dialog box to repeat the ClustalW calculation
   - If it is not a ClustalW alignment, just select OK in the Alignment Views dialog box to regenerate the windows.

Setting reference sequences

In linear Line Wrap mode, you can keep reference sequences at the top of the list and compare them to others. Only the topmost of the scrollable sequences can be made into a reference sequence.

To set reference sequences
1. Ensure that the MSA editor display is in linear Line Wrap mode.
   Click the Line Wrap icon if necessary.
   A pin icon will be visible to the right of the topmost sequence name.
2. Click on the pin icon to fix the position of the reference sequence.
   The sequence is now stationary. It will remain at the top of the list while the other sequences are scrolled underneath. A new pin icon appears to the left of the sequence under the reference sequence.
The MSA Text view

Note. Further sequences can be locked as reference sequences whenever a pin icon appears. You may first need to change the sequence display order to place the required sequence below the existing reference sequences.

Renaming aligned sequences

After a sequence alignment, it is often useful to rename aligned sequences.

To rename an aligned sequence

1. Select a sequence name by double-clicking on it.
   The Rename Sequence dialog box is displayed.
2. Type a name in the New sequence name text box.
3. Select OK to save.

The MSA Text view

The MSA Text view displays the alignments in standard ClustalW form. Under the alignment there is an identity line, which indicates with symbols where there are identities and similarities in the alignment. The view includes a custom header showing a score for the alignment, together with additional parameters.

Note. The settings and parameters reported in the MSA Text view are only valid following a ClustalW alignment.

The text multiple alignment can be saved to disk as a text file.

Tip. The MSA Text view is enabled by default. To disable it, uncheck Multiple Alignment in the Text Display panel on the Alignment Views dialog box.

The appearance of the MSA Text view be modified in a number of ways, using the Text Display tab on the Multiple Alignment Options dialog box.
Note. These settings also affect the MSA Pairwise view.

To modify the appearance of the MSA Text view
1. Click the Prefs icon on the toolbar in the MSA window. The Multiple Alignment Options dialog box is displayed.
2. Select the Text Display tab.
3. If you want to show identical residues in the consensus line, select the Show identities check box, and then choose the required Identities marker from the radio button and text box options.
4. If you want to show similar residues in the consensus line, select the Show similarities check box, and then choose the required Similarities marker from the radio button and text box options.
5. If you want to stop the ends of aligned sequences from being padded with the “.-” symbol, select the Suppress “.-” at ends check box.
6. Enter the number of residues per line in the Line length text box.
7. Choose a numbering style for the alignments, by selecting one of the Numbering radio buttons.
8. Select Apply to see the effects of your changes on any open MSA windows.
9. Select OK to save the changes and close the dialog box.
Alternatively, select Defaults to reset the default settings, or Cancel to close the dialog box without saving.
The MSA Pairwise view

The MSA Pairwise view displays all pairwise combinations of input sequences aligned with each other. Each pairwise alignment includes a summary report of the percentage identity, percentage similarity, and number of gaps. For two sequences there is a single alignment, for three sequences there are three alignments, for four sequences there are six alignments, and so on. The alignments are derived from the complete multiple alignment, with all other sequences removed and all residual gaps closed. This approach closely matches the results of an independent pairwise sequence alignment.

To enable the MSA Pairwise view
1. Click the Views icon on the toolbar in the MSA window.
   The Alignment Views dialog box is displayed.
2. Check Pairwise Alignments in the Text Display panel.
3. Click OK.

Note. You can enable and disable any combination of the results tabs at one time by setting the required options before selecting OK.

The MSA Matrix view

The MSA Matrix view displays a table that shows the similarity and identity scores between pairs of sequences in an alignment. These scores are generated as part of the process of generating pairwise alignments between sequences.

To enable the MSA Matrix view
1. Click the Views icon on the toolbar in the MSA window.
   The Alignment Views dialog box is displayed.
2. Check Identity/Similarity Matrix in the Text Display panel.
3. Click OK.

Note. You can enable and disable any combination of the results tabs at one time by setting the required options before selecting OK.

For nucleic acid alignments only the identity scores are relevant. However, for protein alignments the identity and similarity are distinct. Iden-
tity is the absolute relationship between sequences, for example 100% identity between two sequences means that they are absolutely identical. However, similarity refers to amino acids that are chemically, or rather biologically, related. For example two hydrophobic residues would score higher than a hydrophobic and a hydrophilic residue. Scoring of similarity is done according to the similarity/substitution matrices that are used to generate the alignment.

The MSA Picture view

The MSA Picture view displays the multiple alignments in high resolution PDF form. When printed on a laser printer, the quality of this window is to publication standard. The aligned sequences can be copied and pasted as graphics into other applications, for example word processors or graphical editing applications.

**Tip.** The MSA Picture view is enabled by default. To disable it, uncheck Multiple Alignment in the Picture Display panel on the Alignment Views dialog box.

You can format the contents of the MSA Picture view, using the Picture Fonts and Picture Shading tabs on the Multiple Alignment Options dialog box.

To modify the appearance of the MSA Picture view

1. Click the Prefs icon on the toolbar in the MSA window.

The Multiple Alignment Options dialog box is displayed.
The MSA Picture view

2. Select the **Picture Fonts** tab.
3. Use the **Set** button to choose alternative font styles, sizes and weights for the different types of text.
4. Enter a title for the window in the **Title** text box.
5. Choose a numbering style for the alignments, using one of the **Numbering** radio buttons.
6. Type in the required number of residues per line in the **Line Length** text box.
7. Select **Apply** to see the effects of your changes on any open MSA editor windows.
   
   If you need to cancel the changes, select **Cancel**. If you need to reset the defaults, select **Defaults**.
8. Select the **Picture Shading** tab.
9. If you want similar residues to be treated as identities for the purpose of shading, select the **Treat similarities as identities** check box.
10. Choose the appearance of residue highlighting for identities, using the controls in the **Identities** panel.

![Multiple Alignment Options](image)

11. Choose the appearance of residue highlighting for similarities and mismatches, using the controls in the **Similarities** and **Mismatches** panels.
12. Select **Apply** to see the effects of your changes on any open MSA editor windows.

13. Select **OK** to save the changes and close the dialog box.

Alternatively, select **Defaults** to reset the default settings, or **Cancel** to close the dialog box without saving.

**To modify the consensus line in the Picture view**

1. To view the options for calculating and displaying the consensus line, select the **Consensus** tab in the **Multiple Alignment Options** dialog box.

The consensus line controls are the same for the MSA Editor, Text and Picture views. For detailed instructions, refer to “Calculating the consensus line” on page 126 and “Displaying the consensus line” on page 129.

**The MSA Guide Tree view**

The MSA Guide Tree view displays a guide tree for aligned sequence results when four or more sequences are aligned together.

**To enable the MSA Guide Tree view**

1. Click the **Views** icon on the toolbar in the MSA window.

The **Alignment Views** dialog box is displayed.

2. Check **Guide Tree** in the **Picture Display** panel.

3. Click **OK**.

The tree shows the relationships that ClustalW generates when doing pairwise alignments. These are displayed as a phylogram or a cladogram, with the distances between nodes shown in phylogenetic units. As an approximate guide, a value of 0.1 corresponds to a difference of 10% between two sequences.

**Note.** These guide trees may not be true phylogenetic trees.

You can use the toolbar buttons and the **Tree Viewer Options** dialog box to select a tree shape, root the tree and edit its appearance. You can also print and save the guide tree.

**The consensus sequence window**

The consensus can be used to generate a sequence in a standard MacVector sequence window. You can use this sequence in a variety of ways - for example, to perform BLAST searches.
The consensus sequence window

The sequence is generated using the settings on the **Consensus** tab of the **Multiple Alignment Options** dialog. Any spaces are ignored, resulting in a continuous sequence of the matching residues.

**To display a consensus sequence window**

1. Click the **Views** icon on the toolbar in the MSA window. The **Alignment Views** dialog box is displayed.
2. Select the **Create Consensus Sequence** check box.
3. Type a name for the sequence in the text box.
4. Select **OK**.
Using the NCBI Entrez Database

Overview

This chapter describes how to locate and extract complete sequences from the Entrez database maintained by NCBI. You can do the following:

- find sequences by querying any of the Entrez database fields
- find MEDLINE abstracts in the Entrez database using any of the Entrez database fields
- extract sequences and abstracts from the database to the desktop, or save them to disk.

Refer to "Subsequence analysis" on page 196, for details of subsequence searching.


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Using the NCBI Entrez Database

The Entrez database

The Entrez database, produced by the National Center for Biotechnology Information (NCBI), provides access to DNA and protein sequences and related bibliographic information. The sequence data include the complete nucleotide and protein sequence data from the GenBank, EMBL, DDBJ, PIR, PRF, PDB, SWISS-PROT, dbEST and dbSTS databases, as well as data from U.S. and European patents. Entrez also contains a subset of the MEDLINE database, including references and abstracts which are cited in the sequence databases and other related MEDLINE records.

MacVector can search the Entrez database on the Internet. The data is updated on a daily basis, and the service is available free of charge.

Refer to Appendix A, “Setting up NCBI's Entrez and BLAST Services”, for details of accessing the database on the Internet.

Searching the Entrez Database

Choosing the database

The MacVector interface enables you to search the different databases available through the NCBI separately, providing you with access to the full range of information available.

Note. Not all of the databases available through the NCBI are sequence databases (e.g. Index of NCBI web pages).

The precise list of databases you can search is updated from the NCBI's servers each time you launch an Entrez search.

To choose the database

1. Choose Database | Internet Entrez Search.

The Internet Entrez Browser window is displayed.
2. Select the database you want to search using the **Database** drop-down menu.

Among the most useful databases available are:

**Protein sequence record**

The protein entries in the *Entrez* search and retrieval system have been compiled from a variety of sources, including SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq.

**Core nucleotide db**

The core nucleotide database contains records for all *Entrez* nucleotide sequences that are not found within the EST or GSS divisions of GenBank. These include sequences from all the remaining divisions of GenBank, NCBI Reference Sequences (RefSeqs), Whole Genome Shotgun (WGS) sequences, Third Party Annotation (TPA) sequences, and sequences imported from the *Entrez* Structure database.

**GSS db**

The GSS database contains all records found within the Genome Survey Sequence division of GenBank. GSS records contain first-pass single-
read genomic sequences and rarely include annotated biological features.

The GSS division contains (but is not limited to) the following types of data:

- random "single pass read" genome survey sequences.
- cosmid/BAC/YAC end sequences
- exon trapped genomic sequences
- Alu PCR sequences
- transposon-tagged sequences

**EST db**

The EST database contains all records found within the Expressed Sequence Tags division of GenBank. EST records contain first-pass single-read cDNA sequences and include no annotated biological features.

**Full nucleotide db**

The full nucleotide database is a superset of the Core nucleotide db, GSS db and EST db.

**Performing the search**

The set of three drop-down menus enables you to define a single or combined (Boolean) annotation search of the selected database. The two *All Fields* drop-down menus contain a list of all possible search categories for the database you are searching.

The other drop-down menu contains logical operators that enable you to perform more complicated searches using two criteria. For example, you can extract all ribosomal protein sequences from the organism “*Canis*” using the following settings:

- “canis*” in the *organism* field
- the *And* operator in the central drop-down menu
- “ribosomal” in the *indexed words* field.

**To perform a single category search**

1. Choose a search category in the first *All Fields* drop-down menu.
2. In the adjacent text box, type the required search text.
Searching the Entrez Database

Only one string is allowed as a search query for each field, except for the entry definition field, when separate search strings can be separated by a single space.

3. Ensure that the logical operator drop-down menu is set to None.
4. Select Search to perform the search.

During the search, the Search button label changes to Stop, allowing you to stop the search.

If MacVector finds any matches to your query, it will list them in the top text panel. The Title, Document ID and the Relevance of the match to your search text are listed for each match.

**Note.** If you save the sequence as a file the Document ID number will be used to name the file (see “Saving sequences to file” on page 155).

To perform a combined category search
1. Choose a search category in the first All Fields drop-down menu.
2. In the adjacent text box, type the required search text.

Only one string is allowed as a search query for each field, except for the entry definition field, when separate search strings may be separated by a single space.
3. Choose a logical operator for combining the two fields from the logical operator drop-down menu.

4. Choose a search category in the second All Fields drop-down menu.

5. In the adjacent text box, type the required search text.

6. Select Search to perform the search.

During the search, the Search button label changes to Stop.

If MacVector finds any matches to your query, it will list them in the top text panel. The Title, Document ID and the Relevance of the match to your search text are listed for each match.

**Note.** If you save the sequence as a file the Document ID number will be used to name the file (see “Saving sequences to file” on page 155).

### Viewing details of search results

The top text panel of the Internet Entrez Browser window contains a scrollable list of the matches to your query.

**To view details of search results**

1. Select the required results in the results panel, by clicking or shift-clicking.

2. Select the Details button.

The relevant NCBI web page is displayed in the bottom text panel of the Internet Entrez Browser window.

### Extracting information from the Entrez database

When you have a set of results from your search, you can do the following:

- extract one or more sequences into individual Sequence windows
- save one or more sequences into individual sequence files
- extract abstracts into individual Text windows
- save abstracts as individual text files
- extract abstracts cited in sequence annotations

### Extracting sequences to the desktop

**To extract sequence search results into sequence windows**

1. Select the required results in the results panel, by clicking or shift-clicking.
Searching the Entrez Database

2. Select the **To Desk** button.
   The selected sequences will appear on the desktop in individual Sequence windows.

**Tip.** Instead of using the **To Desk** command, you can extract a sequence just by double-clicking on the entry in the results panel.

**Saving sequences to file**

**To save sequence search results into sequence files**

1. Select the required results in the results panel, by clicking or shift-clicking.

2. Select the **To Disk** button.
   A dialog box is displayed, enabling you to choose a folder in which to store the sequence files.

3. To create a new folder, select the **New** button and type in a name.

4. Select **Choose**.
   Each sequence is saved to its own file. The file is named using the Document ID number of the sequence.

**Extracting abstracts to the desktop**

**To extract MEDLINE search results into text windows**

1. Select the required results in the results panel, by clicking or shift-clicking.

2. Select the **To Desk** button.
   The selected abstracts will appear on the desktop in individual text windows.

**Tip.** Instead of using the **To Desk** command, you can extract an abstract just by double-clicking on the entry in the results panel.

**Saving abstracts to file**

**To save MEDLINE search results into files**

1. Select the required results in the results panel, by clicking or shift-clicking.

2. Select the **To Disk** button.
   A dialog box is displayed, enabling you to choose a folder in which to store the abstract files.

3. To create a new folder, select the **New** button and type in a name.
4. Select **Save**.
Each abstract is saved to its own file. The file is named using the **MEDLINE ID** number of the abstract.

**Extracting MEDLINE abstracts cited in sequence annotations**

If you have extracted a sequence that cites a MEDLINE reference, you can retrieve the MEDLINE abstract directly from the Annotations view of the sequence.

To extract MEDLINE abstracts from a sequence window
1. Ensure that the required sequence is displayed in the active window.
2. Select the **Annotations** tab.
3. Scroll through the annotations and identify the MEDLINE reference.

MacVector retrieves the MEDLINE reference from the *Entrez* database, and the abstract is displayed in a new text window.
Overview

This chapter describes how to:

• calculate sequence property analyses
• perform base composition analysis
• display and manage the results of a sequence property profile calculation
• find open reading frames using user-designated start and stop codons
• find regions of a nucleic acid that are likely to code for protein according to Fickett’s TESTCODE algorithm
• create codon preference plots to locate regions that have a high probability of coding for a highly expressed protein

Refer to Chapter 5, “Working with Sequences”, for details about opening and editing sequence files before analysis.
Calculating Sequence Properties

Sequence Properties

MacVector contains many methods for analyzing the properties and composition of proteins and nucleic acid sequences. Detailed information about these analyses is available in Chapter 17, “Understanding Protein and DNA Analysis”.

Protein sequence property profiles

The available methods for predicting sequence properties for protein sequences are summarized in the following sections.

Antigenicity

Hopp-Woods  The Hopp-Woods scale was designed to predict the locations of antigenic determinants in a protein, assuming that the antigenic determinants would be exposed on the surface of the protein and thus would be located in hydrophilic regions. Its values are derived from the transfer free energies for amino acid side chains between ethanol and water.

Parker  Predicts the location of antigenic determinants by finding the area of greatest local hydrophilicity. It is based on the Hopp-Woods method, but differs in that it uses a modified hydrophilicity scale, based on the HPLC retention times of model peptides.

Protrusion  Uses the Protrusion Index, which is an antigenic scale based on a study of proteins with known 3D structure. The tendency of each residue to be located in a protruding region of the protein can be calculated using this method.

Welling  Calculates a statistical score, where the antigenicity value for each residue is calculated as the log of the quotient between its percentage in a sample of known antigenic regions and its percentage in average proteins.

Antigenic Index  Uses a modified version of the Jameson and Wolf algorithm, which combines hydrophilicity, surface, flexibility and secondary structure predictions to produce a composite surface contour of a protein.
Sequence Properties

Flexibility

Protein flexibility  The Karplus and Schulz method measures the flexibility of each residue and assigns each residue a class (rigid, intermediate, or mobile) based on the flexibility of the residue and its two neighbours. Flexibility is a useful predictive parameter of antigenicity.

Secondary Structure

Chou-Fasman  Uses the tendency of an amino acid to appear in a given secondary structure in known X-ray structures to predict unknown structures.

Robson-Garnier  Predicts secondary structure based upon the effect that each amino acid has on the conformational state of its neighbours.

Hydrophobicity

Fauchere-Pliska  Uses a hydrophobicity scale based on the experimental octanol/water partition of the N-acetyl-amino-acid amides of each residue at neutral pH. Each hydrophobicity value is expressed with respect to glycine (which scores 0), so positive values indicate a greater hydrophobicity than glycine.

Janin  A method based on the accessibility of residues, with a molar fraction (%) of the occurrences of buried (< 20 Angstroms exposed surface area) and exposed (> 20 Angstroms exposed surface area) residues being derived.

Kyte-Doolittle  The Kyte-Doolittle scale is the most commonly used hydropathy scale. Its values are assigned using a combination of the water-vapor transfer free energies for amino acid side chains and the preference of amino acid side chains for interior or exterior environments. Small adjustments are made to the final values based on the experience of the authors.

Manalavan  A method based on a 'bulk hydrophobicity character', as the hydrophobicity of an individual amino acid residue is modified by the presence of other residues within an 8 Angstrom radius.
Sweet-Eisenberg uses a consensus scale comprising four other hydrophobicity scales, including the Janin scale and the von Heijne scale. The method averages the four scales to reduce the effect of outlier values on the predictions.

von Heijne uses a scale that reflects the estimated free energies of transfer of residues when moving from an alpha-helix in water to an alpha-helix in a non-polar phase (with no hydrogen bonding capacity).

Hydrophilicity

Hopp-Woods: The Hopp-Woods scale was designed to predict the locations of antigenic determinants in a protein, assuming that the antigenic determinants would be exposed on the surface of the protein and thus would be located in hydrophilic regions. Its values are derived from the transfer free energies for amino acid side chains between ethanol and water.

Amphipilicity

Amphipilicity Helix: Uses the periodicity in the protein’s hydrophobicity in alpha-helical regions to calculate amphiphilicity.

Amphipilicity Sheet: Uses the periodicity in the protein’s hydrophobicity in beta sheet regions to calculate amphiphilicity.

Transmembrane

Argos Helix: Predicts membrane-bound, helical sequence regions, based on data from proteins which are known to interact with membranes.

von Heijne Helix: Weights the contribution from each residue, so that the residues in the central apolar region of the bilayer dominate the prediction. However, good noise reduction is also obtained.
Sequence Properties

Goldman-Engelman-Steitz (GES)  The GES scale was developed in order to identify possible transmembrane helices in a protein. The scale values are the sums of hydrophobic and hydrophilic components for each amino acid. Hydrophobic components are derived from the free energy of water-oil transfer for the side chains; hydrophilic components take into consideration the free energy for inserting charged groups into a bilayer and the free-energy contributions from hydrogen-bonding with water and with backbone carbonyl groups (if the residues can form such bonds when participating in a helical structure).

Surface  This profile was designed to predict which regions of a protein are most likely to lie on the protein’s surface, based on knowledge of which amino acids are more likely to be found on the surface of proteins of known structure. It is based on the work of Janin et al (1978) and Emini et al (1965).

General  This profile generates a listing of the amino acid composition.

MW  This profile calculates the protein molecular weight.

pI  This calculates the pH at which the protein has a net charge of zero.

Nucleic acid sequence property profiles

The following types of sequence property profile calculation are available for nucleic acid sequences:

- percentage base composition with respect to any combination of nucleotides
- mono-, di-, and trinucleotide frequencies
- melting or dissociation temperature of a nucleic acid sequence or oligo, including a running average for the sequence
- Coding preference plots
Window size

The window size determines the number of residues that are grouped together for analysis. For example, a window size of 3 will result in the sequence being analyzed in blocks of 3 residues, throughout the length of the sequence.

The percentage base composition is calculated on the basis of the window size that you specify in the Base Composition Analysis dialog box.

You can select a different window size for each profile algorithm.

Note. You cannot change the window size for the following protein algorithms: Protein flexibility, von Heijne transmembrane flexibility, and Argos transmembrane flexibility. These methods use a fixed window size.

Performing protein sequence analyses

Protein analyses are accessible by choosing Analyze | Protein Analysis Toolbox. This item is enabled when a protein sequence file is the active window.

When a protein sequence is the active window, one or more analyses can be performed for the sequence.

To perform a protein sequence analysis

1. Choose Analyze | Protein Analysis Toolbox.

The Protein Analysis Toolbox dialog box is displayed.
Performing protein sequence analyses

2. Scroll through the **Protocol** list and select the profile you want to calculate.

When a profile is selected, a brief description of it is displayed in the text panel to the right of the **Protocol** list.

3. Select the **List** and **Plot** check boxes as necessary, for a text listing or graphical view of the results.

To select or deselect all checkboxes in a column, hold down the **option** key while clicking on any checkbox in the column.

4. Repeat steps 2 and 3 for each profile you want to calculate.

5. Choose the portion of the sequence that you want to analyze by typing in the sequence numbers that bracket the region in the **Region** text boxes. Alternatively, select a region from the feature selector drop-down menu to the right of the text boxes.

**Note.** Some profiles require a minimum sequence length, or window, for their calculation. A selection smaller than this value is the most common cause of the calculation failing.

6. If you want to use MacVector default settings, select **Defaults**.

7. Select **OK** to perform the analysis.

**Note.** The **OK** button will only be enabled if at least one **List** or **Plot** checkbox is selected.
Viewing Protein Analysis Toolbox results

The results of the analysis are displayed automatically. Plots generated by selecting the Plot check box are displayed in a graphical Toolbox Plot window. Numerical results generated by selecting the List check box are displayed as tables in a scrollable Toolbox List text window.

Further viewing options, and saving and printing results are described in Chapter 3, “General Procedures”.

Protein Toolbox Lists

There are a few points to note about protein Toolbox Lists:

- each table column is in units specific to the analysis
- column abbreviations are explained at the top of the list
- the table may print incorrectly with large numbers of profiles. This will depend on your printer page width settings.
Performing protein sequence analyses

Protein Toolbox Plots

There are a number of points to note about protein Toolbox Plots:

- when using the zoom facility (see “To magnify an area of the graphic” on page 32), scales are dynamically recalculated to maximize the range
- the secondary structure plots have two parts: running average plots for the helix, coil and turn structures; and structure prediction bars across the top of the plot
- when both Robson-Garnier and Chou-Fasman plots are calculated, a consensus plot is also generated. The running average values are the product of the normalized values from each individual plot,
and structure prediction bars are only drawn where both methods agree.

To navigate in the Protein Toolbox Plot window
1. To magnify a selected region, click and drag in the window
2. To return to the whole plot, double-click anywhere in the window
3. To zoom in, press the Up-arrow key (or Command and + keys) to zoom by a factor of 2, or press Shift+Up-arrow to zoom to individual residues
4. To zoom out, press the Down-arrow key (or Command and - keys) to zoom by a factor of 2, or press Shift+Down-arrow to zoom all the way out
5. To shift to the right, press the Right-arrow key to shift 10% to the right, or press Shift+Right-arrow to shift 80% to the right
6. To shift to the left, press the Left-arrow key to shift 10% to the left, or press Shift+Left-arrow to shift 80% to the left.

Nucleic acid analyses are accessible by choosing Analyze | Base Composition, Analyze | ORF Analysis or Analyze | Nucleic Acid Analysis Toolbox. These items are enabled when a nucleic acid sequence file is the active window.

Nucleic acid analyses are accessible by choosing Analyze | Base Composition, Analyze | ORF Analysis or Analyze | Nucleic Acid Analysis Toolbox. These items are enabled when a nucleic acid sequence file is the active window.
Performing nucleic acid sequence analyses

Nucleic acid analyses are accessible by choosing Analyze | Base Composition, Analyze | ORF Analysis or Analyze | Nucleic Acid Analysis Toolbox. These items are enabled when a nucleic acid sequence file is the active window.

Performing base composition analysis

When a DNA sequence is the active window, one or more profiles can be calculated for the sequence’s base composition.

To perform a base composition analysis

1. Choose Analyze | Base Composition from the menu. The Base Composition Analysis dialog box is displayed.
2. Select one or more check boxes in the List panel to generate a text listing of the required mono-, di- and trinucleotide frequencies and temperature information.
3. Select the Base composition check box to generate percentage composition plots.
4. Specify the required composition plots by selecting Options in the Plot panel. See “Specifying base composition plots” on page 168 for details of this procedure.
5. Select the **temperature profile** check box to generate a running average of the sequence Tm or Td.

6. Specify temperature profile parameters in the **Temperature Parameters** panel as follows:
   - choose Tm or Td from the **Calculate** drop-down menu
   - choose the duplex type from the adjacent drop-down menu
   - enter a value in the **nucleic acid conc.** text box
   - enter a value in the **cation conc.** text box.

7. Type a value in the **window size** text box for the number of residues over which properties are calculated.

8. Select the required check boxes in the **Histograms** panel to generate histograms for di- and trinucleotide repeats along the sequence.

9. Specify the required histograms by selecting **Options** to the right of each check box. See "Specifying nucleotide frequency histograms", p 8-15, for details of this procedure.

10. If required, you can restrict the analysis to a portion of the sequence, by typing in the sequence numbers that bracket the region in the **Region** text boxes. Alternatively, select a region from the feature selector drop-down menu to the right of the text boxes.

11. Select **OK** to perform the analysis. Alternatively, select **Defaults** to restore the default settings, or **Cancel** to close the dialog box without performing the analysis.

### Specifying base composition plots

When base composition plots are generated, MacVector can generate up to six separate graphs, each with a maximum of four plots.

![Base Composition Plotting Options](image)

**To specify base composition plots**
Performing nucleic acid sequence analyses

1. Choose **Analyze | Base Composition** from the menu.
   The **Base Composition Analysis** dialog box is displayed.

2. Select **Options** in the **Plot** panel.
   The **Base Composition Plotting Options** dialog box is displayed.

3. Enter the required composition plot for the first graph, either by typing one or more base characters in the **Panel 1 / Plot 1** text box, or by selecting the arrow button to the right of the text box and choosing from the drop-down menu.

   When a valid entry is made, the following changes occur:
   - a second text box appears, under the **Plot 2** position
   - a new text box appears in the **Panel 2** row, at the **Plot 1** position.

   The **Panel** rows refer to separate graphs, the **Plot** columns to plots on each graph.

4. Enter additional composition plots as required.
   Each time an extra plot is specified for a given panel, a new text box appears under the next plot column, up to the maximum 4 plots. When the first plot for a panel is specified, a new row appears, up to the maximum 6 panels. The following picture shows a typical specification of the composition plots.

5. To change the color of each plot, do one of the following:
   - Click on the arrow button to the right of the color swatch, and choose from the displayed color palette
Calculating Sequence Properties

- Click on the color swatch itself, and use the system Color Picker; this is useful for fine control.

6. Select OK to dismiss the **Base Composition Plotting Options** dialog box.

7. Select OK to generate the plots.

### Specifying nucleotide frequency histograms

The frequency of occurrence of di- and trinucleotides is visualized in MacVector using a histogram plot, where each histogram bar represents the number of occurrences of the selected set of nucleotides, within a region of the sequence whose length is determined by the number of bars you choose to plot on the histogram.
Performing nucleic acid sequence analyses

To specify nucleotide frequency histograms

1. Choose Analyze | Base Composition.

The Base Composition Analysis dialog box is displayed.

2. Select Options in the Histograms panel next to the dinucleotide or trinucleotide check box.

Depending on which Options was selected, either the Dinucleotide or Tri-nucleotide Histogram Options dialog box is displayed.

3. Enter either the 2 or 3 letters that represent the di- or trinucleotide sequence in the Panel text boxes.

Up to 6 histograms can be generated for dinucleotides and trinucleotides. As each histogram is specified, a new panel row appears, up to the maximum allowed.

4. Enter the number of bars required for each histogram in the Plot text box.

The sequence being analyzed is split into equal parts, and the number of occurrences of the nucleotide sequence in each part is represented by a bar on the histogram.

5. If you need to change the bar color, do one of the following:
   - Click on the arrow button to the right of the color swatch, and choose from the displayed color palette
   - Click on the color swatch itself, and use the system Color Picker; this is useful for fine control.

6. Select OK to dismiss the Histogram Options dialog box.

7. Select OK to generate the histograms.
Searching nucleic acids for coding regions

**ORF analysis**

Open reading frame (ORF) analysis enables you to find:

- open reading frames using user-designated start and stop codons
- regions of a nucleic acid that are likely to code for protein according to Fickett’s TESTCODE algorithm.

To run the analysis, a nucleic acid sequence window must be the active window. If you want to define your own start and stop codons, you must edit the genetic code that you are using. See Chapter 11, “Using Transcription and Translation Functions”, for further details of this procedure.

**Finding open reading frames and coding regions**

The ORF analysis and Fickett’s coding region algorithm are initiated on the same dialog box, and can be performed as a single analysis. Refer to Chapter 17, “Understanding Protein and DNA Analysis”, for further details of Fickett’s algorithm.

To find an open reading frame

1. Choose **Analyze | Open Reading Frames** from the menu.
   The **Open Reading Frame Analysis** dialog box is displayed.
2. Select the **start/stop codons** check box.
Performing nucleic acid sequence analyses

Tip. You may want to define your own start and stop codons. Refer to Chapter 11, “Using Transcription and Translation Functions”, for details of this.

3. Specify the minimum amino acid length that you want to consider to be a valid open reading frame in the **min. # of amino acids** text box.

Tip. The majority of proteins are larger than 75 amino acids. However, eukaryotic exons can be as small as 20 to 25 amino acids.

4. Select check boxes for treating the sequence ends as start and stop codons. The options are:
   - **5' ends are starts**
   - **3' ends are stops**
   - **Codons after stops are starts**

Normally all three boxes should be checked, because MacVector defines an open reading frame as a region flanked by a start codon and a stop codon. If your sequence fragment lies in the middle of a coding region and contains no start or stop codons, for example, MacVector will not report any open reading frames unless you have checked both **5' ends are starts codon** and **3' ends are stops**. The **Codons after stops are starts** option is recommended for eukaryote sequences where introns are common. Where there are internal coding exons, it finds that the longest possible ORFs, ensuring that the entire exon is captured.

5. Select the genetic code that contains the start and stop codons you want to use from the **genetic code** drop-down menu.

6. You can restrict the search to a certain region of the sequence by typing in the sequence numbers that bracket the region in the **Region** panel, or by selecting a region from the features table drop-down menu at the right of the text boxes.

7. You can restrict the analysis to one or both strands, by selecting the appropriate item from the **strand** drop-down menu.

8. Select **OK** to perform the analysis.

Tip. You can perform a coding region analysis at the same time (see “To find a coding region using Fickett’s method” on page 174). The results will include only those ORFs that are found by both methods.
After the analysis is complete, the **Open Reading Frame Analysis Display Options** dialog box is displayed. Its use is described in “Displaying ORF and coding region search results” on page 175.

**To find a coding region using Fickett’s method**

1. Choose **Analyze | Open Reading Frames** from the menu. The **Open Reading Frame Analysis** dialog box is displayed.
2. Select the **Fickett’s method** check box.
3. Type in the **min. DNA length**. This should be at least 200 for best results.
4. Select a value for the coding probability from the **min. coding probability** drop-down menu. Fickett’s method assigns any region that has a coding probability below 0.29 as “non-coding” and any region with a coding probability greater than 0.92 as “coding.” Regions with intermediate probabilities are assigned “no opinion.” You can include these borderline regions with the “coding” regions by setting the probability of coding at successively lower numbers.

**Note.** Fickett’s method is independent of start and stop codons and it cannot distinguish between frames. It simply reports regions where the cutoff value is exceeded.

5. You can restrict the search to a certain region of the sequence by typing in the sequence numbers that bracket the region in the **Region**
Performing nucleic acid sequence analyses

panel, or by selecting a region from the features selector drop-down menu to the right of the text boxes.

6. You can restrict the analysis to one or both strands, by selecting the appropriate item from the **strand** drop-down menu.

7. Select **OK** to perform the analysis.

**Tip.** You can perform an open reading frame analysis at the same time (see “To find an open reading frame” on page 172). The results will include only those ORFs that are found by both methods.

After the analysis is complete, the **Open Reading Frame Analysis Display Options** dialog box is displayed. The use of this dialog box is described in the next section.

**Displaying ORF and coding region search results**

The results of a reading frame analysis or coding region search can be displayed in several ways:

- as a text listing
- as a linear map
- as an annotated sequence.

If you have performed both types of analysis at the same time, the combined results are displayed.

Each display type can be saved to disk or printed when the corresponding view is active. Refer to Chapter 3, “General Procedures”, for further details.

![Open Reading Frame Analysis](image)

**To display ORF and coding region search results**

1. The **Open Reading Frame Analysis Display Options** dialog box is displayed on completion of each analysis. To display this dialog box at other times, choose **Analyze | Open Reading Frames** when any open reading frame display result window is active.
2. Select List ORFs by to display a text window that contains a list of the open reading frames / coding regions found. The associated drop-down menu enables you to specify whether the list should be ordered according to position in the sequence or according to the length of the open reading frames / coding regions.

3. Select ORF map to display a map window that shows a linear map of the positions of the ORFs / coding regions.

4. Select ORF-annotated sequence to display an annotated sequence window that shows the nucleic acid sequence and the translated sequences of any ORFs or coding regions found by the analysis.

5. Select OK to display the requested results.

6. If you select a single ORF in the ORF map the sequence will be highlighted in the sequence window. This allows you to very easily create a feature for an ORF.

Performing Nucleic Acid Analysis Toolbox analyses

The coding preference plots in the Nucleic Acid Analysis Toolbox enable you to locate regions in a sequence that have a high probability of coding for a highly expressed protein. Where such regions are found, the plots also help you to determine which reading frame is the coding frame.

MacVector offers a range of algorithms for calculating coding preferences and locating protein-coding regions in a sequence:

- Open reading frames
- G+C % composition
- Fickett’s TestCode
- Uneven positional base frequencies
- Positional base preference
- Gribskov codon preference
- Staden codon preference
- MacVector codon preference

Refer to “Base composition methods” on page 389 and “Codon preference and codon bias tables” on page 393, for details of the calculations. Most algorithms generate three plots, one for each possible starting codon position. These can be displayed separately, or combined in a single panel to accentuate the differences between plots. You can display
Performing nucleic acid sequence analyses

the results of several different analyses in aligned panels, and control the colors of the different plots.

To run this analysis, a nucleic acid sequence window must be the active window, and a codon bias file for the organism under investigation must be available. Several codon bias files are provided with MacVector.

Generating plots with the Nucleic Acid Analysis Toolbox

To generate nucleic acid analysis plots

1. Choose Analyze | Nucleic Acid Analysis Toolbox from the menu

   The Nucleic Acid Toolbox dialog box is displayed.

2. Scroll through the Algorithm list and select the checkbox for an algorithm you want to use.

   When an algorithm is highlighted, a brief description is displayed below the Algorithm list, and the options for it are displayed in the Parameters panel.

   Tip. You can browse through descriptions and parameters for the algorithms without selecting their check boxes. Simply click on a name in the algorithm list to display this information.

3. In the Parameters panel, adjust the settings as required.

   Window size is set independently for each algorithm. Increasing the window size gives smoother, clearer graphs, but reduces the definition
of start and stop locations and can obscure short coding regions. For more details, refer to the individual algorithm entries in “Coding regions” on page 388.

4. Repeat steps 2 and 3 to add more algorithms, as required.

To remove an algorithm from analysis, reselect its check box. To select or deselect all the algorithms, hold down the option key while clicking on any checkbox.

5. To choose the correct codon bias table, select the Codon Bias File: button in the Options panel. A file selection dialog box is displayed; select the required file, normally from the Codon Bias Tables folder.

6. To select the genetic code, choose from the genetic code drop-down menu in the Options panel.

Tip. To modify which codons are used as starts and stops, you can create your own genetic code. See “Modifying genetic codes” on page 240.

7. From the drop-down menu in the Plotting options panel, choose either:
   - Combined plots - to combine the plots into a single panel
   - Plot separately - to plot each frame in a separate panel

8. To adjust the display of codons in the plots, select as required the checkboxes for:
   - Show start codons
   - Show stop codons
   - Show rare codons

To specify where the codons should appear in the display, choose Above, Center or Below from the Location drop-down menu. They will only be displayed where separate plots have been selected and where frame-specific data has been generated.

9. To restrict the search to a certain region of the sequence, type in the sequence numbers that bracket the region in the Region panel.

Alternatively, choose a region from the features table drop-down menu at the right of the text boxes.

10. Select Options if you want to alter the plot colors, codon symbols, or the definition of rare codons. See “To set the nucleic acid analysis toolbox plot options” on page 179.

11. Select OK to generate the plot.
Performing nucleic acid sequence analyses

Alternatively, select the **Defaults** button to restore the default settings, or select **Cancel** to close the dialog box without performing any analyses.

Plot colors, codon symbols, and the definition of rare codons are specified using a separate dialog box, **Nucleic Acid Toolbox Plotting Options**.

**To set the nucleic acid analysis toolbox plot options**

1. In the **Nucleic Acid Toolbox** dialog box, select the **Options** button. The **Nucleic Acid Toolbox Plotting Options** dialog box is displayed.

   ![Nucleic Acid Toolbox Plotting Options](image)

2. In the **Plot Colors** panel, set colors as required. You can do this either by selecting an arrow button and choosing from the drop-down color palette, or by clicking on the color swatch and using the system Color Picker.
   - **Single** sets the color for all separate plots
   - **Frame +1 / -1** sets the color for the first frame in a combined plot, **Frame +2 / -2** the second, and **Frame +3 / -3** the third.

3. In the **Codon Appearance** panel, set the symbols and colors for **Start**, **Stop** and **Rare** codons, using the appropriate drop-down menus and arrow buttons.

4. In the **Define rare codons as less than or equal to** panel, select a radio button to choose the way rare codons will be defined:
   - **% of all codons**
   - **% of synonymous codons** - i.e. all codons that generate the same amino acid as that codon.

   Then type the required threshold value in the selected text box. (The default setting is **5.0% of synonymous codons**. If **% of all codons** is selected, the default value is **0.1%**.)

5. Select **OK** to apply the settings.
Alternatively, select the **Defaults** button to restore the default settings, or **Cancel** to close the dialog box without saving the settings.

**Viewing Nucleic Acid Analysis Toolbox results**

The results window is a scrollable window containing the plots from the selected analyses in aligned panels. In combined-plots mode, the results of each analysis are displayed in a single panel. In separate-plots mode, results for each reading frame is displayed on a separate graph, so there will be either three or six panels, depending on whether you specified one or both strands.

The open reading frames plot always combines the three frames in a single color-coded panel, showing markers for starts, stops and rare codons, and bars representing open reading frames that exceed the minimum length. When you select separate-plots mode, the starts, stops and rare codons are also displayed in the frame results panels of your other analyses.
Prominent peaks in the graph indicate regions that exhibit a codon preference. Such regions are very likely to code for highly expressed proteins. For more details, see “Interpreting coding preference plots” on page 396.

To inspect potential coding regions, you will usually need to select a region of the plot and display it at greater magnification. When you select a region, all the results plots are re-scaled, keeping them aligned.

**To navigate in the Nucleic Acid Analysis Toolbox results window**

- **To magnify a selected region**, click and drag in the window
- **To return to the whole plot**, double-click anywhere in the window
- **To zoom in**, press the Up-arrow key (or Command and + keys) to zoom by a factor of 2, or press Shift+Up-arrow to zoom to individual residues
- **To zoom out**, press the Down-arrow key (or Command and - keys) to zoom by a factor of 2, or press Shift+Down-arrow to zoom all the way out
- **To shift to the right**, press the Right-arrow key to shift 10% to the right, or press Shift+Right-arrow to shift 80% to the right
Calculating Sequence Properties

- **To shift to the left,** press the **Left-arrow** key to shift 10% to the left, or press **Shift+Left-arrow** to shift 80% to the left.

**Tip.** You can check for keyboard shortcuts at any time during a session. Choose **Help | MacVector Keyboard Shortcuts** to display a listing of all MacVector shortcuts.

**Selecting ORFs from Nucleic Acid Analysis Toolbox plots**
If you click any open reading frame in the coding preference plot results window, the corresponding residues are selected in the sequence editor window.

**Tip.** Click on an ORF, go to the sequence editor window, choose **Analyze | Translation**, and you can quickly create a protein sequence from the selected region.

If you want more control in the selection of the ORF, click on any line of stop and start codons, instead of on the ORF bar. If there are several possible start codons, you do not have to choose the longest ORF - click just to the right of whichever start codon you want to use. The selected ORF is outlined, and its residues highlighted in the sequence editor window.

The following figure shows two possible selections for an ORF:

**Note.** If you choose separate-plots mode, you can select ORFs from any graph where codons are displayed.
This chapter provides guidelines on working with the sequence analysis methods in MacVector, including:

- automatic and manual restriction site searching
- proteolytic site searching
- nucleic acid motif searching
- protein motif searching.

Refer to “Searching nucleic acids for coding regions” on page 172, for information about searching nucleic acid sequences for coding regions.

Restriction enzyme sites

You can use restriction enzyme files to:

- locate restriction enzyme cut sites in a nucleic acid sequence
- predict the fragments that would result from single or multiple digests using up to six enzymes.

To use this functionality, a nucleic acid sequence window must be the active window, and a restriction enzyme file must be available.

Searching for restriction enzyme sites

Two types of restriction enzyme site searching are available: automatic and manual.

With both types of search you can, if required, select a subset of the available enzymes from the file you are using. Before you perform the analysis, open the restriction enzyme file and click in front of the name of any enzyme you want to select. A check mark appears to mark your choice. If you save the restriction enzyme file, the selections are saved with the file. See “Selecting enzymes for site analysis” on page 69.

Automatic searching

Sequences in the active Sequence window can now be scanned automatically for restriction enzymes, with the cut sites found displayed in the Sequence Map view. The settings for this feature are specified in the Map View preferences dialog box.
Restriction enzyme sites

To set up automatic restriction enzyme site searching

1. Select MacVector | Preferences from the main menu, then click the Map View icon on the preferences dialog, or click the Prefs icon on the Sequence window toolbar when a Map view is selected.

The Map View preferences dialog box is displayed.

Note. You can also access the Map View preferences dialog using Options | Map View Options from the menu.

2. Ensure that the Automatic RE Analysis box is checked.

By default, MacVector automatically analyses nucleic acid sequences for restriction enzyme sites using the specified enzyme file and displays the cut sites in the Sequence Map view. To switch off this feature, uncheck the Automatic RE Analysis box.

3. Click the Set Enzyme File button to choose an alternative restriction enzyme file for the automatic analysis.

By default, the file /MacVector 10.5/Restriction Enzymes/Common Enzymes is used.

4. Optionally, click the Open button to open the selected enzyme file in the background.

This shortcut enables you to access the enzyme file and modify the enzymes selected within it more quickly.

5. Select one of the following options

   • Choose Use all enzymes to perform the automatic search using all of the enzymes in the specified enzyme file.

   • Choose Only use selected enzymes to perform the automatic search using only those enzymes that are selected in the specified enzyme file.

6. Use the Do not scan if sequence is over setting to turn off automatic searching for larger sequences.

   The restriction enzyme search is relatively fast but the graphical display of the results can take a long time for very large sequences. The default value is 50kb.

7. Use the Maximum number of cut sites setting to screen out restriction enzymes that cut your sequence too frequently.

   This option can reduce search times and screen clutter. The default is six sites, but you might want to reduce this if you routinely use relatively large sequences.
Searching for Sites and Motifs

8. Click OK to save your settings and close the Map View dialog box or Cancel to close the dialog box without saving your changes.

When automatic restriction enzyme analysis is switched on and the active Sequence window contains and nucleic acid sequence, then the sequence is searched for restriction enzyme cut sites automatically and any that are located are displayed in the Sequence Map view.

Moreover, if the Only use selected enzymes option was specified, all open Sequence Map views are updated dynamically when changes are made to the enzyme selection in the specified enzyme file. This makes it easy to identify compatible restriction sites between two sequences (e.g. a gene and a vector), for click cloning (see “Click cloning” on page 191).

Manual searching

To search for restriction enzyme sites manually

1. Make the required nucleic acid Sequence window active.
2. Specify whether to analyze the sequence as a circular or linear molecule by clicking the Topology icon in the Sequence window toolbar until it is set correctly for your sequence.
3. Choose Analyze | Restriction Enzyme from the main menu.

The Restriction Enzyme Analysis dialog box is displayed.
4. Click the Enzyme File button to choose the restriction enzyme file that will be used for the analysis.

A file selection dialog box is displayed, enabling you to choose the file.
5. Choose the required file from the scrolling list and select Choose.
Restriction enzyme sites

6. You can restrict the search to a certain region of the sequence by typing in the base numbers that bracket the region in the Region panel, or by selecting a region from the feature selector drop-down menu to the right of the text boxes.

7. Select your search criteria by choosing from the Search Using drop-down menu as follows:
   - choose all enzymes to use all the enzymes in the selected enzyme file
   - choose selected enzymes to use only the enzymes that you have selected within the enzyme file
   - choose other criteria to display further options

Note. When the other criteria search is performed, all enzymes in the file are used, not only the selected ones.

8. If you have chosen other criteria, do one or more of the following:
   - to restrict the search to enzymes whose recognition sites are a certain size, type the size limits into the site size text boxes. MacVector accepts values from 1 to 100. Leave both boxes empty if you do not want to restrict the search by site size.
   - to limit the search to enzymes that leave 5' ends, 3' ends, or blunt ends when they cut the sequence, select the appropriate option from the end structure drop-down menu.
   - to restrict the search to enzymes that cut the sequence a limited number of times, type the upper limit in the number of cuts text box. MacVector accepts values from 1 to 20. Leave the text box empty if you do not want to restrict the search by number of cuts.

9. Select OK to perform the analysis.
When the analysis is completed, the **Restriction Enzyme Analysis Display** dialog box is displayed. The use of this dialog box is described in the following section.

**Filtering manual restriction site search results**

Manual restriction site search results can be filtered as follows:

- by the number of cuts an enzyme makes
- by the end structure of cut fragments
- by restriction site size
- by the residue range in which the cut site occurs.

To filter restriction site search results

1. The **Restriction Enzyme Analysis Display** dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example, to change the display parameters, choose **Analyze** | **Restriction Enzyme** when any restriction analysis display result window is active.

2. To restrict the displays to enzymes that cut the sequence a limited number of times, select the **# of cuts** checkbox and type a number from 1 to 20 to set the upper limit for number of cuts.

3. To restrict the displays to enzymes that leave 5' ends, 3' ends, or blunt ends when they cut the sequence, select the appropriate option from the **end structure** drop-down menu.
Restriction enzyme sites

If you restricted the search stage to one end type, this drop-down menu will be dimmed.

4. To restrict the search to enzymes whose recognition sites are a certain size, select the site size checkbox and type the size limits into the min and max text boxes. MacVector accepts values from 1 to 100.

5. To restrict the displays to enzymes that do not cut within a given region, select the no cuts checkbox, then either type in the numbers of the bases that bracket the region, or choose a region from the features table drop-down menu at the right of the text boxes.

6. To restrict the displays to enzymes that cut only within a given region, select the cuts only checkbox, then either type in the numbers of the bases that bracket the region, or choose a region from the features table drop-down menu at the right of the text boxes.

7. To restrict the displays to enzymes that cut only within a given reading frame, select the cuts only checkbox, then choose a frame number from the phase drop-down menu.

Tip. If you want to specify a reading frame but not limit the displays to a region, select ALL 1-... from the features table drop-down menu.

8. Select the Display Options as required.

These options are described in the next section.

9. Select OK to display the results.

Displaying manual restriction site search results

You can display manual restriction site search results in a number of ways:

- as a list of enzymes that cut the sequence
- as a list of enzymes that did not cut the sequence
- as a restriction map
- as an annotated sequence
- as a list of the predicted fragments for each digest.

One or more of the displays can be generated at once.
Each display type can be saved to disk or printed when its window is active. Refer to “Saving graphics” on page 33, for further details.

To display restriction site search results

1. The Restriction Enzyme Analysis Display dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example, to change the display parameters, choose Analyze | Restriction Enzyme when any restriction analysis display result window is active.

2. Select the filter options as required. These are described in the previous section.

3. Select the List cutters by check box to display a list of the enzymes that cut the sequence and the locations of the cuts. Use the drop-down menu to choose how to order the list: by name, by position of cuts, or by number of cuts.

4. Select the List non-cutters check box to display a list of the enzymes that were considered in the search but were found not to cut the sequence.

5. Select the Restriction map check box to display the locations of the cut sites in a graphical form. The appearance of this display can be changed interactively using the Graphics Palette. Refer to “Editing the general map appearance” on page 56, for further details.

6. Select the Annotated sequence check box to display an annotated sequence display with the cut sites displayed at their proper locations along the sequence. The appearance of this display can be changed interactively using the Text Display preferences dialog box. Refer to “Formatting the annotated sequence display” on page 35, for further details.
7. Select the **Frag predictions** check box to display fragment predictions for the digests. The enzymes used are those that have satisfied the **Filter Options**.

Use the drop-down menu to choose from the following:
- **single** for single digests
- **double** for double digests
- **both** for single and double digests
- **all combined** for digests using up to six selected enzymes.

8. Select **OK** to display the results.

### Click cloning

Click cloning can be used to duplicate or design cloning experiments in the lab. You can construct new DNA molecules from existing clones and vectors by selecting restriction enzyme sites, copying the intervening fragment and then pasting it into a target molecule.

Click cloning can be performed using either manual or automatic restriction enzyme searches, along with standard **Edit | Copy** operations to digest fragments and **Edit | Paste** operations to clone or ligate the fragments into a vector.

MacVector recognizes sticky or blunt ends and only allows the cloning of fragments if these are compatible, preserving sites and feature information, where needed.

**To perform click cloning with automatic restriction enzyme search**

1. Open the sequence files containing the gene or fragment you want to clone and the vector sequence you want to clone into.

2. Ensure that **Automatic RE Analysis** is checked on and the appropriate enzymes are selected in the specified enzyme file. See “**To set up automatic restriction enzyme site searching**” on page 185.

3. Check that there are suitable restriction sites surrounding the gene or fragment you want to clone.

   If the enzyme file being used for the automatic restriction enzyme search is open, then you can select different enzymes in the file to see if they are present in both sequences.

4. Select suitable flanking sites around the gene or fragment, by clicking on one, then holding down `<shift>` and selecting the other.

5. Select **Edit | Copy** from the menu.
6. Make the vector sequence window active and select the enzyme sites you want to paste the fragment into.

7. Select Edit | Paste from the menu.

As long as the sites are compatible, the fragment will be cloned into the specified sites. The fragment will be flipped automatically if required. If the sites are not compatible, then MacVector will notify you but give you the option of continuing with the cloning operation.

Instead of using the Automatic RE Analysis tool, you may wish to use Filtering restriction site results instead (see “To filter restriction site search results” on page 188). This, more powerful search tool, allows you to chose only enzymes that cut in a particular site (i.e. the MCS) or only enzymes that do not cut in the gene you want to clone.

Proteolytic enzyme sites

You can use proteolytic enzyme files to:

- locate cleavage sites for proteolytic agents in a protein sequence
- predict the fragments that would result from single or multiple digests using up to six proteolytic agents.

To use this functionality, a protein sequence window must be the active window, and a proteolytic enzyme file must be available.

Although we use the term proteolytic enzyme throughout this section of the manual, the analysis can locate cleavage sites for any proteolytic agents—whether enzymes or chemical agents—that cleave a protein in a sequence-specific manner.

Searching for proteolytic enzyme sites

If required, you can select a subset of the available enzymes from the file you are using. Before you perform the analysis, open the proteolytic enzyme file and click in front of the name of any enzyme you wish to select. A check mark appears to mark your choice. Save the proteolytic
Proteolytic enzyme sites

enzyme file to save the selections. See “Selecting enzymes for site analysis” on page 69, for further details.

To search for proteolytic enzyme sites
1. Make the required amino acid sequence window active.
2. Choose Analyze | Proteolytic Enzyme.
   The Proteolytic Enzyme Analysis dialog box is displayed.
3. Select Enzyme File to choose the restriction enzyme file that will be used for the analysis.
   A file selection dialog box is displayed, enabling you to choose the file.
4. Choose the required file from the scrolling list and select Choose.
5. You can restrict the search to a certain region of the sequence by typing in the sequence numbers that bracket the region in the Region panel, or by selecting a region from the feature selector drop-down menu to the right of the text boxes.
6. Select your search criteria by choosing from the Search Using drop-down menu as follows:
   • choose all enzymes to use all the enzymes in the selected enzyme file
   • choose selected enzymes to use only the enzymes that you have selected within the enzyme file.
7. Select OK to perform the analysis.
   When the analysis is completed, the Proteolytic Enzyme Analysis Display dialog box is displayed. The use of this dialog box is described in the following section.
Filtering proteolytic site search results

The proteolytic site search results can be filtered as follows:

- by the number of cuts an enzyme makes
- by cut site size
- by the residue range in which the cut site occurs.

To filter proteolytic site search results

1. The **Proteolytic Enzyme Analysis Display** dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example, to change the display parameters, choose **Analyze** | **Proteolytic Enzyme** when any proteolytic analysis display result window is active.

2. To restrict the displays to enzymes that cut the sequence a limited number of times, select the **# of cuts** checkbox and type a number from 1 to 20 to set the upper limit for number of cuts.

3. To restrict the search to enzymes whose recognition sites are a certain size, select the **site size** checkbox and type the size limits into the **min** and **max** text boxes. MacVector accepts values from 1 to 100.

4. To restrict the displays to enzymes that do not cut within a given region, select the **no cuts** checkbox, then either type in the numbers of the bases that bracket the region, or choose a region from the features table drop-down menu at the right of the text boxes.

5. To restrict the displays to enzymes that cut only within a given region, select the **cuts only** checkbox, then either type in the numbers...
of the bases that bracket the region, or choose a region from the features table drop-down menu at the right of the text boxes.

6. Select the **Display Options** as required.

These options are described in the next section.

7. Select **OK** to display the results.

**Displaying proteolytic site search results**

You can display the proteolytic enzyme site search results in a number of ways:

- as a list of enzymes that cut the sequence
- as a list of enzymes that did not cut the sequence
- as a proteolytic map
- as an annotated sequence
- as a list of the predicted fragments for each digest.

One or more of the displays can be generated at once.

Each display type can be saved to disk or printed when its window is active. Refer to Chapter 3, “General Procedures”, for further details.

**To display proteolytic site search results**

1. The **Proteolytic Enzyme Analysis Display** dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example, to change the display parameters, choose **Analyze** | **Proteolytic Enzyme** when any restriction analysis display result window is active.

2. Select the **List cutters by** check box to display a list of the enzymes that cut the sequence and the locations of the cuts.
Use the drop-down menu to choose how to order the list: by name, by position of cuts, or by number of cuts.

3. Select the **List non-cutters** check box to display a list of the enzymes that were considered in the search but were found not to cut the sequence.

4. Select the **Proteolytic map** check box to display the locations of the cut sites in a graphical form. The appearance of this display can be changed interactively using the Graphics Palette. Refer to “*Editing the general map appearance*” on page 56, for further details.

5. Select the **Annotated sequence** check box to display an annotated sequence display with the cut sites displayed at their proper locations along the sequence. The appearance of this display can be changed interactively using the **Text Display** preferences dialog box. Refer to “*Formatting the annotated sequence display*” on page 35, for further details.

6. Select the **Frag predictions** check box to display fragment predictions for the digests. The enzymes used are those that have satisfied the **Filter Options**.

Use the drop-down menu to choose from the following:

- **single** for single digests
- **double** for double digests
- **both** for single and double digests
- **all combined** for digests using up to six selected enzymes.

7. Select **OK** to display the results.

**Subsequence analysis**

You can scan a nucleic acid or protein sequence for known consensus sequences and motifs that are defined in a subsequence file. A subsequence may consist of one, two or three parts, with the permitted gap between each part defined, as well as the allowed mismatch for each part in a search. Refer to “*Subsequence files*” on page 73, for further information.

To use this functionality, a Sequence window must be the active window, and a subsequence file must be available.
Subsequence analysis

Searching for motifs

If required, you can select a subset of subsequences from the list of all subsequences in the file. Before the motif search, open the subsequence file and click in front of the name of any subsequence you want to select. A check mark appears to mark your choice. Save the subsequence file to save the selections. See “Selecting subsequences for searches” on page 74, for further details.

To search for motifs

1. Make the required Sequence window active.
2. If you are analyzing a nucleic acid sequence, specify whether to analyze the sequence as a circular or linear molecule by clicking the Topology icon in the Sequence window toolbar until it is set correctly for your sequence.
3. Do one of the following:
   - choose Analyze | Nucleic Acid Subsequence for a nucleic acid sequence
   - choose Analyze | Protein Subsequence for a protein sequence.

The appropriate Analysis dialog box is displayed.
4. Select the Subsequence File button to choose the subsequence file that will be used for the analysis.

A file selection dialog box is displayed, enabling you to choose the file.
5. Choose the required file from the scrolling list and select Choose.
6. You can restrict the search to a certain region of the sequence by typing in the base numbers that bracket the region in the Region panel,
or by selecting a region from the feature selector drop-down menu at
the right of the text boxes.

7. Select your search criteria by choosing from the **Search Using**
drop-down menu as follows:
   - choose **all subsequences** to use all the subsequences in the selected
     subsequence file
   - choose **selected subsequences** to use only the subsequences that
     you have selected in the subsequence file.

8. Select **OK** to perform the analysis.

When the analysis is completed, the appropriate Display dialog box is
displayed. The use of this dialog box is described in the following sec-
tion.

**Filtering subsequence search results**

The subsequence search results can be filtered as follows:
   - by the frequency of the motif occurrence
   - by the residue range in which the motif occurs.

![Nucleic Acid Subsequence Analysis Display](image)

**To filter subsequence search results**

1. The Display dialog box is displayed on completion of each analysis.
   To display this dialog box at other times, for example, to change the
display parameters, do one of the following:
Subsequence analysis

- choose Analyze | Nucleic Acid Subsequence when any nucleic acid subsequence display result window is active
- choose Analyze | Protein Subsequence when any protein subsequence display result window is active.

2. To restrict the displays to subsequences that occur a limited number of times, select the # of sites checkbox and type a number from 1 to 20 to set the maximum number of occurrences.

3. To restrict the displays to subsequences that do not occur within a given region, select the no sites checkbox, then either type in the numbers of the bases that bracket the region, or choose a region from the features table drop-down menu at the right of the text boxes.

4. To restrict the displays to subsequences that occur only within a given region, select the sites only checkbox, then either type in the numbers of the bases that bracket the region, or choose a region from the features table drop-down menu at the right of the text boxes.

5. For nucleic acids, to restrict displays to subsequences that cut only within a given reading frame, select the sites only checkbox, then choose the frame from the phase drop-down menu.

Tip. If you want to specify a reading frame but not limit the displays to a region, select ALL 1- ... from the features table drop-down menu.

6. Select the Display Options as required.

These options are described in the next section.

7. Select OK to display the results.

Displaying subsequence search results

You can display the nucleic acid subsequence search results in a number of ways:

- as a list of the subsequences found
- as a list of the subsequences not found
- as a subsequence map
- as an annotated sequence
- as a list of the sequence sizes found between subsequence occurrences.
One or more of the displays can be generated at the same time. Each display type can be saved to disk or printed when its window is active. Refer to Chapter 3, “General Procedures”, for further details.

To display subsequence search results

1. The Display dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example, to change the display parameters, do one of the following:
   - choose Analyze | Nucleic Acid Subsequence when any nucleic acid subsequence display result window is active
   - choose Analyze | Protein Subsequence when any protein subsequence display result window is active.
2. Select the List sites by check box to display a list of the subsequences found and the locations of the sites.
   Use the drop-down menu to choose how to order the list: by name, by position of sites, or by number of sites.
3. Select the List sites not found check box to display a list of the subsequences that were considered in the search but were not found in the sequence.
4. Select the Subsequence map check box to display the locations of the cut sites in a graphical form. The appearance of this display can be changed interactively using the Graphics Palette. Refer to “Editing the general map appearance” on page 56, for further details.
5. Select the Annotated sequence check box to display an annotated sequence display with the sites displayed at their proper locations along the sequence. The appearance of this display can be changed interactively using the Text Display preferences dialog box. Refer to
Subsequence analysis

“Formatting the annotated sequence display” on page 35, for further details.

6. Select the **Frag predictions** check box to display sequence sizes between specified motifs. The motifs used are those that have satisfied the **Filter Options**.

Use the drop-down menu to choose from the following:

- **single** for sizes between a single motif
- **double** for sizes between each pair of motifs
- **both** for sizes of both the single and double options
- **all combined** for sizes using up to six selected motifs
Overview

MacVector provides the following tools to design primers and screen for likely primers and probes:

- primer design using Primer3
- screening a nucleic acid sequence for likely PCR primer pairs
- screening a nucleic acid sequence for likely sequencing primers or hybridization probes
- screening a protein sequence for the least degenerate oligos that can serve as hybridization probes
- screening sequencing and PCR primer sequences for specific characteristics.

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Primer and Probe Design

Primer design
MacVector now includes an automated primer design module based on Primer3. The module has been designed to be easy to use - you can create a new primer in as little as three mouse clicks using the default settings - whilst retaining all of the powerful features of Primer3.

As well as designing primer pairs, this module can also be used to:
- test pairs of primers
- design matching primers for a known primer
- design hybridization primers for use in realtime PCR analyzes and similar techniques.

Designing primers using Primer3

Amplify Feature/Region
The simplest way to design primers with MacVector is to select a particular feature or region of a sequence and design primers either side of it, to amplify the selection.

To design a primer using the Amplify Feature/Region method
1. Open the sequence file you want to analyze.
2. Select a feature of the sequence in the Map tab or the Features tab. Alternatively, select a region of the sequence in the Editor tab.

By default, primers are chosen from a 200 bp long region on either side of the feature or region selected.

3. Choose Analyze | Primers | Primer Design (Primer3) from the menu.

Note. When the Primer Design (Primer3) dialog box opens it is populated with information about the feature or region you selected in the active sequence window. You can populate the dialog box with information about an alternative feature in the active sequence using the feature selector drop-down menu, or you can specify an alternative region by providing residue numbers manually.

4. Ensure that Amplify Feature/Region is selected from the design method drop-down list.
5. Click OK.

The default primer design settings will be used to design the new primer. These will produce a ranked list and graphical map of the top five primer pairs that will amplify the selected feature or region, using the optimum values for the primer Tm and %GC. However, by modify-
Primer design

When the advanced parameters (see “Advanced primer design settings” on page 210) it is possible to tune these, and other values, to obtain the best primers for non-standard situations.

Region to Scan

Alternatively, you can specify a region within which the amplified product should lie. Simply choose the size of product you want and Primer3 will design primers to amplify products of that size, from anywhere in that region.

To design a primer using the Region to Scan method

1. Open the sequence file you want to analyze.
2. Select a feature of the sequence in the Map tab or the Features tab. Alternatively, select a region of the sequence in the Editor tab.
3. Choose Analyze | Primers | Primer Design (Primer3) from the menu.

Note. When the Primer Design (Primer3) dialog box opens it is populated with information about the feature or region you selected in the active sequence window. You can populate the dialog box with information about an alternative feature in the active sequence using the feature selector drop-down menu, or you can specify an alternative region by providing residue numbers manually.

4. Select Region to Scan from the design method drop-down list.
5. Click OK.

By default this method will design primers to produce products between 100 and 300 bases in length. However, you can change the minimum and maximum Product size values to design primers to produce shorter or longer products.

Flanking Regions

Finally, you can design primers by specifying two flanking regions and selecting left and right primers from these. This is similar to the Amplify Feature/Region method but it is much more flexible, as you can specify more precisely the region that each primer is selected from.

To design a primer using the Flanking Regions method

1. Open the sequence file you want to analyze.
2. Select the feature of the sequence that defines the flanking regions you are interested in on the Map tab or the Features tab. Alternatively, select the region of the sequence that defines the flanking regions you are interested in on the Editor tab.
Primer and Probe Design

3. Choose Analyze | Primers | Primer Design (Primer3) from the menu.
4. Select Flanking Regions from the design method drop-down list.
The values for the left region and the right region are populated based on the sequence selection you made above. These initial values define flanking regions of zero length.
5. Modify the start point of the left region and the end point of the right region until they encompass the regions you want to choose primers from.
6. Click OK.

Note. There are no limits on the size of the regions that can be used, however, if they are very short then it may not be possible to find suitable primers with the default settings.

Testing primers using Primer3

You can also use the primer design module to test primer sequences. You can test existing primer pairs to ensure that they match and produce the expected product. Alternatively, you can provide one primer and use the primer design module to design a suitable matching primer for a specified region or sequence, or one that produces a product of a specified size.

To test an existing primer pair
1. Open the sequence file you want to analyze.
2. Choose Analyze | Primers | Primer Design (Primer3) from the menu.
3. Check the Use this primer option for both primers, then paste or type the existing primer sequences into the appropriate Primer Sequences edit boxes.
4. Select Region to Scan from the design method drop-down list
5. Ensure that the Region to Scan values entirely encompass the area that contains the expected product. If in doubt, select the entire sequence.
6. Set the Product size values generously, e.g. between 0.5kb below and 0.5kb above the product size you would expect.
7. Click OK.

To design a new primer to match an existing primer for a specified feature or region
1. Open the sequence file you want to analyze.
Primer design

2. Select the feature of the sequence that you want to amplify in the Map tab or the Features tab. Alternatively, select the region of the sequence that you want to amplify in the Editor tab.

3. Choose Analyze | Primers | Primer Design (Primer3) from the menu.

4. Check the Use this primer option for the existing primer, then paste or type the existing primer sequence into the corresponding Primer Sequences edit box.

5. Ensure that the Find primer option is selected for the primer you want to design.

6. Ensure that Amplify Feature/Region is selected from the design method drop-down list.

7. Click OK.

MacVector will test the existing primer, then design suitable matching primers. Alternatively, to design a matching primer to produce a product of a specified size, use the following procedure.

To design a new primer to match an existing primer for any product size

1. Open the sequence file you want to analyze.

2. Choose Analyze | Primers | Primer Design (Primer3) from the menu.

3. Check the Use this primer option for the existing primer, then paste or type the existing primer sequence into the corresponding Primer Sequences edit box.

4. Ensure that the Find primer option is selected for the primer you want to design.

5. Select Region to Scan from the design method drop-down list.

6. Click OK.

By default this method will design a matching primer to produce products between 100 and 300 bases in length. However, you can change the minimum and maximum Product size values to design primers to produce shorter or longer products.

Real-time primer design

Many real time PCR techniques use a third oligonucleotide that will anneal inside the desired product. This makes it possible to track the progress of the PCR amplification using one of the many proprietary
technologies available. The primer design module enables you to design these.
You can design an internal primer suitable for use with a pair of existing primers, design external primers to suit an existing internal primer, or design all three primers from scratch.

To design a pair of external primers and a suitable internal hybridization primer from scratch

1. Open the sequence file you want to analyze.
2. Select the feature of the sequence you want to monitor in the Map tab or the Features tab. Alternatively, select the region of the sequence you want to monitor in the Editor tab.

By default, primers are chosen from a 200 bp long region on either side of the feature or region selected.

3. Choose Analyze | Primers | Primer Design (Primer3) from the menu.

Note. When the Primer Design (Primer3) dialog box opens it is populated with information about the feature or region you selected in the active sequence window. You can populate the dialog box with information about an alternative feature in the active sequence using the feature selector drop-down menu, or you can specify an alternative region by providing residue numbers manually.

4. Ensure that Amplify Feature/Region is selected from the design method drop-down list.

Tip. Alternatively, use the Flanking Regions design method to gain more control over the regions from which the primers are chosen.
5. Ensure that the **Find primer** option is selected for both external primers.
6. Check the **Hybridizing Primer Sequence** box.
7. Ensure that the **Find Hybridizing Primer** option is selected.
8. Click **OK**.

To scan for a suitable internal hybridization primer for a pair of existing external primers

1. Open the sequence file you want to analyze.
2. Choose **Analyze | Primers | Primer Design (Primer3)** from the menu.
3. Check the **Use this primer** option for both primers, then paste or type the existing primer sequences into the appropriate **Primer Sequences** edit boxes.
4. Select **Region to Scan** from the design method drop-down list
5. Ensure that the **Region to Scan** values entirely encompass the area that contains the expected product. If in doubt, select the entire sequence.
6. Set the **Product size** values generously, e.g. between 0.5kb below and 0.5kb above the product size you would expect.
7. Check the **Hybridizing Primer Sequence** box.
8. Ensure that the **Find Hybridizing Primer** option is selected.

**Note.** You should ensure that the advanced primer design settings on the **Hybridization Primer** tab are set to values appropriate for the primer technology you are using. The documentation supplied with your primer technology should contain this information.

9. Click **OK**.

To scan for a suitable pair of external primers for an existing internal primer

1. Open the sequence file you want to analyze.
2. Select the feature of the sequence you want to monitor in the **Map** tab or the **Features** tab. Alternatively, select the region of the sequence you want to monitor in the **Editor** tab.

By default, primers are chosen from a 200 bp long region on either side of the feature or region selected.
3. Choose **Analyze | Primers | Primer Design (Primer3)** from the menu.
Primer and Probe Design

**Note.** When the **Primer Design (Primer3)** dialog box opens it is populated with information about the feature or region you selected in the active sequence window. You can populate the dialog box with information about an alternative feature in the active sequence using the feature selector drop-down menu, or you can specify an alternative region by providing residue numbers manually.

4. Ensure that **Amplify Feature/Region** is selected from the design method drop-down list.

5. Check the **Hybridizing Primer Sequence** box.

6. Check the **Test this primer** option for the hybridizing primer, then paste or type the existing internal primer sequence into the **Hybridizing Primer Sequence** edit box.

7. Click **OK**.

**Advanced primer design settings**

The default primer design parameters are set such that most primer design experiments can be run successfully without the need to make any changes. However, all of the parameters can be accessed and modified using the **Advanced options** button on the **Primer Design (Primer3)** dialog box.

The following tabs, containing groups of related primer design parameters, are displayed.

**Tip.** Users familiar with Primer3 can hover the mouse over parameters on these tabs to display tooltips with information about which underlying Primer3 parameters they correspond to. See “**Primer design**” on page 369 for further information.

**Characteristics**

On this tab you can select minimum, maximum and optimum values for **Length**, **Percent G+C**, and **Tm**. Primer3 will always try to design primers that match the optimum values specified as closely as possible.

**Note.** The optimum value for these parameters does not need to be midway between the minimum and maximum values.

The **GC Clamp** is the number of G and/or C residues you want to include at the 3’ end of each primer. **Maximum Poly-X** limits the number of consecutive residues of the same type that can appear in a primer sequence. The **Maximum difference in Tm between primers** setting enables you to ensure that primers are closely matched. By default, the allowed differ-
Primer design

ence is set to a quite high value, however, Primer3 will still usually find well-matched primers.

Primer Binding

On this tab you can set values for Primer vs Primer, 3’ end vs 3’ end and Primer vs Product which control the maximum acceptable binding between the primers and the product. The units of these parameters are defined internally by Primer3. Increase the values to make the primer design experiment less sensitive to binding. This may be necessary if you are scanning a short sequence and cannot find suitable primers. To remove Primer vs Product filtering altogether, set the value to -1.

Check the Allow ambiguous residues box if the sequence you are analyzing contains ambiguous residues. Optionally, specify the maximum number of ambiguous residues you are willing to accept in the primers using the Allowed Ns in primers edit box.

Reaction Conditions

On this tab you specify the reaction conditions you use in the lab. They will affect the reported Tm of the primer binding to the template.

Hybridization Primer

On this tab you can set alternative basic parameters for hybridization primer scans. See “Characteristics” on page 210 for further details about the parameters on this tab.

Note. These parameters are completely independent from the basic parameters used for external primers. So, if you modify the basic parameters used for external primers on the Characteristics tab, then those changes will not be reflected on the Hybridization Primer tab.

Misc.

On this tab you can control the number of primer pairs that are reported. The default is 5 but you can choose up to 100.

Note. The Graphical map output can only display the top 25 primers (see “Graphical map” on page 213).

Analyzing primer design results

Initially, the results of primer design experiments are presented in the Primer Design Display dialog box. This summarizes the number of primers found, the number that were rejected, the number of suitable pairs of
primers found and the number that were rejected. This information is divided into four sections.

- A summary of the number of individual primers that were considered and how many were accepted.
- A summary of the reasons why the rejected primers were discarded. This section is useful for tuning the analysis if the default values do not produce acceptable primers.
- A summary of the number of pairs of primers that were considered and how many were rejected,
- A summary of the reasons why any rejected primer pairs were discarded.

Note. If your primer design experiment included the design of an internal hybridization primer, then statistics for this will also be included in these summaries.

The Primer Design Display dialog also provides a choice of three display options, which enable you to view the detailed results of the primer design experiment in different ways.

To select display options
1. Check the boxes adjacent to the display options you want to see.
   You can select as many of the different options as you like.
2. Click OK.

Note. The selection you make the first time the Primer Design Display dialog box appears is retained throughout your MacVector session.

Primer3 output

The Primer3 output option displays the raw output from Primer3. See “Primer3 output file format” on page 380 for a description of the syntax of this output.
Primer design

Graphical map

The **Graphical map** option displays the primer pairs identified, the internal hybridization primers identified (if this option was selected) and the predicted product or amplicon in a new Primer3 map window, along with any existing features of the sequence.

Like all other map views in MacVector, you can control the appearance of the Primer3 map using the Graphics Palette.

The Primer3 map window and the parent sequence window are linked so, if you click on any graphical object in the Primer 3 map window, then the corresponding region is selected in the parent sequence window.

Spreadsheet

The **Spreadsheet** option displays the primer sequences and detailed statistics about them in a new Primer3 spreadsheet window.

You can copy and paste individual sequences, entire lines, or the whole spreadsheet into other documents, including Excel spreadsheets and others that can import tab separated values. This makes it easy to distribute the primer sequence information to your oligonucleotide synthesis service. You can also save the Primer3 spreadsheet directly, as a comma separated value (CSV) file.

![Primer3 Spreadsheet](image)

You can also sort any of the columns in the Primer3 spreadsheet. By default the results are sorted by rank each pair has been given. So, the first primer pair listed is the most suitable and so on. However, you can also sort the results by Tm or %GC values. To do this click on the heading cell or the appropriate column.

The Primer3 map and spreadsheet windows are linked so, if you select a primer in the Primer3 spreadsheet window, then that primer feature is selected in the Primer3 map window and in the parent sequence window. Similarly, if you select a primer feature in the Primer3 map win-
dow, then it is also selected in the Primer3 spreadsheet window and the parent sequence window.

**PCR primer pairs**

MacVector provides an automated means of screening a nucleic acid sequence for PCR primer pairs. Refer to “Primers and probes” on page 369, for details of the method used.

**Screening for PCR primer pairs**

To use this functionality, a nucleic acid sequence must be active.

![Find PCR Primer Pairs dialog box](image)

**To screen for PCR primer pairs**

1. Choose **Analyze | Primers | PCR Primer Pairs**.
   
The **Find PCR Primer Pairs** dialog box is displayed.

2. In the **Scan criteria** panel, do one of the following:
   - select **product size** if the entire nucleic acid sequence is known and you want to find primer pairs that amplify a product of a certain size in a given region
   - select **two flanking regions** to scan if each primer must lie in a certain region, or if you do not know the sequence of the product you
PCR primer pairs

want to amplify but do know the sequences of the regions flanking the required product.
The choice made affects the other text boxes in this panel.

3. If you selected **product size**, then do the following:
   - enter the base numbers that bracket the region you want to scan by typing them in the **Region to scan** text boxes, or by selecting a region from the feature selector drop-down menu, to the right of the boxes.
   - enter the range of product sizes that you want to amplify in the **Product size** text boxes.

4. If you selected **two flanking regions**, then do the following:
   - enter the base numbers that bracket the region where the forward primer should be found by typing them in the **Forward primer (5') region** text boxes, or by selecting a region from the features table drop-down menu at the right of the boxes.
   - enter the base numbers that bracket the region where the backward primer should be found by typing them in the **Backward primer (3') region** text boxes, or by selecting a region from the features table drop-down menu at the right of the boxes.

5. Type the required range of primer lengths in the **length** text boxes.

6. Type the required percentage range of primer G+C content in the **percent G+C** text boxes.

7. Type the required range of primer Tm values in the **Tm (°C)** text boxes.

8. Type the IUPAC code for the two nucleotides that you want to appear at the 3' end of the primers in the **3' dinucleotide** text box.

9. Use the **primer vs. primer (any)** drop-down menu to specify the maximum number of consecutive bonds of any type that you will allow the primer to form with itself (hairpin formation) or with another primer (dimer formation).

10. Use the **primer vs. primer (G-C)** drop-down menu to specify the maximum number of consecutive G-C bonds that you will allow the primer to form with itself (hairpin formation) or with another primer (dimer formation).
11. Use the 3'-end vs. 3'-end drop-down menu to specify the maximum number of consecutive bonds that you will allow for the formation of “primer dimers.” Primer dimers can lead to amplification of the primers alone instead of the required product.

12. Use the 3'-end vs. product drop-down menu to specify the maximum number of consecutive bonds you will allow between the 3' end of a primer and the product it amplifies. This is used to eliminate primers that may bind to alternate sites tightly enough to result in false priming and amplification of the wrong product(s).

13. Type in the sum of the concentrations of the two primers at the start of the amplification reaction in the total initial primer conc. (µM) text box.

14. Type in the monovalent cation (Na and K) concentration of the reaction mixture in the monovalent cation conc. (mM) text box.

15. Select OK to perform the screen. Alternatively, select Defaults to restore the default settings, or Cancel to close the dialog box without performing the screen. At the end of the screen, the PCR Pairs Display dialog box is displayed.

The PCR Pairs Display dialog box contains statistical information about the PCR primer pair screening: how many were considered, how many
eliminated, and the reasons for rejection. If no pairs were found, look at these statistics to determine the most common cause of rejection. This can help you decide which setup parameters to modify.

Displaying PCR primer screen results

The **PCR Pairs Display** dialog box provides a number of options for displaying the results of the screen.

**To display the results of a PCR primer pairs screen**

1. The **PCR Pairs Display** dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example to change the display parameters, choose **Analyze | Primers | PCR Primer Pairs** when any PCR pairs display result window is active.

2. Select the **pair Tm difference** checkbox to restrict the displays to those primer pairs whose Tm’s differ by less than the amount chosen in the associated drop-down menu.

3. Select the **list of pairs** checkbox to see a list of the primer pairs, and information about them and the products they amplify: Tm, G+C content, length, location, and the optimum annealing temperature.

4. Select the **graphical map of pairs** checkbox to see a graphical representation of the pairs and the products they amplify.

5. Select **OK** to display the results.

PCR primer pair characteristics

For any input pair of PCR primers, MacVector can generate useful information about the primers and the product. This enables you to assess primer pairs from sources other than the MacVector primer design functions. The calculated characteristics include:

- length and G-C percentage of each sequence
- average Tm, derived from the reverse complement of the primer
- primer pair Tm difference
- self-dimer and dimer formation
- hairpin formation
- self-duplex and duplex formation
- binding site information
- product formation.

To use this functionality, a DNA sequence must be the active window.
To calculate PCR primer pair characteristics

1. Choose Analyze | Primers | Test PCR Primer Pair.

The PCR Characteristics dialog box is displayed.

2. Enter the sequence of the first primer in the pair into the Primer 1 text box, using the standard IUPAC one-letter codes. The sequence order must be 5’ to 3’.

3. Enter the sequence of the second primer in the pair into the Primer 2 text box, using the standard IUPAC one-letter codes. The sequence order must be 5’ to 3’.

Tip. You might want to cut and paste text into both Primer 1 and Primer 2 boxes. You can do this by storing text in the Text Editor. You can then switch to the Text Editor to copy new text to the clipboard, before pasting into MacVector.

4. If required, select the Parameters button to adjust the parameters used to control the tests that generate the characteristics. See “To modify the PCR primer test parameters” on page 221, for further details.
5. You can restrict the search for unwanted binding sites to a certain region of the active sequence you are testing primers against, by typing in the base numbers that bracket the region in the **Region** text boxes, or by selecting a region from the features table drop-down menu at the right of the text boxes.

6. Do one of the following:
   - select **Apply** to run the analysis and display results in the information text boxes. This leaves the **PCR Characteristics** dialog box displayed.
   - select **OK** to close the **PCR Characteristics** dialog box and display a text window of the same name that duplicates the results shown in
the information text boxes. It also shows aligned sequences and product sequences.

Note. If a text window of the correct name is already open, then the contents will simply be updated. Because the contents are only updated when the OK button is clicked, it is possible to display information in the text window that is out of step with the PCR Primer Pair dialog information boxes.

**PCR primer test parameters**

The characteristics are calculated from a series of tests, and you can modify the test parameters. The parameters are accessible from the PCR Characteristics dialog box, and include several parameters used on the Find PCR Primer Pairs dialog box (see “Screening for PCR primer pairs” on page 214).
To modify the PCR primer test parameters

1. Choose Analyze | Primers | Test PCR Primer Pair.

The PCR Characteristics dialog box is displayed.

2. Select the Parameters button.

The PCR Primer Parameters dialog box is displayed.

Note. The Primer binding parameters are used to identify regions of the target sequence that the primer can potentially bind to.

3. Select Allow Ambiguous residues to let primer sequences contain characters other than A, C, T, and G, and to accept matches between ambiguous bases in the test sequence.

4. Use the Mismatches drop-down menu to specify the maximum number of residues by which the primer can differ from the target sequence.

5. Use the 3’-end clamp drop-down menu to specify the number of contiguous residues at the 3’ end that must bind with the target.

Note. The Contiguous bonds allowed parameters are used to screen the primer for self-annealing artifacts that might interfere with the reaction.

6. Use the primer vs. primer (any) drop-down menu to specify the maximum number of consecutive bonds of any type that you will allow the primer to form with itself (hairpin formation) or with another primer (dimer formation).

7. Use the primer vs. primer (G-C) drop-down menu to specify the maximum number of consecutive G-C bonds that you will allow the
Primer and Probe Design

primer to form with itself (hairpin formation) or with another primer (dimer formation).

8. Use the 3’-end vs. 3- end drop-down menu to specify the maximum number of consecutive bonds that you will allow for the formation of primer dimers.

Primer dimers can lead to amplification of the primers alone instead of the required product.

9. Use the 3’-end vs. product drop-down menu to specify the maximum number of consecutive bonds you will allow between the 3’ end of a primer and the product it amplifies.

This is used to check for primers that may bind to alternate sites tightly enough to result in false priming and amplification of the wrong product(s).

10. Type in the sum of the concentrations of the two primers at the start of the amplification reaction in the total initial primer conc. (µM) text box.

11. Type in the monovalent cation (Na and K) concentration of the reaction mixture in the monovalent cation conc. (mM) text box.

Note. Steps 10 and 11 only affect the Tm calculation.

12. Select OK to return to the PCR Characteristics dialog box.

Alternatively, select Defaults to restore the default settings, or Cancel to close the dialog box without saving the parameters.

Sequencing primers and hybridization probes

MacVector provides functionality for screening a nucleic acid sequence for likely sequencing primers or hybridization probes of up to about 40 nucleotides.
Screening for sequencing primers or probes

To use this functionality, a nucleic acid sequence window must be the active window.

To screen for sequencing primers or probes

1. Choose **Analyze | Primers | Sequencing Primers/Probes**.

The **Find Sequencing Primers/Probes** dialog box is displayed.

2. In the **Region to scan** panel, enter the base numbers that bracket the region you want to scan by typing them in the text boxes, or by selecting a region from the features table drop-down menu at the right of the boxes.

3. Choose the strand to scan from the **strand** drop-down menu.

4. Type the required range of primer/probe lengths in the **length** text boxes.

5. Type the required percentage range of primer/probe G+C content in the **percent G+C** text boxes.

6. Type the required range of primer/probe Tm values in the **Tm (°C)** text boxes.
7. Type the IUPAC code for the two nucleotides that you want to appear at the 3' end of the primer/probe in the 3' dinucleotide text box.

8. Use the primer vs. primer (any) drop-down menu to specify the maximum number of consecutive bonds of any type that you will allow the primer/probe to form with itself (hairpin formation) or with another primer/probe molecule (dimer formation).

9. Use the primer vs. primer (G-C) drop-down menu to specify the maximum number of consecutive G-C bonds that you will allow the primer/probe to form with itself (hairpin formation) or with another primer/probe molecule (dimer formation).

Use the 3'-end vs. 3'-end drop-down menu to specify the maximum number of consecutive bonds that you will allow for the formation of dimers.

10. This is important for sequencing primers; for hybridization probes, choose the largest value.

11. Use the primer vs. sequence drop-down menu to specify the maximum number of consecutive bonds you will allow between the primer/probe secondary binding sites and the target sequence. There are two options associated with this:
   • for a sequencing primer, you would usually select the radio button that specifies 3'-end only.
   • for a hybridization probe, you would usually select the radio button that specifies the entire primer.

12. In the ...over the region text boxes, type the base numbers that bracket a comparison region, or select a region from the features table drop-down menu to the right of the boxes.

This is the region of the sequence that you want to compare with the potential primer/probe to eliminate those that bind to alternate sites.

13. Choose the strand to use in the comparison from the strand drop-down menu.

14. Type in the sum of the concentrations of the two primers at the start of the amplification reaction in the total initial primer conc. (µM) text box.

15. Type in the monovalent cation (Na and K) concentration of the reaction mixture in the monovalent cation conc. (mM) text box.

16. Select OK to perform the screen.
Sequencing primers and hybridization probes

Alternatively, select **Defaults** to restore the default settings, or **Cancel** to close the dialog box without performing the screen.

At the end of the screen, the **Primers/Probes Display** dialog box is displayed.

The **Primers/Probes Display** dialog box contains statistical information about the scan for primers/probes: how many were considered, how many eliminated and the reasons for rejection. If none were found, look at these statistics to determine the most common cause of rejection. This can help you decide which setup parameters to modify.

**Displaying sequencing primer or probe screen results**

The **Primers/Probes Display** dialog box provides a number of options for displaying the results of the screen.

**To display the results of a sequencing primer or probe screen**

1. The **Primers/Probes Display** dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example to change the display parameters, choose **Analyze | Primers | Sequencing Primers/Probes** when any Primers/Probes display result window is active.

2. Select the **Tm (°C)** checkbox to restrict the displays to those primers/probes whose Tm’s lie within the range specified in the text boxes.
3. Select the **percent G+C** checkbox to restrict the displays to those primers/probes whose G+C percentage lies within the range specified in the text boxes.

4. Select the **list of primers/probes** checkbox to see a list of the primers or probes and information about them: Tm, G+C content, length, and location.

5. Select the **map of primers/probes** checkbox to see a graphical representation of the sequencing primers or hybridization probes.

6. Select **OK** to display the results.

**Sequencing primer characteristics**

For any sequencing primer, MacVector can generate useful information about its properties and binding characteristics. This enables you to assess sequencing primers from sources other than the MacVector screening function. The calculated characteristics include:

- length and G-C percentage of each sequence
- average Tm, derived from the reverse complement of the primer
- self-dimer formation
- hairpin formation
- self-duplex formation
- binding site information.

To use this option, a DNA sequence must be the active window.
Sequencing primers and hybridization probes

To calculate sequencing primer characteristics

1. Choose Analyze | Primers | Test Sequencing Primers/Probes.

The Sequencing Characteristics dialog box is displayed.

2. Enter the primer sequence into the Primer text box, using the standard IUPAC one-letter codes. The sequence order must be 5’ to 3’.

3. If required, select the Parameters button to adjust the parameters used to control the tests that generate the characteristics. See “To modify the sequencing primer test parameters” on page 229, for further details.

4. You can restrict the search for unwanted binding sites to a certain region of the active sequence you are testing primers against, by typing in the base numbers that bracket the region in the Region text boxes, or by selecting a region from the feature selector drop-down menu to the right of the text boxes.

5. Do one of the following:

   • select Apply to run the analysis and display results in the information text box. This leaves the Sequencing Characteristics dialog box displayed.

   • select OK to close the Sequencing Characteristics dialog box and display a text window of the same name that duplicates the results.
shown in the information text box. It also shows aligned sequences and product sequences.

Note. If a text window of the correct name is already open, then the contents will simply be updated. Because the contents are only updated when the OK button is clicked, it is possible to display information in the text window that is out of step with the PCR Primer Pair dialog boxes.

Sequencing primer test parameters

The characteristics are calculated from a series of tests, and you can modify the test parameters. The parameters are accessible from the Sequencing Characteristics dialog box, and include several parameters used on the Find Sequencing Primers/Probes dialog box (see “Screening for sequencing primers or probes” on page 223).
Sequencing primers and hybridization probes

**Note.** The shared parameters are linked, so changes in one dialog box will be reflected in the other.

To modify the sequencing primer test parameters

1. Choose **Analyze | Primers | Test Sequencing Primer/Probe.**

2. Select the **Parameters** button.

3. Select **Allow Ambiguous residues** to let primer sequences contain characters other than A, C, T, and G.

4. Use the **Mismatches** drop-down menu to specify the maximum number of residues by which the primer can diverge from the target sequence.

5. Use the **3’-end clamp** drop-down menu to specify the number of residues at the 3’ end that must bind with the target.

This is used to reduce the number of potential binding sites.

6. Use the **primer vs. primer (any)** drop-down menu to specify the maximum number of consecutive bonds of any type that you will allow the primer to form with itself (hairpin formation) or with another primer (dimer formation).

7. Use the **primer vs. primer (G-C)** drop-down menu to specify the maximum number of consecutive G-C bonds that you will allow the primer to form with itself (hairpin formation) or with another primer (dimer formation).
8. Use the 3'-end vs. 3'-end drop-down menu to specify the maximum number of consecutive bonds that you will allow for the formation of “primer dimers.”

Primer dimers can lead to amplification of the primers alone instead of the required product.

9. Type in the sum of the concentrations of the two primers at the start of the amplification reaction in the total initial primer conc. (µM) text box.

10. Type in the monovalent cation (Na and K) concentration of the reaction mixture in the monovalent cation conc. (mM) text box.

Note. Steps 9 and 10 only affect the Tm calculation.

11. Select OK to return to the PCR Primer Pair dialog box. Alternatively, select Defaults to restore the default settings, or Cancel to close the dialog box without saving the parameters.

Least degenerate hybridization probes

MacVector provides a way to find an oligo to use as a hybridization probe for a gene whose exact DNA sequence is not known, provided the amino acid sequence is known. The process used is as follows:

- reverse-translate the amino acid sequence;
- scan the resulting DNA sequence to find a region that is not very degenerate;
- use this region to make probes, thus minimizing the number of oligonucleotide probes that would have to be synthesized.

Refer to “Screening a protein to find a hybridization probe” on page 387, for more details of this process.

Screening for least degenerate hybridization probes

To use this functionality, a protein sequence window must be the active window.

Before you run the analysis, you may want to reduce the degeneracy of the resultant DNA sequence by eliminating certain codons from the genetic code used for the reverse translation. Your knowledge of the codon preferences of the organism of interest will be your best guide in
Least degenerate hybridization probes

eliminating codons. Refer to “Reducing the degeneracy of a genetic code” on page 243 for details of this procedure.

To find least degenerate hybridization probes

1. Choose Analyze | Reverse Translation.
2. Type the residue numbers that bracket the region you want to scan in the Region text boxes, or select a region from the features table drop-down menu at the right of the boxes.
3. Select the genetic code to be used from the genetic code drop-down menu.
4. Select the Probe list check box to generate a list of least-ambiguous oligonucleotide probes for the specified region.

The output lists the sequence of each probe, the corresponding amino acid sequence, the percent G+C content of the probe, and the dissociation temperature of the probe-DNA complex.
5. If you are generating a probe list, limit the range of probe sizes by entering values in the minimum and maximum text boxes.
6. Select the Annotated sequence check box to display the degenerate nucleic acid sequence that results from reverse translating the protein. The appearance of this display can be changed interactively by choosing Customize | Format Annotated Display. Refer to “Formatting the annotated sequence display” on page 35, for further details.
7. Select the New DNA check box to create a new DNA sequence window for the degenerate sequence created by reverse translating the protein sequence. Type a name for the new sequence in the text box.
8. Select OK to perform the screen.
Screen results

If you have displayed an annotated sequence, the amino acid that results from translation is shown beneath the degenerate nucleic acid sequence. Note that this amino acid sequence may not match the original protein because certain degenerate codons could code for more than one amino acid. For example, serine has six codons, four of the form TCN and two of the form AGY. These two forms reduce to the single degenerate codon WSN. If this degenerate codon is then retranslated, it will be assigned the “unknown” amino acid X, because WSN can expand to any of the following:

- TCN = Ser
- ACN = Thr
- AGY = Ser
- AGR = Arg
- TGY = Cys
- TGA = End
- TGG = Trp.
Overview

This chapter describes how to transcribe and translate DNA sequences to protein. You can assemble the required sequence for transcription by choosing particular features such as introns and exons. MacVector provides flexible facilities for translating and reverse translating sequence data, according to the chosen genetic code. In addition to using the genetic codes supplied with MacVector, you can also create your own codes.

The reverse translation functionality is described in “Least degenerate hybridization probes” on page 230.

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Using Transcription and Translation Functions

Transcribing and translating DNA to protein

MacVector enables you to transcribe a DNA sequence, and translate the resulting mRNA into an amino acid sequence using a selected genetic code. MacVector can also display a codon usage table for the translation.

Using the Translation Analysis dialog box, you can transcribe and translate a DNA sequence in a single operation. Alternatively, using the Generate Transcript dialog box, you can first perform a transcription analysis and then translate. This option is particularly useful if you require the mRNA sequence itself, or if you want to assemble the transcription sequence from discontinuous segments of DNA.

Both the Transcription Analysis and the Generate Transcript dialog boxes allow you to specify the segments to be transcribed by choosing exon, intron, RNA or coding sequence (CDS) features.

All segments are assumed to be on the same strand, so you cannot merge segments from the plus and minus strands in the same transcription. To run a transcription or translation analysis, a nucleic acid sequence window must be the active window.

Generating a transcript

An entire sequence of DNA in the sequence window can be transcribed automatically by clicking on the sequence type indicator icon on the toolbar. The indicator icon will change from DNA to RNA. Click on the icon again to reverse the transcription.

Alternatively, to transcribe one or more features in the sequence, use the Generate Transcript dialog box.

Note. MacVector detects features by their feature table entries. It does not predict exons, so you must first ensure that the appropriate features have been defined.

Specifying a CDS feature

This is the initial default method. When you choose the Single CDS method and select a range of the current DNA sequence, MacVector lists all CDSs found within the range. The CDS features for both strands are listed together; those on the minus (complementary) strand are marked with a blue ‘C’. Each CDS may contain several exons; these are listed on successive lines within the CDS feature entry. Only one CDS feature can be selected for transcription, because only a single CDS can contribute to a mRNA molecule.
Transcribing and translating DNA to protein

Note. A CDS feature may or may not include the terminal stop codon, depending on the preference of the submitting author.

**Specifying exons**

When you choose **Specify exons**, and select a strand and a range of the current DNA sequence, MacVector lists all exons found on that strand within the range. Initially all exons are checked, so they will be included in the transcription. However, you can deselect exons to prevent them from being transcribed. This is useful where a gene has alternate splice sites.

The default assembly order is from 5’ to 3’ on the selected strand. Therefore the exons are listed in ascending order of residue numbering on the plus strand, and in descending order on the minus strand. You can change the order of assembly by moving items in the list.

**Tip.** Rearranging the order of exons may be useful if the gene has been cloned into circular DNA, and the origin of the circular DNA is inside the gene.

**Specifying introns**

When you choose the **Specify introns** method, and select a strand and a range of the current DNA sequence, MacVector lists all introns found on that strand within the range. In contrast to MacVector’s treatment of exons, all introns are initially unchecked, so they will be excluded from the transcription. If you select any introns, they will be transcribed.

**Specifying RNA features**

When you choose the **Specify RNA features** method, and select a strand and a range of the current DNA sequence, MacVector lists all RNA features found on that strand within the range. Initially all features are unchecked, so they will be excluded from the transcription. If you select any RNA features, they will be transcribed. You can also change the order of assembly, by moving items in the list.

**Preserving features**

When the mRNA window is created, the relevant features from the DNA sequence’s feature table can be copied into it. Where a feature lies partially within a selected range, it will be split and copied as a feature fragment. If several segments have been transcribed, there can be many such feature fragments in the resulting mRNA sequence. MacVector lets you choose how much feature information to include in the transcribed sequence: all the features, just the features that define the
selected segments, or no features at all. The default is to include only the defining features. If no features are copied, the mRNA window will contain only the residues.

To transcribe DNA features into mRNA

1. Choose Analyze | Generate Transcript from the menu.
   The Generate Transcript dialog box is displayed.

2. From the Method drop-down menu, choose one of the following methods for selecting segments to transcribe:
   - Single CDS
   - Specify exons
   - Specify introns
   - Specify RNA sequences
   The appropriate features are displayed in a list.

3. To enter a range in the sequence, type the appropriate sequence numbers in the From and to text boxes.
   You will be selecting features for transcription within this range.

4. From the Strand menu, select plus or minus.
If you have selected the **Single CDS** method, CDS features of both strands are listed and this menu is not available.

5. Do one of the following, depending on the transcription method you chose in step 2:
   - **Specify exons**: Deselect any listed features that you do not want transcribed by clicking in their check boxes.
   - **Single CDS**: Select a CDS feature for transcription from the list. The selected feature is highlighted.
   - **Specify introns** or **Specify RNA sequences**: Select any listed introns that you want transcribed by clicking in their check boxes.

6. If you chose **Specify exons** or **Specify RNA features**, you can change the order of assembly of the listed features. To move a feature, click on the double arrow at the left of the line. Holding down the mouse button, drag the feature to its new position in the list, and then release the mouse button.

As the line is dragged, a dashed outline follows the cursor, and an arrow at the left of the list shows where the insertion position will be if the mouse is released.

7. From the **mRNA Feature Table** drop-down menu, choose which features should be copied into the transcribed sequence: **Copy all features**, **Default**, or **Do not copy features**.

8. Type a name for the transcribed sequence in the **mRNA Sequence Name** text box.

9. Select **OK** to perform the transcription and display the resulting mRNA sequence.

### Translating DNA or mRNA to protein

The **Translation Analysis** dialog box is used to control the translation of a nucleic acid sequence and to set up displays of the resulting protein sequence. The nucleic acid sequence can be either DNA, or the mRNA output from a transcription analysis (see “To transcribe DNA features into mRNA” on page 236). If you are using a DNA sequence, select one or more segments for translation, using the **Segment(s) to translate** text box or the feature selector drop-down menu. If you have a transcribed mRNA sequence, you will normally translate the entire sequence.

For instructions on choosing and modifying genetic codes, see “Genetic codes” on page 240.
To translate a nucleic acid sequence to protein

1. Choose **Analyze** | **Translation** from the menu.

   The **Translation Analysis** dialog box is displayed.

2. If you do not want to translate the entire sequence, select the parts of the sequence to translate, by doing one of the following:
   - type the required ranges in the **Segment(s) to translate** text box
   - select the required features from the feature selector drop-down menu to the right of the box.

   When you are typing ranges, there must be a slash (/) between the numbers of a range, and a semicolon (;) after each specified range. For segments on the minus (reverse complement) strand, type the base numbers with reference to the plus strand.

   **Note.** If two or more segments are specified, they must be listed in the order that they are to be translated. If several segments on the minus strand are specified, the first segment to be translated will be the segment with the highest number.

3. Choose the genetic code to use for the translation from the **genetic code** drop-down menu.

   For instructions on choosing and modifying genetic codes, see “**Genetic codes**” on page 240.

4. Choose the reading frame from the **phase** drop-down menu.
Transcribing and translating DNA to protein

The reading frame is relative to the start of the first segment entered in the **Segment(s) to translate** box. In almost all cases, the phase should be set to 1.

5. Choose which strand to translate from the **strand** drop-down menu. If regions are entered from the features table, the strand is set automatically to the strand corresponding to the last feature that was used.

6. To create a new protein sequence window containing the protein sequence specified by the translation settings, select the **Create new protein** check box. Type a name for the protein sequence in the text box.

**Note.** The protein sequence will only include the translations of the specified segments.

7. To generate a table displaying the frequency of each of the 64 codons in the selected sequence, select the **Codon Usage Table** check box.

8. Select the **Annotated sequence with translation** check box to generate an annotated sequence of the DNA and translated protein. This always displays the portion of the nucleic acid sequence specified in the **Region to Display** text boxes. The displayed sequence can be further controlled as follows:
   - select the **as specified in...** radio button to see the translation only for those segments specified in the **Segment(s) to translate** box
   - select the **of entire display region** radio button to see the translation of the whole display region. With this option, you can choose the number of reading frames translated from the **no. of frames** drop-down menu.

9. You can restrict the display to a certain region of the sequence by typing in the base numbers that bracket the region in the **Region to Display** text boxes, or by selecting a region from the feature selector drop-down menu to the right of the text boxes.

This enables you to display more than just the translated portion of the sequence, for example, if you want to show regulatory regions upstream from the coding region.

**Note.** The annotated sequence will show all bases in the specified region, not just those which have been translated.

10. Select **OK** to perform the translation and display the requested results.
Genetic codes

Genetic codes are used for translating and reverse translating sequence data. MacVector enables you to:

- designate a default genetic code for performing auto-translations
- create a new genetic code
- “delete” codons from a genetic code to reduce the amount of degeneracy when reverse translating an amino acid sequence to a DNA sequence.

Autotranslations are controlled by the annotated sequence formatting. When enabled, they display selected features with a translation beneath the main sequence display.

Refer to “Formatting the annotated sequence display” on page 35, for further information.

Selecting a genetic code

The default genetic code can be changed at any time. If an annotated sequence window is open at the time you make the change, any autotranslations that are present will be updated immediately to reflect the new genetic code settings. However, the results of previous analyses will not be changed. For example, if you translate a DNA sequence and create a new protein sequence window, the amino acids in the protein sequence will not change if you subsequently change the genetic code.

To select a genetic code

1. Choose Options | Modify Genetic Codes from the menu.
   The Genetic Code dialog box is displayed.
2. Choose a code from the genetic code drop-down menu.
3. Select OK to remove the Genetic Code dialog box.

Modifying genetic codes

Creating and editing codes is done using the Genetic Code dialog box.
At the left side of the dialog box is a chart with 64 cells representing the 64 codons. The labels on the left side of the chart are for the first nucleotide of the codon, the labels on top for the second nucleotide, and the labels on the right for the third nucleotide. Each cell of the chart contains the three-letter abbreviation of the amino acid coded for by the codon controlling that cell. To the right of the chart is a graphic that looks like a stylized tRNA anticodon loop. When you click on a cell, the codon corresponding to the selected cell will be displayed beneath the loop.

Adding a new genetic code

New genetic codes can be added at any time.

To add a new genetic code

1. Choose Options | Modify Genetic Codes.

The Genetic Code dialog box is displayed.

2. Choose the code that will be used as a basis for the new one from the genetic code drop-down menu.

3. Type the name of the new genetic code in place of the selected code, then select the button labeled with a plus sign (+) in the upper right corner of the dialog box.

Note. The new code will not be saved permanently until you select OK.

4. Modify the code as required.
Refer to the procedure “To modify a genetic code” on page 242.

5. Select OK to save the changes.

Note. The new code will become the default code, unless you select an alternative one before closing the Genetic Code dialog box.

Modifying a genetic code

The selected genetic code can be modified at any time. We recommend that any modifications to codes supplied with MacVector are done on a copy of the code. See “To add a new genetic code” on page 241, for details of how to copy a code.

To modify a genetic code

1. Choose Options | Modify Genetic Codes.

The Genetic Code dialog box is displayed.

2. Choose the code to be modified from the genetic code drop-down menu.

3. Select a cell whose assignment you want to change.

4. Scroll down the amino acid list on the right of the dialog box, and double-click on the amino acid you want to be coded for in the highlighted cell. If the codon does not code for an amino acid (for example, TAA, TAG, and TGA in the universal code) choose *** instead of an amino acid name.

The contents of the highlighted cell change to reflect the new assignment.

5. To designate any of the 64 codons to be a start or stop codon, select the appropriate cell, then select the start or stop button above the stylized tRNA anticodon loop.

6. Repeat steps 3 through 5 for each change you require.

7. Select OK to save the changes.

Deleting a genetic code

A genetic code can be deleted at any time. This should be done with caution, because the only way you can restore it is to re-enter it by hand or to reinstall MacVector.

To delete a genetic code

1. Choose Options | Modify Genetic Codes.

The Genetic Code dialog box is displayed.
Genetic codes

2. Choose the code to be deleted from the genetic code drop-down menu.

3. Select the button labeled with a minus sign (-) in the upper right corner of the dialog box.

4. Select OK to delete the code permanently.

Note. If you select Cancel, the code will not be lost, even if you have done step 3 above.

Reducing the degeneracy of a genetic code

When you perform a reverse translation, you can eliminate codons from consideration to reduce the degeneracy of the DNA sequence that will result from the reverse translation. This can be useful, for example, when you know that your organism uses only two of the six standard serine codons in the universal genetic code.

To reduce the degeneracy of a genetic code

1. Choose Options | Modify Genetic Codes.

The Genetic Code dialog box is displayed.

2. Choose the code to be modified from the genetic code drop-down menu.

3. Select a codon that you want to ignore.

4. Scroll to the end of the amino acid list on the right of the dialog box, and select “- - -”.

5. Repeat steps 3 and 4 for each codon you want to ignore in the reverse translation.

6. Select OK to save the changes.

Each codon marked as “- - -” will not be considered when performing reverse translations.
Using Transcription and Translation Functions
Overview

MacVector provides a number of routines for aligning and comparing sequences. For sequence similarity, a matrix comparison is the method of choice for obtaining an overall picture of how the sequences are related, and is the subject of this chapter.

The MacVector sequence alignment routines use elements of the matrix comparison method as a rapid filter before the actual alignment.

Refer to Chapter 13, “Aligning Sequences”, for information about scoring matrix methods for sequence comparison and alignment, and sequence comparisons using the Entrez database.
Pustell DNA matrix

The MacVector implementation of DNA matrix analysis uses techniques developed by Pustell. The method enables you to:

- look for regions of similarity between two nucleic acid sequences using a dot matrix plot
- look for direct or inverted repeats within a single nucleic acid sequence
- display matching regions as either diagonal lines or as diagonally arranged characters whose values indicate the degree of similarity

An outline of the matrix analysis and the parameters used are presented in Chapter 18, “Understanding Sequence Comparisons”. The match and mismatch scores used in scoring the comparison can be reassigned, or new scoring matrices can be created and edited. See “Scoring matrix files” on page 78, for further details.

Performing a DNA matrix analysis

To use this functionality, one or more nucleic acid sequences must be open, a window containing a nucleic acid sequence must be the active window, and a nucleic acid scoring matrix file must be open or available to the program.

The output is a two-dimensional plot, with the residues of one sequence along the X-axis, and the residues of the comparison sequence along the Y-axis.

Before running this analysis, we recommend that you read Chapter 18, “Understanding Sequence Comparisons”, to understand the implications of changing the scoring parameters.
To perform a Pustell DNA matrix analysis

1. Choose Analyze | Pustell DNA Matrix from the menu. The DNA Matrix Analysis dialog box is displayed.

2. In the left-hand scrolling list, select a sequence to display along the X-axis.

3. In the right-hand scrolling list, select a sequence to display along the Y-axis. The name that is highlighted in each list is the sequence assigned to that axis.

4. You can limit the analysis to a region of each of the sequences by typing in the numbers that bracket the region in the X-Region and Y-Region text boxes, or by selecting a region from the feature selector drop-down menu that appears to the right of the text boxes.

Tip. If you are comparing a very long sequence with a very short one, the procedure will need less memory if you display the shorter sequence on the x-axis.

5. Click the Scoring Matrix button to choose a scoring matrix file. A standard dialog box is displayed, enabling you to search for and select the file to use.

6. Enter a window size in the window size text box.
Whenever MacVector finds an exact match, it examines the segment (or window) of the aligned sequences that surround the matching region. The length of the segment is the value typed in for window size.

7. Enter a minimum score in the **min. % score** text box.

Whenever MacVector finds an exact match, it computes a total score for the window using the match / mismatch scores in the scoring matrix. It then determines a percent score by dividing the window’s score by the score that would occur if all of the bases in the window matched. If this percent score equals or exceeds the value of **min. % score**, the window is saved.

8. Choose a value from the **Hash Value** drop-down menu.

The hash value is a measure of how long an exact match between two sequences must be before MacVector will attempt to score and align that matching region. A hash value of 1 is the most sensitive, 6 is the least sensitive.

**Tip.** For most comparisons, start with a hash value of 6, because it is unusual for two sequences to possess significant similarity without having regions of that size that match exactly.

9. Choose a value from the **jump** drop-down menu.

When the **jump** setting is 1, the hash value represents the number of bases in a row that must match perfectly. When the **jump** setting is 3, the hash value is the number of triplets in a row whose first base must match perfectly. When the **jump** setting is **both**, matching regions found for both **jump** settings are scored.

**Tip.** For the initial analysis stage, we recommend that the **jump** setting be set to **both**.

10. Use the **strand** drop-down menu to choose whether to compare the plus strands of the two sequences (++), the plus strand of the X-axis sequence with the reverse complement strand of the Y-axis sequence (+–), or both.

**Tip.** For the initial analysis, we recommend that you select **both**.

11. Select **OK** to perform the analysis.

Alternatively, select **Defaults** to restore the default settings, or **Cancel** to close the dialog box without performing the analysis.

When the analysis is complete, the **DNA Matrix Analysis** display dialog box is displayed. This dialog box is described in the following section.
Displaying DNA matrix analysis results

You can use the DNA Matrix Analysis display dialog box to filter results and display the following:

- matrix map
- aligned sequences

To display DNA matrix analysis results

1. The DNA Matrix Analysis display dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example to change the display parameters, choose Analyze | Pustell DNA Matrix when any matrix analysis display result window is active.

2. You can limit the display to a region of each of the sequences by typing in the numbers that bracket the region in the X-axis and Y-axis text boxes, or by selecting a region from the feature selector dropdown menu that appears to the right of the text boxes.

3. If you chose both for the analysis strand setting, you can now choose to display the comparison of the plus strands only (++) , the plus strand of the X-axis sequence with the reverse complement strand of the Y-axis sequence (+–), or both. If you limited the initial analysis, you will not be able to display the other types of comparisons.

4. If you set jump to both in the analysis, you can now separately display the matches found for the jump setting of 1 and the jump setting of 3, or you can look at both simultaneously. If you limited the initial analysis, the drop-down menu for the jump setting will be disabled.

5. Enter a value for the window size in the window size text box.
This can have any value starting from the window size used in the original analysis stage up to 60.

6. Enter a value for the score in the min. % score text box.

This can have any value starting from the min. % score used in the original analysis stage up to 100.

7. Select the Matrix map check box to display a dot matrix plot of the results of the comparison.

8. Use the drop-down menu at the end of the Matrix map check box to choose how you want to display the matching regions.

The line setting displays matching regions as diagonal lines. The character setting displays matching regions as diagonally arranged characters. The character used indicates the percent match at that point along the diagonal. “A” represents 100 percent match, and each succeeding letter is two percent less, so “B” is 98 percent, “C” is 96 percent, and so on. A listing of the letter codes can be found in Appendix C, “Reference Tables”.

9. Select the Aligned sequences check box to see a display of the X-axis sequence aligned with all of the regions of the Y-axis sequence that meet or exceed the min. % score.

10. Select OK to display the results.

**Pustell protein matrix**

The MacVector implementation of protein matrix analysis uses techniques developed by Pustell. The method enables you to:

- look for regions of similarity between two protein sequences using a dot matrix plot
- look for repeats within a single protein sequence
- display matching regions as either diagonal lines or as diagonally arranged characters whose values indicate the degree of similarity

An outline of the matrix analysis and its parameters is presented in Chapter 18, “Understanding Sequence Comparisons”. The match and mismatch scores used in scoring the comparison can be reassigned, or new scoring matrices can be created and edited. See “Scoring matrix files” on page 78, for further details.
Performing a protein matrix analysis

To run this analysis, one or more protein sequences must be open, a window containing a protein sequence must be the active window, and a protein scoring matrix file must be open or available to the program. The output is a two-dimensional plot, with the residues of one sequence along the X-axis, and the residues of the comparison sequence along the Y-axis.

Before using this functionality, we recommend that you read Chapter 18, “Understanding Sequence Comparisons”, to understand the implications of changing the scoring parameters.

To perform a Pustell protein matrix analysis
1. Choose Analyze | Pustell Protein Matrix from the menu. The Protein Matrix Analysis dialog box is displayed.
2. In the left-hand scrolling list, select a sequence to display along the X-axis.
3. In the right-hand scrolling list, select a sequence to display along the Y-axis.
   The name that is highlighted in each list is the sequence assigned to that axis.
4. You can limit the analysis to a region of each of the sequences by typing in the numbers that bracket the region in the X-Region and Y-
Comparing Sequences using Pustell Matrix Analysis

Region text boxes, or by selecting a region from the feature selector drop-down menu that appears to the right of the text boxes.

Tip. If you are comparing a very long sequence with a very short one, the procedure will need less memory if you display the shorter sequence on the x-axis.

5. Click the Scoring Matrix button to choose a scoring matrix file.
A standard dialog box appears, enabling you to search for and select the file to use.

6. Enter a window size in the window size text box.
Whenever MacVector finds an exact match, it examines the segment (or window) of the aligned sequences that surround the matching region. The length of the segment is the value typed in for window size.

7. Enter a minimum score in the min. % score text box.
Whenever MacVector finds an exact match, it computes a total score for the window using the match / mismatch scores in the scoring matrix. It then determines a percent score by dividing the window’s score by the score that would occur if all of the residues in the window matched. If this percent score equals or exceeds the value of min. % score, the window is saved.

8. Choose a value from the Hash Value drop-down menu.
The hash value is a measure of how long an exact match between two sequences must be before MacVector will attempt to score and align that matching region. A hash value of 1 is the most sensitive, 2 is the least sensitive.

Tip. For most comparisons, start with a hash value of 2, because it is unusual for two sequences to possess significant similarity without having regions of that size that match exactly.

9. Select OK to perform the analysis.
Alternatively, select Defaults to restore the default settings, or Cancel to close the dialog box without performing the analysis.

When the analysis is complete, the Protein Matrix Analysis display dialog box is displayed. This dialog box is described in the following section.

Displaying protein matrix analysis results

You can use the Protein Matrix Analysis display dialog box to filter results and display the following:

- matrix map
• aligned sequences.

To display protein matrix analysis results
1. The Protein Matrix Analysis display dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example to change the display parameters, choose Analyze | Pustell Protein Matrix when any matrix analysis display result window is active.

2. You can limit the display to a region of each of the sequences by typing in the numbers that bracket the region in the X-axis and Y-axis text boxes, or by selecting a region from the features table drop-down menu that appears to the right of the text boxes.

3. Enter a value for the window size in the window size text box. This can have any value starting from the window size used in the original analysis stage up to 60.

4. Enter a value for the score in the min. % score text box. This can have any value starting from the min. % score used in the original analysis stage up to 100.

5. Select the Matrix map check box to display a dot matrix plot of the results of the comparison.

6. Use the drop-down menu at the end of the Matrix map check box to choose how you want to display the matching regions. The line setting displays matching regions as diagonal lines. The character setting displays matching regions as diagonally arranged characters. The character used indicates the percent match at that point along the
Comparing Sequences using Pustell Matrix Analysis

diagonal. “A” represents 100 percent match, and each succeeding letter is two percent less, so “B” is 98 percent, “C” is 96 percent, “a” is 50 percent, and so on. A listing of all the letter codes can be found in Appendix C, “Reference Tables”.

7. Select the Aligned sequences check box to see a display of the X-axis sequence aligned with all of the regions of the Y-axis sequence that meet or exceed the min. % score.

8. Select OK to display the results.

Pustell protein and DNA matrix

The MacVector implementation of matrix comparison enables you to compare protein and DNA sequences. You can:

• look for regions of similarity at the amino acid level between a nucleic acid sequence and a protein sequence using a dot matrix plot
• display matching regions as either diagonal lines or as diagonally arranged characters whose values indicate the degree of similarity

An outline of the matrix analysis and its parameters is presented in Chapter 18, “Understanding Sequence Comparisons”. The match and mismatch scores used in scoring the comparison can be reassigned, or new scoring matrices can be created and edited. See “Scoring matrix files” on page 78, for further details.

Performing a protein / DNA matrix analysis

To run this analysis, at least one nucleic acid sequence file and one protein sequence file must be open, and a window containing a sequence must be the active window. A protein scoring matrix file must be open or available on disk.

When performing this analysis, MacVector first translates the nucleic acid sequence in the three reading frames of one of the DNA strands (if the strand options of ++ or +– are chosen) or in all six reading frames (if both strands are chosen). The comparison is then performed on the resulting amino acid sequences. The output is a two-dimensional plot, with the residues of the protein sequence along the X-axis, and the residues of the translated DNA sequence along the Y-axis. The aligned sequence display shows each aligned sequence from all the reading frames.
Before running this analysis, we recommend that you read Chapter 18, “Understanding Sequence Comparisons”, to understand the implications of changing the scoring parameters.

To perform a Pustell protein / DNA matrix analysis

1. Choose Analyze | Pustell Protein & DNA from the menu.

The Protein & DNA Matrix Analysis dialog box is displayed.

2. In the left-hand scrolling list, select a protein sequence to display along the X-axis. Only protein sequences appear in this list.

3. In the right-hand scrolling list, select a DNA sequence to display along the Y-axis. Only DNA sequences appear in this list.

4. You can limit the analysis to a region of each of the sequences by typing in the numbers that bracket the region in the X-Region and Y-Region text boxes, or by selecting a region from the feature selector drop-down menu that appears to the right of the text boxes.

5. Click the Scoring Matrix button to choose a scoring matrix file.

A standard dialog box is displayed, enabling you to search for and select the file to use.

6. Enter a window size in the window size text box.

Whenever MacVector finds an exact match, it examines the segment (or window) of the aligned sequence that surrounds the matching region. The length of the segment is the value typed in for window size.
7. Enter a minimum score in the **min. % score** text box.
Whenever MacVector finds an exact match, it computes a total score for the window using the match / mismatch scores in the scoring matrix. It then determines a percent score by dividing the window’s score by the score that would occur if all of the bases in the window matched. If this percent score equals or exceeds the value of **min. % score**, the window is saved.

8. Choose a value from the **Hash Value** drop-down menu.
The hash value is a measure of how long an exact match between two sequences must be before MacVector will attempt to score and align that matching region. A hash value of 1 is the most sensitive, 2 is the least sensitive.

**Tip.** For most comparisons, start with a hash value of 2, because it is unusual for two sequences to possess significant similarity without having regions of that size that match exactly.

9. Use the **strand** drop-down menu to choose whether to use the plus strand of the DNA sequence (++), the reverse complement strand (+−), or both.

**Tip.** For the initial analysis, we recommend that you choose **both**.

10. Choose the genetic code for the translation of the DNA from the **genetic code** drop-down menu.

11. Select **OK** to perform the analysis.
Alternatively, select **Defaults** to restore the default settings, or **Cancel** to close the dialog box without performing the analysis.

When the analysis is complete, the **Protein & DNA Matrix Analysis** display dialog box is displayed. This dialog box is described in the following section.

### Displaying protein / DNA matrix analysis results
You can use the **Protein & DNA Matrix Analysis** display dialog box to filter results and display the following:
- matrix map
aligned sequences.

To display protein / DNA matrix analysis results

1. The Protein & DNA Matrix Analysis display dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example to change the display parameters, choose Analyze | Pustell Protein & DNA when any matrix analysis display result window is active.

2. You can limit the display to a region of each of the sequences by typing in the numbers that bracket the region in the X-axis and Y-axis text boxes, or by selecting a region from the features table drop-down menu that appears to the right of the text boxes.

3. If you chose both for the analysis strand setting, you can now choose to display the comparison of the protein with the plus strand only (++) or the reverse complement strand only (+–), or both at once. If you limited the initial analysis, you will not be able to display both types of comparisons.

4. Enter a value for the window size in the window size text box. This can have any value starting from the window size used in the original analysis stage up to 60.

5. Enter a value for the score in the min. % score text box. This can have any value starting from the min. % score used in the original analysis stage up to 100.

6. Select the Matrix map check box to display a dot matrix plot of the results of the comparison.
7. Use the drop-down menu at the end of the **Matrix map** check box to choose how you want to display the matching regions. The **line** setting displays matching regions as diagonal lines. The **character** setting displays matching regions as diagonally arranged characters. The character used indicates the percent match at that point along the diagonal. “A” represents 100 percent match, and each succeeding letter is two percent less, so “B” is 98 percent, “C” is 96 percent, “a” is 50 percent, and so on. A listing of all the letter codes can be found in Appendix C, “Reference Tables”.

8. Select the **Aligned sequences** check box to see a display of the protein sequence aligned with all of the regions of the translated DNA sequence that meet or exceed the **min. % score**.

9. Select **OK** to display the results.
Overview

MacVector enables you to align both protein and DNA sequences from the following sources:

- sequences stored locally in any format recognized by MacVector
- sequences in the online NCBI databases accessible via the Internet.

Several methods are used to perform alignments:

- alignment with a scoring matrix pre-filter
- BLAST searches on the NCBI server
- multiple alignment using the ClustalW algorithm.

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Aligning sequences using a folder search

This method performs a search on sequences stored in a single folder, and enables you to:

- perform an optimal alignment between two nucleic acid sequences using scoring matrices and taking gaps into account
- perform an optimal alignment between a protein query sequence and either a protein sequence or a nucleic acid sequence, using scoring matrices and taking gaps into account
- view the alignments of a group of related sequences with the query sequence in a multiple-alignment format.

To perform alignments, a Sequence window must be the active window. A scoring matrix appropriate for the sequence must be open or available on the hard disk.

The scoring matrix pre-filter

MacVector uses a scoring matrix to pre-filter the sequences under consideration. An outline of the scoring matrix and its parameters is presented in Chapter, “Understanding Sequence Comparisons”. The match and mismatch scores used in the scoring can be reassigned, or new scoring matrices can be created and edited. See “Scoring matrix files” on page 78, for further details.

When it finds a match, MacVector computes an initial score for the matching sequence. You have the choice of using this score as a ranking to reject sequences before alignment. Alternatively, you can perform an optimal alignment on each sequence that passes a cut-off score, introducing gaps and indels if required, and calculate an optimal score to use as the basis for acceptance or rejection.

Aligning sequences in a folder

User folders may contain both MacVector files and text files, but all the text files must be in either GCG format or in one of the IUPAC formats. Do not place GCG files and IUPAC-format files in the same folder. Text files that use the Staden codes for ambiguous nucleotides will not
Aligning sequences using a folder search

be compared properly, so they should not be present in a folder that is being searched.

To align sequences using a scoring matrix pre-filter

1. Choose **Database | Align to Folder**. The **Align to Folder** dialog box is displayed.
2. Click **Folder to Search**.
   
   A standard dialog box appears that enables you to locate and select the folder containing sequence files.
3. Choose a value from the **hash value** drop-down menu.
   
   The **hash value** is a measure of how long an exact match must be between two sequences before MacVector will attempt to score and align that matching region. For protein sequences, a hash value of 1 is the most sensitive, and 2 is the least sensitive. For DNA sequences, a hash value of 1 is the most sensitive, and 6 is the least sensitive.

   **Tip.** For most comparisons, start with a hash value of 2 for a protein query sequence, or 6 for a DNA query sequence. This is because it is unusual for two sequences to possess significant similarity without having regions of those sizes that match exactly.
4. Enter the number of matching sequences to retain in the **scores to keep** text box.
Aligning Sequences

If the program finds more matches than this, the list will be trimmed by dropping sequences with the lowest scores.

5. Choose from the **processing** drop-down menu whether an optimal alignment should be done on-the-fly or at the end of the database search, as follows:

   - choose **none** to save the sequences in order of the initial score. If more matches are found than you wanted to keep, the sequences with the lowest initial scores are dropped. At the end of the search, MacVector performs an optimal alignment of each of the saved sequences with the query sequence. Indels and gaps are introduced if they will improve the optimized score. The matches are then listed by optimized score in the results windows.

   - choose **align** to perform an optimal alignment for any sequence whose initial score exceeds a minimum cut-off score. The matching sequences are saved in order of the optimized score rather than the initial score. If more matches are found than you wanted to keep, the sequences with the lowest optimized scores are dropped.

6. Click **Scoring Matrix** to choose a scoring matrix file.

   A standard dialog box is displayed so that you can select the file to use. Make sure that you choose a protein scoring matrix for a protein query sequence, and a DNA scoring matrix for a DNA query sequence.

7. You can limit the query sequence to a region of the entire sequence by typing in the numbers that bracket the region in the **Region** text boxes, or by selecting a region from the feature selector drop-down menu that appears to the right of the text boxes.
Aligning sequences using a folder search

8. If you have a protein query sequence, select the Align to DNA check box to compare the query only to the nucleic acid sequences that are in the folder.

Note. The Align to DNA check box only appears when you have a protein query sequence.

Each nucleic acid sequence is translated on-the-fly in all six reading frames and the resulting amino acid sequences are compared with the protein query sequence. Do not select this check box if you want to compare the query with protein sequences.

9. If the Align to DNA check box is selected, use the genetic code drop-down menu to choose the genetic code that will be used to translate the nucleic acid sequences in the folder.

10. Select OK to perform the alignment.

Alternatively, select Defaults to restore the default settings, or Cancel to close the dialog box without performing the analysis.

When the analysis is complete, the Folder Sequence Query display dialog box is displayed. This dialog box is described in the following section.

Displaying alignment results

You can use the Folder Sequence Query display dialog box to filter results and display the following:

- a text file describing the analysis
Aligning Sequences

- a horizontal map displaying the aligned sequences represented as horizontal bars
- an aligned sequence display.

To display sequence alignment results

1. The Folder Sequence Query display dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example to change the display parameters, choose Database | Align to Folder when any analysis display result window is active.

2. You can limit the displays to a single match or to a consecutive subset of the ordered matches by typing the numbers in the Entries to show text boxes.

3. You can limit the region that will be scored by typing in the numbers that bracket the region in the Score Rgn text boxes, or by selecting a region from the feature selector drop-down menu that appears to the right of the text boxes.

The numbers are with reference to the query sequence.

4. You can limit the region that will be displayed by typing in the numbers that bracket the region in the Display Rgn text boxes, or by selecting a region from the feature selector drop-down menu that appears to the right of the text boxes.

The numbers are with reference to the query sequence. The display region must lie within the score region.

5. Check Description list to display a list of the saved matches in order of optimized score.

6. Check Horizontal map to display a graphical representation of the alignments.
Aligning sequences using a folder search

7. Check **Aligned sequences** to display the optimized alignments between the query sequence and each of the saved matching sequences.

8. Select **OK** to generate the results displays.

The results windows

An example Horizontal Map window is shown below.

The Description List window shows the locus name of the sequence, its optimized and initial scores, and the first part of the sequence entry’s
Aligning Sequences

definition line, if one was present. An example Description List window is shown below:

![Description List Window](image1)

The Aligned Sequence window shows the residue-by-residue comparison of each aligned sequence with the query sequence. An example of the Aligned Sequence window is shown below:

![Aligned Sequence Window](image2)
Aligning sequences using a folder search

The exact appearance of this window is controlled by options on the Aligned Display preferences dialog box, accessed by choosing MacVector | Preferences from the menu, then clicking the Aligned Display icon on the preferences dialog. See “Formatting the aligned sequence display” on page 38, for further details.

For any sequence in the folder, only one match can be reported. If a folder sequence yields more than one match to the query sequence, only the best match is shown in the results window.

Searching Align to Folder results

The Results search available on the Find dialog box enables you to find specified text within the results windows from analyses such as Align to Folder. See “Searching results” on page 113.

Additional information

When searching a user folder, there are certain very rare situations that would cause MacVector to treat some protein sequence files in the folder as if they were nucleic acid sequences and vice versa. This is because of the way that MacVector differentiates sequence types. If a sequence file contains no information about the type of molecule present (this occurs with line or old-format GCG files), MacVector attempts to classify the sequence by counting the number of A, C, G, T, and U characters and non-printing ASCII characters in the first line of the sequence data. If any non-printing ASCII characters (null characters or control characters) are found in the first line of the sequence data, MacVector assumes that the file is not a sequence file and will not attempt to compare it to the query sequence. If there are no non-printing characters, it checks to see if the count of ACGTU characters exceeds 90 percent of the total characters in the line. If so, the sequence is assumed to be a nucleic acid sequence. Otherwise it is considered to be a protein sequence. If any of the protein sequences in the user folder you are searching are line or GCG files, and have an unusual amino acid composition (abnormally rich in alanine, cysteine, glycine, or threonine), they may be mis-tagged as nucleic acid sequences. Alternatively, if there are any nucleic acid sequences present that contain an unusually high percentage of ambiguous base assignments in the first part of the sequence, they may be misclassified as protein sequences and thus not compared with the query sequence. Such misclassifications will probably occur only for very short sequences with unusual compositions.
Aligning sequences using the BLAST search algorithms

MacVector enables you to use the BLAST (Basic Local Alignment Search Tool) heuristic search algorithm to search sequence databases available on the National Center for Biotechnology Information (NCBI) server, to find sequences that are similar to either nucleic acid or protein query sequences. Currently, homologs to a query sequence can be determined using the following search types:

- **BLASTN** - compares a nucleotide query sequence against a nucleotide sequence database.
- **BLASTP** - compares an amino acid query sequence against a protein sequence database.
- **BLASTX** - compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.
- **TBLASTN** - compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands).
- **TBLASTX** - compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

All of the above search types use the newest BLAST algorithms (BLAST 2; Altschul et al. 1997). All except TBLASTX offer gapped alignment searching. Because they yield more biologically meaningful results, gapped searches are enabled by default.

These BLAST search programs ascribe significance to their findings using the statistical methods of Karlin and Altschul (1990) or (1993). For a discussion of basic issues in similarity searching of sequence databases, see Altschul et al. (1994); for information about the BLAST 2 programs, see Altschul et al. (1997).

PSI-BLAST, PHI-BLAST, and 2-sequence BLAST searches are not currently available.

The BLAST programs have been tailored for sequence similarity searching, and are not generally useful for motif style searches.

The BLAST analysis also enables you to:
Aligning sequences using the BLAST search algorithms

- extract matching sequences from the database to the desktop, or save them to a newly created folder on your hard drive;
- extract the annotation and features table sections of the sequence homologs that you have identified;
- extract MEDLINE abstracts from your sequence homologs and save them to a newly created folder on your hard drive.

**Note.** NCBI request that authors should cite Altschul *et al.* (1997) when reporting results using gapped BLAST searches.

**Using the BLAST searches**

MacVector can directly perform BLAST searches of the sequence databases available on the NCBI server over the Internet.

Refer to Appendix A, “Setting up NCBI’s Entrez and BLAST Services”, for details of setting up the Internet access.

To perform a BLAST search, a nucleic acid or a protein Sequence window must be the active window.

The query sequence cannot exceed 100,000 residues. If your sequence is longer than this, you will be required to specify a region of the sequence to be used as the query.

**Performing a standard BLAST search**

The MacVector interface to the BLAST server has been designed to give the same results as the NCBI web page interface. To accomplish this, a standard search is provided on a dialog box with few options.

To perform a standard BLAST search

1. Choose **Database | Internet BLAST Search.**
While the connection is being made to the NCBI server, an information box is displayed. The **Internet BLAST Search** dialog box is then displayed.

**Note.** If any part of the connection process fails, an appropriate error message is displayed and the entire BLAST search is aborted.

2. Choose a search program from the **Program** drop-down menu.  
MacVector only displays program options relevant to the current query sequence type.

3. Choose a database to search from the **Database** drop-down menu.  
MacVector only displays databases relevant to the current search program you have requested.

4. Choose a threshold score for the BLAST search from the **Expect** drop-down menu.  
Lower **Expect** thresholds are more stringent, leading to fewer chance matches being reported.

5. If you do not want a gapped search, click in the **Perform gapped alignment** checkbox to deselect this option.

6. You can limit the query sequence to a region of the entire sequence.  
One way to do this is to type, in the **Region** text boxes, the numbers of the first and last residues in the region. Another way is to select a region from the feature selector drop-down menu to the right of the text boxes.

7. Click **More choices** to expand the dialog box and modify the default settings.  
The extra settings are described in “Performing an advanced BLAST search” on page 271.

**Note.** If a **Fewer Choices** button is displayed, the expanded form of the **Internet BLAST Search** dialog box is already displayed.

8. Select **OK** to perform the BLAST search.  
Alternatively, select **Defaults** to restore the default settings, or **Cancel** to close the dialog box without performing the analysis.

An information box indicates the elapsed time since the BLAST search was started, and the estimated time for the completion of the job. You can click **Close** to dismiss this dialog. Once dismissed the progress of the Blast job can be monitored in the Job Manager. See Appendix B,
Aligning sequences using the BLAST search algorithms

“Using the Job Manager” for more details about the Job Manager. There is also a Stop button which will cancel the BLAST Search.

When the search is complete, the Stop button changes to View.

9. Click View or use the Job Manager to access the results of the completed BLAST search.

Performing an advanced BLAST search

MacVector enables you to change the standard BLAST search options, by expanding the Internet BLAST Search dialog box.

![Internet BLAST Search dialog box]

To perform an advanced BLAST search

1. Choose Database | Internet BLAST Search.

While the connection is being made to the NCBI server, an information box is displayed. The Internet BLAST Search dialog box is then displayed.

Note. If any part of the connection process fails, an appropriate error message is displayed and the entire BLAST search is aborted.

2. Set the Program, Database, and Expect values, as described in “To perform a standard BLAST search” on page 269.

3. Click More choices to expand the dialog box.

The dialog box expands to show additional options.

Note. If a Fewer Choices button is displayed, the expanded form of the Internet BLAST Search dialog box is already displayed.
4. The default setting is to perform a gapped alignment. If you do not want this, click in the **Perform gapped alignment** check box to deselect this option.

5. Set the gapped alignment controls as required (these will be disabled if you deselected **Perform gapped alignment** in the previous step):
   - Use the **Open cost** drop-down menu to choose a penalty value for inserting a gap
     A high value will favor alignments with as few gaps as possible (range 3 - 19, default 11)
   - Use the **Extend cost** drop-down menu to choose a penalty value for extending a gap
     A high value will favor alignments with many short gaps over ones with fewer longer gaps (range 1 - 3, default 1)
   - Use the **X-dropoff** drop-down menu to choose a criterion for discarding initially unpromising alignments.
     Lower values will give faster searches, but may miss some significant matches (range 15 - 50, default 50).

6. Choose a scoring matrix for the BLAST program from the **Matrix** drop-down menu.

   **Note.** This item is disabled if the BLASTN program is selected in the program drop-down menu.

7. Select the **Low Complexity Filter** check box to enable filtering.

   This feature uses sequence similarity to mask regions that are non-specific for protein identification. It can eliminate spuriously high scores that reflect compositional bias rather than specific pairwise alignment (e.g., hits against proline-rich regions or poly-A tails). Queries searched with the BLASTN program are filtered with DUST. Other programs use SEG.

8. Choose a genetic code from the **Genetic code** drop-down menu.

   This is the code used to translate the query sequence, and defaults to the standard universal genetic code.

   **Note.** This item is only enabled if the BLASTX program is selected under the program drop-down menu.

9. Choose a value from the **Output descriptions** drop-down menu to restrict the number of short descriptions of matching sequences reported.
Aligning sequences using the BLAST search algorithms

10. Use the **Sorted by** drop-down menu to control the order in which database sequence matches are reported in the output from a BLAST search:

- choose **pvalue** to sort from the most statistically significant to the least statistically significant
- choose **count** to sort from highest to lowest by the number of High Scoring Segment Pairs (HSP) found for each database sequence
- choose **highscore** to sort from highest to lowest by the score of the highest scoring HSP for each database sequence
- choose **total score** to sort from the highest to the lowest by the sum total score of all HSPs for each database sequence.

11. You can limit the query sequence to a region of the entire sequence. To do this, type in the numbers that bracket the region in the **Region** text boxes, or select a region from the feature selector drop-down menu that appears to the right of the text boxes.

12. Select **OK** to perform the BLAST search. Alternatively, select **Defaults** to restore the default settings, or **Cancel** to close the dialog box without performing the analysis.

An information box indicates the elapsed time since the BLAST search was started, and the estimated time for the completion of the job. You can click **Close** to dismiss this dialog. Once dismissed the progress of the BLAST job can be monitored in the Job Manager. See Appendix B, “Using the Job Manager” for more details about the Job Manager.

There is also a **Stop** button which will cancel the BLAST Search.

---

**Note.** At the conclusion of a BLAST search, matching sequences are not automatically retrieved from the remote NCBI server. If you want to perform additional analyses on matching sequences, you must first retrieve them. See “Retrieving matching sequences” on page 277, for further details.

When the search is complete, the **Stop** button changes to **View**.
13. Click **View** or use the Job Manager to access the results of the completed BLAST search.

This is described in the following section.

**Displaying BLAST search results**

You can use the **BLAST Search Results** display dialog box to filter results and display the following:

- a text file listing the search results
- a textual aligned sequence display

A few statistics of the search are displayed in the statistics panel on the left side of the dialog. These include the name, size and build date of the database used. Below these are the best scores that were found, in particular the best (i.e. lowest) SumP(n) and the largest score obtained by a single high-scoring segment pair. Finally, the value of the expect parameter is shown, and the number of matches that were saved and aligned for viewing in the Description list and Aligned sequences windows.

**To display BLAST search results**

1. Ensure that the **BLAST Search Results** display dialog box is displayed.

This dialog box can be displayed by clicking **View** on the **Internet BLAST Search** dialog box after a search has finished. Alternatively, click **View** on the appropriate job in the Job Manager to display the **BLAST Search Results** display dialog box.

2. Select the **Description list** check box to generate an annotated list of the matching sequences, numbered in the order that was specified in the **Sorted by** search parameter.
Aligning sequences using the BLAST search algorithms

Note. If you want to save matching sequences to a new folder, or open sequence windows for them, you must generate a description list.

You can restrict the description list, by typing in appropriate list numbers in the Entries to show text boxes.

3. Select Aligned sequences if you want to display the optimized alignments between the query sequence and each of the matching database sequences.

You can restrict the aligned sequences displayed, by typing in appropriate list numbers in the Entries to show text boxes.

Note. The aligned sequences window will not be created unless the Aligned sequences checkbox is selected.

4. Select OK to close the BLAST Search Results dialog box and display the chosen results.

This button is only activated if at least one of the checkboxes has been selected.

5. If you need to display further results from the same search, ensure that a BLAST search results window is active, and then choose Database | Internet BLAST Search. The BLAST Search Results display dialog box will be displayed again.

The results windows

The Description List window shows you the saved matches in the order determined by the sort by choice on the Internet BLAST Search dialog box. The list shows the following information:

- the document ID of the sequence, usually a Universal Identifier prefaced by the characters gi |
Aligning Sequences

- a short definition of the sequence
- the score of the highest-scoring segment pair
- the smallest sum probability value of the match
- the number of segments that were aligned to the target sequence.

Note. When the Description List window is active, it can be used to extract database results. See “Retrieving matching sequences” on page 277, for further details.

The Aligned Sequences window displays the alignments of the high scoring segment pairs between the query sequence and the database sequence. The appearance of the aligned sequence display can be changed interactively, see Chapter , “General Procedures”, for details.

The aligned sequences output closely follows the traditional BLAST format used by NCBI:
- each matching sequence has a header section describing the accession numbers and definitions of the entry in the searched database
- the individual high-scoring segment pairs are presented with scoring information for each segment
- the actual alignments with the query sequence presented on top and the database sequence presented below.

Searching BLAST results

The Results search available on the Find dialog box enables you to find specified text within the results windows from analyses such as BLAST. See “Searching results” on page 113.
Aligning multiple sequences using ClustalW

Retrieving matching sequences
Two methods are available to retrieve the matching sequences:

- open a new sequence window for each match of interest
- save each match of interest to a new folder on disk.

To create new sequence windows
1. Make the Description List window active.
2. Select the sequences of interest in the description list. You just need to select at least one character on the line containing the sequence for it to be considered selected.
3. Choose Database | Retrieve to Desktop.
   All selected files are placed in their own sequence window.

To save sequences to a folder
1. Make the Description List window active.
2. Select the sequences of interest in the description list.
3. Choose Database | Retrieve to Disk.
   A standard dialog box is displayed, so you can save the sequences to an existing folder or to create a new folder.

Storing BLAST searches
You can save the contents of the Description List and Aligned Sequences windows to text files in the normal way, by choosing File | Save As... while the window you want to save is selected.

Aligning multiple sequences using ClustalW
You can use the ClustalW algorithm (Thompson et al, 1994) to align several nucleotide or amino acid sequences simultaneously. MacVector provides extra graphical features for the alignments:

- both multiple and pairwise alignments can be displayed in standard MacVector text windows
- multiple alignments can be displayed as high quality PDF images that can be exported to other applications
- a summary of the similarity scores between each pair of sequences can be displayed in tabular form
- a consensus sequence can be displayed and saved
• a guide tree of the sequence comparisons can be displayed, showing the relationships between sequences.

To perform a ClustalW alignment, you need two or more sequences of the same type (nucleic acid or protein) open - either in individual Sequence windows or in one or more MSA windows.

**Performing a ClustalW alignment**

The **ClustalW Alignment** dialog box is divided into four panels:

- **Pairwise Alignment**
- **Multiple Alignment**
- **Sequences To Align**
- **Protein Gap Parameters**

The parameters that can be set within each panel vary depending on the type of sequences being aligned and the alignment speed that is selected. To perform your alignment, set parameters in each panel according to your sequence type and alignment requirements.

ClustalW aligns sequences in two stages. The first, pairwise, stage compares each sequence individually with every other sequence. The Pairwise Alignment parameters control the speed and sensitivity of this stage. The second, multiple alignment, stage progressively merges the sequences, starting with the pair that scored highest in the pairwise stage. The Multiple Alignment parameters control this stage.
Aligning multiple sequences using ClustalW

To perform a ClustalW alignment
1. Choose Analyze | ClustalW Alignment from the menu or click the Align icon on the MSA Editor toolbar.

   The ClustalW Alignment dialog box is displayed.

Setting options in the Pairwise Alignment panel
2. If you are aligning protein sequences, choose the protein scoring matrix from the Matrix drop-down menu.
3. Choose from the Alignment Speed drop-down menu:
   • Slow means that the initial pairwise alignments are performed using a full dynamic programming algorithm
   • Fast uses the Wilbur & Lipman (1983) method.

4. For Slow alignments, do the following:
   • enter a value between 0 and 100 in the Open Gap Penalty text box. This is the score that is subtracted when a gap is inserted in an alignment. Increasing the gap opening penalty makes gaps less frequent
   • enter a value between 0 and 10 in the Extend Gap Penalty text box. This is the penalty for extending the gap by 1 residue. Increasing the extend gap penalty makes gaps shorter. Terminal gaps are not penalized.
5. For Fast alignments, do the following:
   - choose a value from the Ktuple drop-down menu. This is the number of consecutive residues that must match the query sequence exactly before MacVector will attempt to score the matching region. Increasing the value speeds the alignment, but if the value is too large, significant homology between sequences may be missed. Throughout MacVector, Ktuple size is also referred to as hash size or word size.
   - enter a value between 1 and 500 in the Gap Penalty text box. This is the penalty for each gap. The parameter has little effect on speed or sensitivity unless extreme values are entered.
   - enter a value between 1 and 50 in the Top Diagonals text box. This value determines the number of Ktuple matches on each diagonal in an imaginary dot-matrix plot. A large value increases sensitivity, a small value makes the alignment faster.
   - enter a value between 1 and 50 in the Window Size text box. This value specifies the window size around each top diagonal. Diagonals that fall inside this window are used in the alignment. Increasing the window size results in a more sensitive, but slower alignment. Decreasing the window size increases the speed, but may result in small regions of homology being missed.

Setting options in the Multiple Alignment panel

6. If you are aligning protein sequences, choose the protein scoring matrix series from the Matrix drop-down menu.

7. Enter a value between 0 and 100 in the Open Gap Penalty text box. This is the score that is subtracted when a gap is inserted in an alignment. Increasing the gap opening penalty makes gaps less frequent.
Aligning multiple sequences using ClustalW

8. Enter a value between 0 and 10 in the **Extend Gap Penalty** text box. This is the penalty for extending the gap by 1 residue. Increasing the extend gap penalty makes gaps shorter. Terminal gaps are not penalized.

9. Enter a value between 0% and 100% in the **Delay Divergent** text box. By delaying the alignment of the divergent sequences until after the most closely related sequences have been aligned, a more accurate alignment is achieved. The default value is 40%, which means that if a sequence is less than 40% identical to any other sequence, its alignment is delayed.

10. Choose from the **Transitions** drop-down menu as follows:
   - **weighted** to give higher weightings to transitions (A <-> G, T <-> C) compared to transversions (A <-> T, A <-> C, G <-> T, G <-> C)
   - **unweighted** to treat transitions and transversions equally.

**Note.** This option is only present for nucleic acid alignments.

**Setting options on the Protein Gap Parameters panel**

If you are aligning protein sequences, you must set a number of additional parameters.

11. Enter a value between 0 and 100 in the **Gap Separation Distance** text box. This allows you to discourage gaps being opened too close together by increasing the open gap penalty for a specific distance from existing gaps. For example, if the gap separation distance is set to 8, the open gap penalty is increased if the new position is within 8 residues of an existing gap.

12. Select the **End Gap Separation** check box to treat end gaps as internal gaps, to avoid gaps opening too close together, as specified by **Gap Separation Distance**. If disabled, end gaps are ignored.

13. Select the **Residue-specific Penalties** check box to modify the gap opening penalty at each position in the alignment or sequence. The **Open Gap Penalty** is multiplied by a residue-specific gap modification factor, as shown in “Residue-specific gap modification factors for ClustalW sequence alignment” on page 436. If there is a mixture of residues at the position, the multiplying factor is the average of all the contributions from each sequence.
14. Select the **Hydrophilic Penalties** check box to increase the chances of a gap opening within a hydrophilic stretch, as defined by **Hydrophilic Residues**.

15. In the **Hydrophilic Residues** text box, enter the IUPAC one-letter code for each residue that is to be considered hydrophilic. Any run of 5 hydrophilic residues is considered to be a hydrophilic stretch.

**Choosing sequences**

16. From the scrolling list in the **Sequences To Align** panel, highlight the sequences that you want to align.

Use `<shift>` click or `<command>` click to toggle selections. Sequences are aligned in the order they appear in the list.

17. If you want to change the order in which sequences are aligned, do the following:

   - put the mouse pointer over the arrow at the left of the sequence you want to move
   - hold the mouse button down and drag the sequence to the required position
   - release the mouse button.

18. Select **OK** to perform the alignment.

A dialog box is displayed, informing you of the progress of the alignment. You can close this and monitor progress using the Job Manager if you prefer. See Appendix B, “Using the Job Manager” for more details about the Job Manager. There is also a **Stop** button which will cancel the ClustalW Alignment. The ClustalW job runs in the background, allowing you to continue to use other functions in MacVector while the job is in progress.

**Note.** While a ClustalW job is in progress you cannot edit the alignments in the original window, nor can you submit additional ClustalW jobs from that window. However, you can submit additional ClustalW jobs from other windows.

When the calculation is complete, the **Alignment Views** display dialog box is displayed. This is described in the following section. The MSA Editor view is also displayed. This is described in “The MSA Editor view” on page 123.

**Displaying ClustalW alignment results**

The **Alignment Views** display dialog box allows you to choose one or more types of results to display.
Aligning multiple sequences using ClustalW

You can choose from the following output options:

- multiple alignment text display
- pairwise alignment text display
- pairwise matrix display
- multiple alignment picture display
- consensus sequence display
- guide tree.

Only guide trees are discussed here. Refer to “The Multiple Sequence Alignment window” on page 116 for a full discussion of the main display options.

The MSA Editor is also displayed with the results; for more information, see “The MSA Editor view” on page 123.

If you want to recalculate the alignment, select Recalculate to return to the ClustalW Alignment dialog box.

The Alignment Views dialog box is displayed on completion of each analysis. To display this dialog box at other times, for example to change the display parameters, choose Analyze | ClustalW Alignment when any ClustalW analysis results window is active or click on the Views icon on the MSA Editor toolbar.
Displaying a guide tree

You can display a guide tree for aligned sequence results when four or more sequences are aligned by ClustalW. The guide tree shows the relationships that ClustalW generates when doing pairwise alignments, and is shown as a phylogram or a cladogram, with the distances between nodes shown in phylogenetic units. As an approximate guide, a value of 0.1 corresponds to a difference of 10% between two sequences.

Example windows:

![Slanted cladogram and Phylogram]

Note. ClustalW guide trees may not be true phylogenetic trees. See Chapter, "Reconstructing Phylogeny", and "Phylogenetic analysis" on page 413.

To display a ClustalW guide tree

1. In the Picture Display panel of the Alignment Views dialog box, select the Guide Tree check box.
2. Select OK to close the dialog box.

The guide tree is displayed in a Tree Viewer window. You can use the toolbar buttons and the Tree Viewer Options dialog box to select a tree shape, root the tree and edit its appearance. You can also print and save the guide tree. For full details, see “The Tree Viewer window” on page 293, “Editing Trees” on page 295, and “Saving and printing tree diagrams” on page 303.
Overview

Phylogenetic trees indicate the evolutionary relationships among sequences. This chapter describes how to generate phylogenetic trees from multiple alignments of nucleic acid or protein sequences, and introduces the different methods for calculating evolutionary distances and building trees. Further information on the methods is presented in “Phylogenetic analysis” on page 413.

This chapter also describes how to use the MacVector Tree Viewer to inspect and edit phylogenetic trees, and how to print and save them. The Tree Viewer can also be used to inspect guide trees for ClustalW multiple alignments.

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Phylogenetic reconstruction

MacVector offers two modes of phylogenetic analysis, depending on the type of output required. Best Tree mode calculates the best tree using a given method. Bootstrap mode repeatedly resamples the data, generating phylogenies from each of the new data sets. A consensus tree of these phylogenies indicates how reproducible the Best Tree analysis is. The following sections describe how to generate phylogenetic trees using different methods, and how to view and save them. For further information on the methods, see “Phylogenetic analysis” on page 413.

To run a phylogenetic analysis, the active window must be a Multiple Sequence Alignment (MSA) window containing four or more sequences.

Position masking

Alignments are statements of homology. We assume that amino acids or nucleotides at the same position have evolved from the same ancestral residue. In practice, however, it is often difficult to have any confidence in this homology for some regions of the alignment, and these should be omitted from the phylogenetic analysis.

MacVector allows you to mask any positions in the alignment, to exclude them from the analysis. This is done using the MSA Editor. When you enable the position masking tool, a blue bar appears above the alignment. By clicking in this bar at the appropriate locations, you can mask particular sites, which appear grayed.

Tip. If you save the multiple alignment as a NEXUS file, the position mask will be saved with it (as an exclusion set in the assumptions block of the file).
Phylogenetic reconstruction

To exclude sites from phylogenetic analysis

By default, position masking is not enabled, and there is no blue bar displayed above the alignment in the MSA Editor view.

1. With the multiple alignment active and MSA Editor view selected, click the *Prefs* icon on the MSA Editor toolbar.

The **Multiple Alignment Options** dialog box is displayed.

2. Select the **Editor** tab, and check the **Show position mask** box to enable this feature.

3. Select **OK**.

The blue position masking bar is displayed above the alignment in the MSA Editor view.

4. Select the positions to mask, using the mouse as follows:
   - to mask a single position, click in the masking bar at that position
   - to mask adjacent positions, click and drag
   - to unmask a position, click on it again
   - to mask all positions, hold down the Option key and click anywhere in the masking bar. Repeat to unmask all positions
Generating phylogenetic trees

This section describes how you can generate a phylogenetic tree from any alignment of four or more nucleotide or protein sequences. You can limit the analysis to a subset of the alignment, if required.

It also describes how you can check the reliability of this tree, by running a bootstrap analysis to display a bootstrap consensus tree for the same data. There are two stages in the generation of trees: calculation of the distances between pairs of sequences, and reconstruction of the phylogeny using the distance information. All building methods proceed by joining the two most similar sequences first, and then adding the other sequences one by one, in order of decreasing similarity.

To generate a phylogenetic tree

1. With the multiple alignment active and the MSA Editor view selected, click the Phylogeny icon on the MSA Editor toolbar.

Alternatively, choose Analyze | Phylogenetic Analyses | Reconstruct Phylogeny.

The Phylogenetic Reconstruction dialog box is displayed.

2. Use the Tree Building Method drop-down menu to choose a method of building trees.

- **Neighbor Joining** is the default. This method makes no assumptions about rates of divergence in different lineages
- The **UPGMA** method assumes that sequences have diverged at a constant rate.
Phylogenetic reconstruction

3. To specify the method for resolving ties, click the Options button in the Tree Building Method panel. The Tree Building Method Options dialog box is displayed; select either the Systematic or Random radio button.

When there are two nodes or sequences equidistant from the current node in the tree:

- the Systematic method resolves the tie by adding the nodes in the order of the aligned sequences. This is the default method.
- the Random method chooses the order randomly.

4. Use the Distance drop-down menu to choose a method of calculating the pairwise distances between sequences.

For nucleotide sequences the options are:

- Absolute (# differences)
- Uncorrected (“p”)
- Jukes-Cantor
- Tajima-Nei
- Kimura 2-parameter
- Tamura-Nei (default)
- LogDet/Paralinear

For protein sequences the options are:

- Absolute (# differences)
- Uncorrected (“p”) (default)
- Poisson-correction

5. To set the distance options, click the Options button in the Distance panel.
The **Distance Options** dialog box is displayed. Options that are not relevant for your chosen **Distance** method will be grayed out and unavailable for selection.

- In the **Gamma Correction** panel, use the radio buttons to turn gamma correction **Off** or **On**, and enter a **Gamma shape parameter** value in the text box if required. Where gamma correction is available, the default value is 0.5.
- In the **Transition:Transversion Ratio** panel, select the **Estimate** radio button to have the ratio estimated from your sequence data, or select **User defined value** and enter the required value in the text box. Where available, the default value is 1.0.
- In the **Treatment of Gaps** panel, select the radio button either to **Ignore all sites containing gaps** or to **Distribute proportionally**. The default is to **Ignore all sites containing gaps**.

Select **OK** to confirm the settings and close the dialog box. You can use the **Defaults** button to restore the default settings.

6. In the **Mode** panel, select **Best tree**.
7. By default, all sequences will be included in the tree, and they are all highlighted in the **Sequences To Include** list. If necessary, you can edit this list:
Phylogenetic reconstruction

- To exclude a highlighted sequence from the tree, hold down the <shift> key and click on the sequence in the list
- To include a sequence that is not highlighted, hold down the <shift> key and click on the sequence
- To include all sequences, select the Select All button
- To exclude all sequences, select the Select None button.

8. Select OK to confirm the settings and perform the analysis.

Alternatively, you can use the Defaults button to restore the default settings, or the Cancel button to close the dialog without retaining the settings.

During the analysis an information box is displayed, showing a progress bar for each calculation stage. When it is complete, the resulting tree is displayed in a Tree Viewer window. You can control its appearance interactively (see “The Tree Viewer window” on page 293).

Invalid distance warning

In some situations, no valid distances can be calculated for a pair of sequences. When this happens, a warning dialog box is displayed:

If you continue, then for each distance that cannot be determined, MacVector will assign the largest distance that was calculated in the matrix. For more details, see “Invalid distances” on page 415.
To generate a bootstrap consensus tree
1. With the tree displayed, click the Calculate icon on the Tree Viewer toolbar.

The Phylogenetic Reconstruction dialog box is displayed.
2. In the Mode panel, select the Bootstrap radio button.
3. Type in the required Number of replications.
The default value is 1000. Reducing this number gives faster but less reliable results.
4. Select OK to perform the analysis.

After the analysis is complete, the consensus bootstrap tree is displayed in the Tree Viewer window. The consensus bootstrap tree only includes nodes that appear consistently when the data is repeatedly resampled. Nodes that occur very frequently in the resampled data are labeled with their percentage occurrence. You can adjust the cutoff points for displaying and labeling nodes (see “Setting the tree display options” on page 301).

The topology of the bootstrap consensus tree will not always match that of the best tree.

For more details of the method and its interpretation, see “Bootstrap- ping” on page 418.
5. To display the original tree, click the Calculate icon and repeat the calculation using Best tree mode.

Displaying existing phylogenetic trees

When a phylogenetic tree has been calculated, it can be saved along with its associated multiple alignment file, and retrieved when the file is opened.

To display an existing phylogenetic tree

1. With the required alignment active and the MSA Editor view selected, click the Phylogeny icon on the MSA Editor toolbar.

The tree is displayed in a Tree Viewer window.

Displaying ClustalW guide trees

The ClustalW alignment algorithm generates a guide tree which is then used to specify the order in which sequences are aligned (see “Aligning multiple sequences using ClustalW” on page 277). This may not be a true phylogenetic tree, because it is based on local pairwise alignments. However, the tree’s appearance may sometimes help you to judge the reliability of a ClustalW alignment.

To display a ClustalW guide tree

ClustalW guide trees are saved with their associated multiple alignment file, and you can view them when the file is retrieved.

1. With the ClustalW alignment active and the MSA Editor view selected, click the Views icon on the MSA Editor toolbar or choose Analyze | ClustalW Alignment from the main menu.

The Alignment Views dialog box is displayed.

2. In the Picture Display panel, select the Guide Tree checkbox, and select OK.

The ClustalW guide tree is displayed in the MSA Guide Tree view.

Note. You cannot recalculate a ClustalW tree. When a ClustalW tree is displayed in the MSA Guide Tree view, the controls for recalculating and deleting are not available.

The Tree Viewer window

The Tree Viewer window can be used to display phylogenetic trees.
Reconstructing Phylogeny

It includes tools for rooting trees to emphasize the direction of evolution, deleting parts of the tree, rotating nodes to re-order the sequences, and formatting the tree diagram for publication.

The Tree Viewer has a toolbar containing icons that are used to perform the following functions:

**Note.** The Tree Viewer toolbar, like all toolbars in MacVector, can be customized. Right-click on the toolbar to access this functionality. The tools described below are those that appear in the default Tree Viewer toolbar. Some of these tools may be absent and other tools may be present depending on your settings.

The **Slanted**, **Rectangular** and **Phylogram** icons enable you to control the type of tree displayed:

- slanted cladogram
- rectangular cladogram
- phylogram

The **Reroot** icon enables you to select an outgroup. See “Rooting” on page 297.

The **Rotate** icon enables you to change the order in which two nodes are arranged on the screen. See “Rotating nodes” on page 300.

The **Focus** icon enables you to display only the part of the tree you have selected. To re-display the whole tree, double-click anywhere in the Tree Viewer window.

The **Delete** icon enables you to delete the part of the tree you have selected. See “Deleting nodes” on page 299.

The **Calculate** icon provides access to the Phylogenetic Reconstruction dialog box, which can be used to recalculate phylogenies.

The **Sort MSA** icon enables you to sort the alignment to match the phylogenetic tree, when the tree has been calculated for a multiple alignment. See “Sorting a multiple aligned sequence to match a tree” on page 300.

The **Font Bigger** and **Font Smaller** icons enable you to adjust the font size in the Tree Viewer window. Each click increases or decreases the font size by one increment.
The Tree Viewer window

The **Line Size** icon enables you to select the weight of the lines in the Tree Viewer display. Click the **Line Size** icon and choose the required line thickness from the drop-down menu.

The **Prefs** icon provides access to the Tree Viewer Options dialog box. See “Setting the tree display options” on page 301 for more information about the options available in this dialog box.

The **Distances** icon enables you to

At the bottom of the Tree Viewer window there are controls for magnification and page mode.

- Use the **page mode** button to either show or hide dotted lines marking the page boundaries for printed output.

- The **view scale** controls let you set a magnification for viewing the tree, either by choosing from a drop-down menu or by typing in a text box. The current viewing magnification is displayed.

**Tip.** The width of a tree display is set to fit the current paper size. If you want to make a tree wider, use **File | Page Setup** to select a larger paper size or a landscape orientation.

**Editing Trees**

You can edit a phylogenetic tree in a Tree Viewer window by rooting, rotating and deleting, using the icons in the toolbar. You can also format the fonts and lines used in the display, and control the display of branch and node labels. When a tree is saved along with the source MSA document, any editing you have applied to it is retained.

**Note.** If you have deleted part of a tree, the phylogeny will have been recalculated, so it no longer represents the full MSA document.

**ClustalW guide trees vs. Phylogenetic trees**

There are important differences in the way MacVector treats ClustalW guide trees and phylogenetic trees. Both can be edited by rooting and rotating, but ClustalW trees cannot be changed by deleting, because they cannot be recalculated. Phylogenetic trees retain all editing and formatting changes, when their associated MSA documents are saved: ClustalW guide trees, on the other hand, lose all editing changes when their MSA documents are saved.
Tree shapes

The same phylogenetic data can be displayed in any of three styles:

- **Slanted cladogram**

![Slanted cladogram diagram]

- **Rectangular cladogram**

![Rectangular cladogram diagram]

- **Phylogram**
Only the phylogram display mode draws branch lengths to scale, indicating evolutionary distance. This mode is not available for bootstrap consensus trees.

**Rooting**

An important property of a tree is its polarity. The neighbor joining method of generating trees makes no assumption about which sequence is closest to the ancestral sequence, and in which direction evolution has proceeded. This can often be clarified by rooting the tree. Rooting affects only the appearance of the tree, not the underlying phylogenetic analysis.

You can recognize an unrooted tree by its first (leftmost) junction, which is always trifurcated. In a rooted tree, the first junction is always bifurcated - it separates either the outgroup from the rest (outgroup rooting) or two aggregates from each other (midpoint rooting).

Consider this *unrooted tree*, generated by neighbor joining:
This tree does not make it clear that the primates are all closely related.
Here is the same phylogenetic analysis, but shown rooted on an out-group (the mouse):

This tree makes it clearer that the ungulate and primate sequences form two well-defined groups.
Where there is no obvious outgroup, *midpoint rooting* may be useful. This method works by sampling each internal node, calculating the sum of all branch lengths under the nodes to the left and right of it. Where these two sums are most nearly equal, the midpoint is identified. By
identifying its “center of gravity”, midpoint rooting helps to distinguish two groups in this tree:

You can root a tree from a selected outgroup by using the Reroot icon. Alternatively you can specify outgroup rooting in the Tree Viewer Options dialog box, in which case MacVector will root the tree on its longest terminal node. To specify midpoint rooting, use the Tree Viewer Options dialog box.

The rooting of trees generated using UPGMA cannot be changed. ClustalW guide trees can be rooted.

To root a tree on an outgroup
1. In the Tree Viewer window, select the required outgroup by clicking on its branch.

   The branch becomes highlighted, and the Reroot icon becomes active.

   Tip. Ensure that only one sequence is highlighted. You can only root the tree on a single sequence.

2. Click the Reroot icon or choose Analyze | Phylogenetic Analyses | Root Tree on Node.

   The tree is shown rooted on the selected outgroup.

   You can also specify outgroup rooting by using the Tree Viewer Options dialog box (see “Setting the tree display options” on page 301).

Deleting nodes

You can delete a node from a phylogenetic tree. The tree is recalculated automatically, excluding all sequences contained in the node.
To delete a node from a tree

1. In the Tree Viewer window, select a node to delete by clicking on it. The node, together with its sub-nodes, becomes highlighted. The Delete icon becomes active.

2. Click the Delete icon or choose Analyze | Phylogenetic Analyses | Delete Node.

The tree is recalculated, with the node deleted.

Note. To undo a deletion, choose Edit | Undo Delete.

Rotating nodes

You can re-order the sequences in any tree diagram by rotating selected nodes through 180°. Rotation flips the order of all sequences contained in the selected node. Rotating affects only the appearance of the tree, not the underlying phylogenetic analysis.

To rotate nodes on a tree

1. In the Tree Viewer window, select a node to rotate by clicking on the branch above it. The node, together with its sub-nodes, becomes highlighted. The Rotate icon becomes active.

Note. The rotate icon only becomes active when more than one sequence has been selected.

2. Click the Rotate icon or choose Analyze | Phylogenetic Analyses | Rotate Node.

The node is rotated, flipping the order of its sequences.

3. Repeat steps 1 and 2 as required to sort the tree.

Sorting a multiple aligned sequence to match a tree

If a phylogenetic tree has been calculated for a multiple alignment, you can sort the alignment to match the tree. This can be a rapid way to sort sequences into phylogenetic groups.

To sort the MSA by tree order

1. With the Tree Viewer window active click the Sort MSA icon or choose Analyze | Phylogenetic Analyses | Sort MSA by Tree.

The multiple alignment is sorted to match the tree sequence order.
Setting the tree display options

The Tree Viewer display can be controlled using the toolbar buttons (see “The Tree Viewer window” on page 293). More advanced settings can be made by using the Tree Viewer Options dialog box.

To set the display options for the Tree Viewer window

1. Click the Prefs icon on the Tree Viewer toolbar. The Tree Viewer Options dialog box is displayed.
2. To adjust the appearance of the sequence name labels, use the font, size, and style drop-down menus in the Name Labels panel.
3. To adjust the appearance of the branch labels, use the controls in the Branch Labels panel:
   - The Visible check box controls whether or not the labels are displayed. By default the check box is selected and the labels are displayed.
   - To change the appearance of the branch labels, use the font, size, and style drop-down menus.
4. When a bootstrap tree is being displayed, you can use the controls in the **Bootstrap Filter** panel to control the display threshold values:

- Use the **Collapse nodes <** text box to set the threshold percentage occurrence for a node to be displayed in the bootstrap tree. Nodes occurring less frequently than this value will be collapsed. You may enter any value from 50 through 100. The default is 50%.

- Use the **Label nodes >** text box to set the threshold for labeling a node’s percentage occurrence in the bootstrap tree. Nodes occurring less frequently than this value will not be labeled. You may enter any value from 50 through 100, provided it is not less than the **Collapse nodes** value. The default is 70%.

The **Bootstrap Filter** controls are disabled when there is no bootstrap tree in the window.

5. To change the rooting of a tree generated using the neighbor joining method, select the appropriate **Rooting** radio button:

- **Unrooted**: the tree will not be rooted. This is the default setting for neighbor joining trees

- **Midpoint rooting**: the tree will be rooted on its center of gravity

- **Outgroup rooting**: the tree will be rooted on its longest branch

If you have already rooted the tree manually using the **Reroot** icon, then selecting this radio button has no effect.

**Rooting** controls are disabled if the current tree was calculated using UGPMA, because that method always roots the tree.

6. To adjust the rule for displaying any branches with calculated lengths that are negative, do one of the following in the **Negative Branch Lengths** panel:

- Select **Collapse to zero** if you want to collapse such branches in the display. This is the default setting

- Select **Mark as dashed line** to indicate negative lengths by dashed lines.

**Negative Branch Lengths** controls are available only when a **phylogram** display has been selected.

7. To display details of the methods used to calculate the phylogeny, select the **Display summary of parameters** check box.

8. Select **OK** to apply the settings and close the dialog box.
Saving and printing tree diagrams

Alternatively, select **Defaults** to restore the program default settings, or **Cancel** to close the dialog box without applying or saving any changes.

**Saving and printing tree diagrams**

Both phylogenetic trees and ClustalW guide trees can be saved as pictures or printed.

**Saving trees**

If a phylogenetic tree has been generated, it will be saved when you save its associated MSA document, so that when you reload the MSA file and select the **Phylogeny** button, a Tree Viewer window will open and display the tree. Any rooting, rotating, deleting and reformatting you have done is retained when you save the MSA document.

In addition, it is often useful to save an image of the tree as it appears in the Tree Viewer window.

To save a phylogenetic tree diagram

1. With the Tree Viewer window active, choose **File | Print**. The **Print** dialog box is displayed.
2. Click the **PDF** button and choose **Save as PDF** from the menu.
3. Navigate to the folder where you want to save the graphic data and choose **Save**.

**Copying trees**

When the Tree Viewer window is active, you can use **Edit | Copy** to copy the tree to the clipboard. You can then paste it into another application as a picture.

**Printing trees**

An important function of the Tree Viewer is to provide publication-quality printed output. To keep the interface simple to use, the phylogenetic tree display is always scaled to fit the width of the selected paper size. You can set the page size with **File | Page Setup**, and view the page boundaries in the Tree Viewer window, using the **page mode** button in the bottom left corner.

Using the controls in the **Tree Viewer Options** dialog box, you can adjust the line width and font sizes so that both large and small trees are presented clearly.
To print a phylogenetic tree
1. With the Tree Viewer window active, choose File | Print.
   The Print dialog box is displayed.
2. Set the printer controls, and select Print to print the tree.
Overview

This chapter describes how to use MacVector to align one or more sequences against a reference sequence so that you can quickly identify differences or similarities. There are two functions available:

- **Sequence Confirmation** lets you align one or more sequence or chromatogram sample files against a reference sequence. This can be used for confirming the sequence of a cloned fragment of DNA or for identifying SNPs or other differences between clones. It is very similar to sequence assembly, except that the alignment always requires a reference sequence to act as an assembly scaffold.

- **cDNA Alignment** lets you align one or more cDNA clones against a reference genomic sequence. This can be used for identifying where exon/intron boundaries lie in cDNA/mRNA sequences.

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Aligning Sequences to a Reference

Align to Reference

Sequence Confirmation

The **Sequence Confirmation** function lets you align one or more sample sequences against a reference sequence. It has many similarities to sequence assembly, except that it requires and takes advantage of the use of a known reference sequence as a scaffold.

You can use this functionality to help you solve a number of typical laboratory problems:

- Confirming the sequence of a cloned fragment
- Sequencing across the ends of a cloned fragment to confirm the junction sequence
- Screening clones from a site-specific mutagenesis experiment to identify successful mutations
- Screening related clones for single nucleotide polymorphisms

The main limitation of this approach is that you must have a reference sequence to act as a scaffold against which the sample sequences can be aligned. You cannot, therefore, use this function to assemble trace files from *de novo* sequencing projects. For these types of tasks MacVector Assembler is a far better tool. See Chapter 16, “Assembler” for a discussion of the features of MacVector Assembler. However, for the less complex resequencing assembly tasks commonly encountered during a molecular biologist’s day, the **Sequence Confirmation** function is ideal.

To align trace files against a template sequence

1. Open the MacVector sequence file you want to use as your template.
2. Chose Analyze | Align to Reference... to create a new Assembly window.

The template sequence is displayed along the top of the new Assembly window.

3. Click the **Add Seqs** icon on the toolbar in the Assembly window, select the sequence files you wish to assemble against your template then click the **Open** button.

Alternatively, select Edit | Add Sequences From File from the main menu, select the sequence files you wish to assemble against your template then click the **Open** button.

**Note.** You can hold down the `<shift>` key to select multiple sequences to import.
MacVector attempts to import all of the selected files into the assembly project. An error message is displayed listing the names of any files that could not be imported.

The Assembler window updates to display the sequences in the order in which they were imported. If trace data is present, this is also displayed in a lower trace panel.

**Note.** Unaligned sequences are displayed in italics starting at residue “1” to alert you to the fact that they have yet to be aligned (or could not be aligned after automated assembly). Italicized sequences are not included in consensus calculations.

4. Click the **Align** icon on the toolbar in the Assembly window. The **Align to Reference Parameters** dialog box is displayed.

5. Ensure that **Sequence Confirmation** is selected from the **Alignment Type** drop-down menu.

6. Click **OK** to perform the alignment using the default parameters. See “Alignment parameters” on page 309 for more information about modifying the alignment parameters.

The calculation may take some time if your sequence is long and you have many trace files in the alignment.

7. Finally you can save the edited reference sequence in MacVector format by choosing **File | Save As...** from the Assembly editor window. You can save the actual sequence confirmation project itself at any time and you will be prompted to save any changes when you close the project window.

**cDNA Alignment**

The **cDNA Alignment** function lets you align one or more cDNA clones against a reference genomic sequence. This can be used for identifying where exon/intron boundaries lie in your cDNA/mRNA sequences.

**To align cDNAs against a genomic sequence**

1. Open the MacVector sequence file you want to use as your template.

2. Chose **Analyze | Align to Reference**... to create a new Assembly window.

The template sequence is displayed along the top of the new Assembly window.
3. Click the **Add Seqs** icon on the toolbar in the Assembly window, select the cDNA sequence files you wish to assemble against your template then click the **Open** button.

Alternatively, select **Edit | Add Sequences From File** from the main menu, select the cDNA sequence files you wish to assemble against your template then click the **Open** button.

**Note.** You can hold down the `<shift>` key to select multiple sequences to import.

MacVector attempts to import all of the selected files into the assembly project. An error message is displayed listing the names of any files that could not be imported.

The Assembler window updates to display the sequences in the order in which they were imported. If trace data is present, this is also displayed in a lower trace panel.

**Note.** Unaligned sequences are displayed in italics starting at residue “1” to alert you to the fact that they have yet to be aligned (or could not be aligned after automated assembly). Italicized sequences are not included in consensus calculations.

4. Click the **Align** icon on the toolbar in the Assembly window.

The **Align to Reference Parameters** dialog box is displayed.

5. Select **cDNA Alignment** from the **Alignment Type** drop-down menu.

6. Click **OK** to perform the alignment using the default parameters.

See “Alignment parameters” on page 309 for more information about modifying the alignment parameters.

The calculation may take some time if your genomic sequence is long and you have many sequences in the alignment.

7. Finally you can save the edited reference sequence in MacVector format by choosing **File | Save As...** from the Assembly editor window. You can save the actual sequence confirmation project itself at any time and you will be prompted to save any changes when you close the project window.

**Alignment algorithms**

During the first step of a **Sequence Confirmation** alignment, the algorithm finds all of the short sequences of perfect **Hash Value** matches and then extends each match left and right to the end of the perfect alignment. This “diagonal” is scored based on how many residues of perfect matches are found. The highest scoring diagonal is then extended past the mismatches at each end to get the alignment generating the best pos-
Alignment parameters

Possible score. The Sensitivity setting determines how far ahead the algorithm looks to find the optimal alignment. An extension is terminated either when the end of one of the sequences is reached, or if the current score is $X_{Dropoff}$ below the best score.

The cDNA Alignment algorithm takes a more complex approach. It needs to deal with multiple segments, so instead of just taking the best diagonal, it keeps Max. Diagonals in an array. Each of these diagonals is extended separately to find the best possible score for each diagonal. The diagonals are then assembled into a segmented alignment by starting with the highest scoring diagonal, then adding the next best scoring diagonal that will fit on either side of it. The process continues through the sorted list of diagonals until all diagonals that can be fitted in the alignment have been accounted for.

Note. The most CPU intensive part of the algorithm is the extension of the perfect diagonals, accounting for mismatches, insertions/deletions etc. With a large number of diagonals, this can take a substantial amount of time, especially if a high Sensitivity value is used.

The Minimum Score for a diagonal is the score which must be exceeded before a diagonal is saved. With the current default of 25, where the residue Match is 2, this means that at least 13 perfect residues are needed before a diagonal is saved.

The default alignment parameter values for cDNA Alignments are different to those for Sequence Confirmation. The Hash value is larger (6) so the algorithm needs at least 6 perfect matches before a diagonal is extended - this speeds the initial search somewhat. The Sensitivity is also reduced (3), to speed up the extensions. The Mismatch and Gap Penalty are higher, to decrease the likelihood of ragged ends. However, it should be noted that this can be disadvantageous if you are aligning trace files of cDNA clones against a genome and, in those circumstances, these values should be reduced to 3 or 4.

Alignment parameters

The following parameters have an impact on the alignment algorithm:

**Match**

(Valid range -100 to 100, default 2). This is the value the algorithm assigns to a match between a sample residue and the reference residue. It should typically be a positive value.

**Mismatch**
Aligning Sequences to a Reference

(Valid range -100 to 100, default -4). This is the value the algorithm assigns to a mismatch between a sample residue and the reference residue. It should typically be a negative value so that it reduces the cumulative match value as the algorithm extends the match between the sequences.

Ambiguous Match

(Valid range -100 to 100, default 0). This is the value the algorithm assigns to an ambiguous match between the sample and reference residues. The default is a neutral value – you can increase this to the match value if you want ambiguous matches to be treated exactly the same as matches. Alternatively, assigning it the same value as the mismatch parameter treats ambiguous matches as full mismatches.

Gap Penalty

(Valid range -100 to 100, default 3). This is the value the algorithm subtracts from the cumulative match score whenever it has to insert a gap character. Unlike some other algorithms, the SNP Assembly algorithm does not distinguish between gap insertions and gap extensions – all gaps are treated as a gap insertion.

Hash Value

(Valid range 1 to 6, default 4). This is the number of bases MacVector uses for the hashing algorithm. A value of 4 (the default) means that MacVector initially only searches for perfect 4 base matches between the reference and sample sequences. Larger values lead to faster searches, but with reduced sensitivity.

Sensitivity

(Valid range 1 to 8, default 4). This value affects what MacVector does when it is extending a match and encounters a mismatch. The value determines how far ahead the algorithm should look to determine whether to insert a gap in either of the sequences or to accept the mismatch. A value of 4 means it looks ahead in all directions until 4 additional mismatches (or the end of the sequence) has been encountered. The larger the value, the more likely the algorithm can handle longer regions of poor quality sequence, but performance will be much slower. The default (or even a smaller value) is quite adequate for most sequencing projects. Only increase this if you have long regions of poor quality sequence that is being poorly aligned using the default parameter.
Alignment parameters

Score Threshold
(Valid range 1 to 1000, default 50). This value controls how MacVector determines that a match is significant. After finding an initial match, MacVector attempts to extend the match in each direction using the match/mismatch and gap penalty scoring parameters. It retains the extended segment that gives the highest score. If the best score exceeds the score threshold, then MacVector considers this to be a significant match and includes the sample sequence in the alignment. If no individual match segment exceeds this score, the sample is treated as “unaligned” and will appear in the alignment view in italics. If you dramatically change the values for match/mismatch, you should alter this to keep it approximately in sync.

X Dropoff
(Valid range 1 to 1000, default 15). This value is used by MacVector to tell it when to give up extending a match. When extending, it keeps track of the best possible match score. It continues to extend the match in each direction, only giving up when the cumulative score falls to less than $X \text{ Dropoff}$ from the best score. If you reduce this to a low value (e.g. “0”) only perfect matches will be generated in the final alignment. You should typically set this to a value that will permit a few mismatches to be incorporated into the alignment to allow for sequencing errors. A smaller value will speed up calculations, but may not correctly align longer regions of poor quality matches.

Max. Diagonals (cDNA Alignment only)
(Valid range 1 - 1000, default 50) This is the maximum number of diagonals that MacVector should keep and evaluate as potential exons during cDNA alignment. If this is set too low, MacVector may miss some short exon sequences. Setting this to a large value will lead to longer computation times.

Minimum Score (cDNA Alignment only)
(Valid range 1 - 1000, default 25) This is the minimum acceptable score for a diagonal to be saved. The default setting of 25, in conjunction with the default residue Match score of 2, means that a diagonal must span at least 13 perfect matches before it is saved for later evaluation.
The Assembly editor

The Assembly editor is used for editing Contigs in Assembler and also as the main editor for **Alignment to Reference**. However, the interface does change somewhat, depending on which function is being used.

The **Alignment to Reference** tool is used for aligning chromatograms with a template and also for aligning cDNA with a genomic template.

The editor only displays the lower panel, showing individual trace files, when trace file data has been imported. Where no trace files are present, the lower panel is hidden.

The Assembly editor is highly interactive. All of the sequence residues can be edited, although there are some restrictions that simplify the use of the editor for sequence confirmation and SNP identification purposes.

The numbering used for the reference sequence is the original numbering. Gaps are not included in the numbering, or in the locations shown in the features table, so that these are directly comparable to the original sequence.

You can edit the reference sequence at any time. To delete a base, simply type the `<space>` character. Visually, this converts it into a gap character but behind the scenes the base is physically deleted from the reference. If all of the sequences that overlap a position have gaps, the position will be closed up and the shared gaps will be deleted.

**Note.** All editing uses overwrite mode rather than insert mode. Hold down the `<option>` key to insert gaps or residues.

You can edit any of the sample sequences (in either the upper or lower pane) in a similar way. As you type, the consensus sequence is updated in real time. If you delete the last non-gap character at a position, the gap will close up as with the reference sequence.

You can copy any selection of more than one residue. There are important differences in the way the reference sequence is copied compared to the consensus or sample sequences:

- when the reference sequence has the primary highlight, the reference sequence and all overlapping features are copied to the clipboard. In this case, all gaps are stripped out so that you see the “real” ungapped sequence if you then paste the copied sequence into another window.
The Assembly editor

- when a sample sequence or the consensus is copied to the clipboard, only the actual sequence characters are copied, in addition, all gaps are retained in the sequence.

If you want to change the reference sequence to match the consensus sequence, select the region of the consensus that you want to copy. Choose Edit | Copy, carefully place the cursor on the first residue in the reference sequence that you wish to replace, then choose Edit | Paste. The first time you do this you will get a warning message, but when you click “Paste Anyway” the reference sequence residues will be replaced by the copied consensus sequence.

To remove a sequence from the alignment, click on the title button to select the entire sequence and press the <delete> key. Most actions are “undoable” to a single level.

Note. If you are editing very large assemblies, certain actions (e.g. deleting one or more sequences) may be slow, while a copy is made of the assembly to allow a later undo.

To delete a residue, overwrite it with either a <space> or a “-” character. The consensus will be updated dynamically. If all overlapping sequences have a gap at that location, the gap will be closed up. Deletions at either end of a sequence are considered “true” deletions and the residues will actually be deleted, rather than being replaced by gaps.

The following keys may be used to edit the alignment:

- any IUPAC letter replaces the base
- any IUPAC + <option> inserts that base
- <space> or ‘-’ replaces the selected base with a gap
- <option> + <space> or <option> + ‘-’ inserts a gap. In the reference sequence, this will insert gaps into all of the reads sequences to maintain the alignment. If this is not what you desired, simply use the <delete> or <backspace> keys to delete the gaps in the read sequences.
- double-click on a read to select the entire sequence, then the left and right cursor keys and will "nudge" the read left or right. This only works for a selection of the entire sequence, and will not nudge exons or introns.
- <delete> or <backspace> physically deletes the selected bases.
Limitations of the Assembly editor

To simplify the use of the Assembly editor, the following restrictions apply:

- Although you can copy any number of residues, pasting is disabled for all sequences.
- You cannot perform a block selection of the sample sequences - only one sample sequence can have residues selected at any one time (although you can select multiple sample sequences to remove them from the assembly).
- You cannot edit the consensus sequence, it is always calculated dynamically. However, you can select and copy residues on the consensus line.

As well as viewing the sequence in the main view, the editor also includes other views, just like the Sequence editor and the MSA editor. These are accessible from the tabs below the toolbar.

The Assembly map view

The Map view displays the features table of the main sequence. It also shows aligned and unaligned reads. This view is connected to the main view so, clicking a feature on the Map view will highlight that sequence in the main view.

Tip. To select a single feature within a multi-segmented feature, hold down the <option> key whilst clicking on that feature.

Colors and fonts

By default, MacVector shows all residues in black Monaco 9 normal font. You can change this by choosing MacVector | Preferences from the main menu, then selecting the Fonts icon. The Editor window font section of this dialog box controls the font used for all of the different sequence editors within MacVector.

In the Assembly editor, certain font variations are used to indicate status:

- Italics – unassembled sequences are shown in italics. They are always positioned at “1” in the alignment. All sequences that are added to an alignment are initially unassembled and will be shown in italics. After assembly, only those sequences that do not have significant alignments with the reference sequence are shown in italics.
The Assembly editor

**Note.** When italics are used, they always apply to the entire sequence. There are no circumstances in which only a subset of a sequence is displayed in italics.

- Gray text – This is used to indicate the regions where a sequence could not be aligned with the reference using the automated assembly algorithm. Grayed-out residues are not considered in consensus calculations.

**Navigation**

**Scroll Bars**

There are three scroll bars in the assembly window. Each will respond interactively to drags on the “thumb” and will scroll the appropriate display incrementally when clicked on the arrows or in the “page scroll” region.

- Alignment Vertical – this scroll bar lets users scroll vertically through the sample sequences that have been added to an assembly. The reference and consensus sequences always stay at the top of the alignment pane. Because each sample sequence has its own line in the display, you may see a lot of “white space” while scrolling through a large assembly.

- Multiple Trace Vertical – this scroll bar lets users scroll vertically through the trace sample sequences that overlap the current Alignment view. This is typically only a subset of the available sample sequences.

- Horizontal – this scroll bar lets users scroll horizontally through the entire alignment.

**Scaling Controls**

These controls affect the way the trace panes are displayed. There are two types:

- Width Control – this is a single control on the toolbar. When dragged, it interactively adjusts the horizontal scaling factor of the trace displays. The default value displays the traces at a scaling factor of one pixel per sample point.

- Vertical Gain – each individual trace pane has a vertical slider in the left-hand pane that controls the vertical scaling of the traces. The default is that the tallest peak in the trace is “100%”. When dragged, it interactively adjusts the vertical scaling factor of the
trace so that users can “zoom in” to more closely examine areas of ambiguity.

Miscellaneous

ShowAsDots mode
The user can toggle the “ShowAsDots” button to switch between two different display modes. In normal mode, all sequences are displayed as expected. When “ShowAsDots” is enabled, residues in the consensus and sample sequences that exactly match the reference sequence are shown as dots. This includes gap characters, but not unaligned sample sequences, spaces or “masked” regions where a sample sequence is not considered to be aligned to the reference. The user can edit “dots” as with any normal residue – if they type a residue that matches the reference, that residue will be displayed as a dot.

Cut/Copy/Paste
Cut is always disabled. Copy is active only when a selection is present and acts on the currently selected sequence as follows:

• If the reference sequence is highlighted, the selected residues in the reference are copied to the clipboard as a nucleic acid sequence. Any overlapping features are NOT copied.

• If the consensus sequence is highlighted, the selected residues in the consensus are copied to the clipboard as a nucleic acid sequence. Any overlapping features are NOT copied.

• If a sample sequence is highlighted (and the reference is not), the selection in that sample sequence is copied to the clipboard as a nucleic acid sequence.

Paste is enabled only when the reference sequence is selected. When you paste into the reference, the residues on the clipboard will overwrite the reference sequence, NOT insert. You can use this feature to copy and paste the consensus sequence into the reference.

Undo
Most operations in the assembly window can be undone, as with other MacVector windows. In particular, you can undo an assembly operation, and also adding/deleting sequences.

Using the Find options

Standard Find
The user can select the “Find” item in the “Edit” menu to bring up the standard Find dialog. This lets users find matching residues in the reference sequence. Note that any gaps in the reference sequence are ignored during the search, so searching for “GATC” will find a match with the sequence “G-AT--C”.

Mismatch Find

The dialog lets the user find mismatches between the reference sequence and the consensus. It is invoked by clicking on the Find button on the toolbar (the image with a pair of binoculars and a "dotted" mismatched base). When the "Find" button is selected, the display changes so that the first mismatch between the reference and consensus is selected and displayed. When “Find Next” is chosen, the display centers on the next mismatch between reference and consensus. If you have multiple assembly windows open, the find dialog is always associated with the one at the front. When a different type of window is selected, the dialog is hidden to help reduce screen clutter.

Saving and loading alignments

File format details

Alignment files are BSML (Biological Sequence Markup Language) XML files. The file contents can be viewed and edited with any standard text editor. The contents of the file largely follow the standard BSML format with some variations as appropriate. There have been a few changes to support sequence assembly:

- The “model-type” is “assembly” rather than “sequence”
- Each sequence in the alignment is stored in standard BSML/DS Gene format – however, the “ID” field of each sequence has specific meaning. The edited reference sequence has ID “REFERENCE_SEQ”, the consensus is “CONSENSUS_SEQ” and each component is labeled “SEQ_0”, “SEQ_1” etc. A copy of the original sequence, along with all features, annotations and feature appearance information is stored in “ORIGINAL_SEQ”.
- Each component sequence has an “interval-loc” entry that defines its location and strand on the alignment. This is a standard BSML model, but is usually associated with features rather than sequences.
- Each component sequence has additional attributes;
Aligning Sequences to a Reference

- “IsAssembled” – “0” for no, “1” for yes
- “ClipLeft” – the extent of the masking at the 5’ end of the sequence.
- “ClipRight” – the start of the masking at the 3’ end of the sequence.

- There are additional attributes stored at the “definitions” level describing the parameters used in the assembly. Most correspond directly to parameters in the assembly dialog:
  - “GapPenalty”
  - “MismatchScore”
  - “MatchScore”
  - “AmbiguousScore”
  - “HashValue”
  - “ScoreThreshold”
  - “XDropOff”
  - “Recursion” – this is shown in the dialog as “sensitivity”
  - “Threshold”

- There are additional attributes stored at the “definitions” level describing the current settings of the assembly window:
  - “ShowDots” – “0” for no, “1” for yes – determines if matches to the reference are shown as dots or standard residue characters.

Saving alignments

The user can save the alignment at any time. The usual rules apply:

- **Save As...** is always enabled. The user can choose to save the alignment under a different name or in a different format.
- **Save** is only enabled if the alignment has been edited since the last save. It is also enabled for new alignments, but will bring up the **Save As...** dialog as a valid file name is not initially specified.

Saving the modified reference sequence

The user can choose **Save As...** at any time and choose **MacVector NA Sequence** format from the **Format** popup menu. This saves the reference
sequence (complete with any annotations and features) in standard MacVector single sequence format.

Loading alignments

The user can open an existing alignment at any time using the **File** | **Open...** command. Currently, alignments files can only be viewed/selected if **All Documents** or **All Readable Documents** is selected in the **Enable** popup menu. After selection, the alignment will load and be positioned at the start of the alignment. The status of the **show dots** tool-bar button is remembered, but all other display information is reset to the default.

**Tutorial**

**Sample Files**

After installing MacVector, you will find example files for this tutorial in the folder;

MacVector/Sample Files/SequenceConfirmation

This contains a single annotated MacVector format file called **SampleSequence** along with a folder containing 32 chromatogram files in SCF format.

**Opening “SampleSequence”**

The first step in the tutorial is to open and explore the sample sequence to be used in the analysis;

1. Select **File** | **Open** and navigate to the **Tutorial Files/Align To Reference/Sequence Confirmation** folder.
2. Select the sequence entitled **SequenceSample** and click on the **Open** button.
A standard MacVector nucleic acid sequence window will open;

3. Click on the **Map** tab to open the Map view.

The sequence has already been annotated with three open reading frames. The tutorial will show how this information is retained throughout the sequence confirmation analysis.

**Opening the Assembly window**

4. Choose **Analyze | Align To Reference** from the main menu.
The Assembly window opens.

This window contains a copy of the original SampleSequence. The actual sequence residues are displayed along the top line of the window. You can click on the Map, Features, Annotations and Text tabs to see exactly the same information that was present in the original sequence.

**Note.** Unlike many MacVector analysis windows, there is no link between the sequence confirmation assembly window and the original sequence.

The Assembly window is split into an upper and a lower pane. We will see that the lower pane is used to display chromatogram information. The relative size of the two panes can be adjusted by clicking and dragging on the vertical splitter control in the right hand border:

Similarly, a vertical splitter control is present in the bottom border that lets you adjust the width of the title pane on the left hand side:

5. Close the original SampleSequence editor window

The window closes, but the Assembly window stays open. This reinforces the fact that the Assembly window contains a copy of the original sequence. This is different from many other MacVector analysis windows, but is necessary to simplify editing and saving in the window.

**Adding Sequences to the Assembly**

1. Click the Add Seqs icon on the toolbar or select Edit | Add Sequences From File from the menu.
2. In the file open dialog, navigate to the folder; Sample Files/SequenceConfirmation/TraceFiles
3. Click on the first file in the folder, scroll to the bottom of the list, then hold down the <shift> key and select the last file in the folder.
4. Finally, click on the Open button to import the entire list of files into the Assembly window.

The Assembly window should be populated with the imported trace files. You can import trace files in ABI, SCF and ALF formats. In addition, you can import plain sequence files in MacVector or FastA formats.

The imported sample sequences appear in the upper pane, with their titles displayed as buttons in the left hand pane, along with an arrow head indicating the direction of assembly. You can click on the buttons to select the entire sample sequence.

The imported sample sequences are initially shown in italics to indicate that they are unaligned. They are also inserted into the assembly at the left-most position. At this stage the consensus sequence is blank because the consensus calculation only considers assembled sequences. We will see the consensus become updated later when we assemble the sequences.
The lower pane shows the actual chromatogram trace displays of the imported sample sequences. If any MacVector plain sequence files were imported, these are not shown in the pane. We will look at these in more detail later.

5. Click on the **Map** tab.

The Map view opens, showing each of the added sample sequences as a pale gray arrow at the beginning of the sequence with italicized labels.

![Map view screenshot](image)

Each sample sequence is displayed as type “Read*”. You can show/hide these on an individual or group basis using the floating features palette window. If you don’t see this window, make it visible by choosing the menu option **Windows | Show Graphics Palette**.
Aligning Sequences to a Reference

Assembling Sequences

1. Return to the Editor view and click the Align icon on the toolbar or select Analyze | Align To Reference... from the main menu.

The Align To Reference Parameters dialog box is displayed.
For most assemblies where the sample files are of good quality and are closely related to the reference, the default parameters are usually ideal. If you want to try other combinations of parameters, you can always revert to the recommended parameters by clicking on the **Defaults** button. Although not used in this tutorial, this dialog also contains the **cDNA Alignment** options.

2. Click on the **OK** button to initiate the assembly calculation.
Once complete, the sequence confirmation window refreshes to display the aligned sequences. Any sequences were not assembled remain italicized and positioned at the beginning of the assembly.

During the assembly, gaps become inserted into the reference sequence. However, these are for display purposes only so that the assembled sample sequences can align appropriately – behind the scenes, the reference sequence is unchanged after the assembly. The only way the reference sequence changes is if you make edits to the sequence directly.

The assembly algorithm understands that the sample sequences may have vector sequence at the ends, or poor quality sequence that interferes with the assembly. To account for this, it will terminate the alignment extension when it encounters poor quality matches. The display indicates those residues that are “masked” (i.e. not included in the assembly) by drawing them in gray rather than black.
3. Click again on the **Map** tab.

The Map view reopens, but this time the sample sequences are shown in their new location on the assembly, and, in addition, they are shown in a darker color with non-italicized labels. This is because assembled sequences are given the type “Read” rather than the “Read*” type assigned to unassembled sequences.

### Navigating in the Assembly window

The Assembly window provides a number of different functions for exploring and selecting sequences and residues in the assembly. You can use these tools to quickly locate differences between the assembled sample files and the reference sequence.
Aligning Sequences to a Reference

1. Click on a base in the reference sequence.
   The base highlights as expected, but, in addition, the equivalent bases in the consensus sequence and aligned sample sequences highlight in gray. The same bases are highlighted in the lower multiple trace pane, and the chromatograms scroll so that the highlighted bases are all aligned at the center of the screen.

2. Click on a base in the consensus sequence.

3. Drag select in the consensus to highlight multiple residues.
   This has a very similar effect to clicking on the reference sequence except that now the consensus sequence gets the primary highlight and the reference sequence shows no selection. However, the aligned sample sequences become highlighted and the chromatograms scroll just as with the reference sequence.
   You can use this feature at any time to realign the chromatogram display to any location in the reference or consensus sequence. When drag-selecting, all of the corresponding residues in the assembled sample sequences become highlighted and the chromatograms scroll and align to the center residue of the selection.

4. Use the horizontal scroll bar to scroll through the sequence.

5. Use the upper vertical scroll bar to scroll down through the assembled sample sequences to keep the appropriate sequences in view as you scroll horizontally through the assembly.
   Each assembled sample sequence gets its own line in the upper pane, so you need to scroll vertically to keep them in view. You can also use the vertical splitter control to view more of the sequences in the upper pane.
   As you scroll through the assembly, the chromatograms in the lower pane scroll appropriately. They key in on the center base in the visible portion of the reference sequence, although the selection does not change and will scroll off the screen as you navigate through the assembly.

6. Click on a base in the one of the assembled sample sequences.
   This selects the residue in the sample sequence and also in the chromatogram. However, the chromatograms do not realign – this lets you edit the sample sequences without the chromatograms constantly realigning.
Identifying Mismatches

1. Click the **Show Dots** icon on the toolbar.

![Show Dots](image)

The upper pane redraws so that any residues in the consensus or assembled sample sequences that match the reference are shown as dots. This has a dramatic visual effect on identifying mismatches between the sample sequences and the reference.

2. Click the **Find Mismatches** icon on the toolbar.

The **Find** floating window is displayed.

![Find](image)

3. Click on the **Find** button.

This searches for the first mismatch between the consensus sequence and the reference sequence. The display refreshes as if you had clicked on that residues in the reference sequence i.e. that residue becomes selected and the chromatograms scroll to that position.

4. Click on the **Find Next** button in the **Find** window to locate the next consensus/reference mismatch. This should occur at residue 998.

5. Examine the chromatograms – again the reference sequence is incorrect: in this case it has a “T” instead of a “C”.

6. Continue clicking on **Find Next**.

You can work your way through the sequence identifying all of the residues where the consensus does not match the reference sequence.

Editing Assemblies

You can edit the reference sequence directly, or any of the assembled sample sequences. You **CANNOT** edit the consensus sequence. This is always calculated dynamically from the overlapping sample sequences.
1. Click the **Find Mismatch** icon on the toolbar to ensure the window is open.

2. Click on the **Find** button to reset the assembly to the first consensus/reference mismatch. This should be around residue 705.

3. Examine the chromatograms – clearly the reference sequence is incorrect: it has a “C” where most of the sample sequences indicate the residue should be a “T”.

4. Type a “T”.

   The residue changes as you would expect. If you have **Show Dots** set, the consensus and sample sequence residues will change to dots to indicate that they now match the reference.

5. Click on **Find Next** a second time and replace the mismatched residue at that position.

6. The third mismatch between the consensus and reference sequence occurs at the start of a region with a number of mismatches between the two sequences. Click on the first mismatched residue in the consensus sequence. Drag-select across the entire region of mismatched bases – about 40 bases or so.

7. Choose **Edit | Copy**, then click on the first mismatched residue in the reference sequence. Choose **Edit | Paste**. A dialog appears, warning you that you are about to overwrite the reference with the copied residues.

   ![Paste Anyway dialog](image)

8. Click on **Paste Anyway** to paste the selected residues over the reference sequence.

   The reference sequence is overwritten with the copied residues. You should use this feature with care; it is designed to simplify copying the consensus sequence to the reference, but if you make a mistake, the reference can become badly corrupted. However, you can always choose **Edit | Undo** to revert the last editing action.

9. Choose **Edit | Undo**, then repeat the last copy/paste operation with the **Show Dots** mode enabled.
Tutorial

The consensus sequence contains dots where it matches the reference, when you copy the consensus, the original residues are copied to the clipboard, not the dots. You can confirm this by pasting into an external text editing application if you wish. You can also paste the residues copied from the consensus line into a new MacVector nucleic acid sequence window.

**Note.** When you copy and paste the consensus, any gaps are carried along with the sequence. When you copy and paste the reference sequence, gaps are removed.

**Saving Assemblies**

1. Choose **File | Save**.

   The standard file **Save** dialog box appears.

2. Choose a suitable location for the file and click on the **Save** button.

   This saves the assembly in MacVector’s assembly format. This is an XML format based on the BSML standard. You can view the contents of the file using a standard text editor (e.g. Microsoft Word) if you want to see how this is formatted.

3. Choose **File | Save As**. This time, choose “MacVector Single Nucleic Acid File” from the **Format** drop-down menu before choosing a file name and location.

   This saves a copy of the reference sequence, complete with all features and any edits you have made.

4. Open the file you have just created.

**Tip.** The easiest way to do this is to select the file name from the "Recent Files" appended to the bottom of the **File** menu.

   The file opens as a standard MacVector nucleic acid sequence file. If you look at the feature list, you will see that the locations of the assembled chromatogram files have been saved in the file as “Read” features. This gives you a record of the traces you used in the sequence confirmation assembly.

**Analyzing the Reference Sequence**

You can perform any MacVector nucleic acid sequence analysis directly on the sequence confirmation assembly reference sequence. There is no need to save the file in MacVector format before running an analysis.
1. If you do not still have it open, open the original SampleSequence nucleic acid file you started with.

2. Choose Analyze | Pustell DNA Matrix from the main menu.

3. Make sure SampleSequence is selected in the left pane and the Assembled sequence in the right pane.

4. Click on OK.

When the calculation is complete, make sure the Matrix Map and Aligned Sequences options are selected in the results display options dialog and click on the OK button.

5. As you scroll through the aligned sequences result window, you should be able to spot the mismatches in the regions where you made the edits in the reference sequence.
Now try a Restriction Enzyme analysis on the assembled sequence and compare the results to an analysis on the original SampleSequence. You should see that the edit you made at residue 703 created an Eco RI restriction site that is not present in the original sequence.
Overview

This chapter describes the Assembler plugin module, including a Quick Start guide and an in depth Tutorial. Additional documentation on the phred, cross_match and phrap algorithms can be found in the MacVector 10.5/Documentation/ folder.

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Introduction

Assembler is an add-on module for MacVector 9.0 or later. It provides an intuitive interface to the industry standard phred, cross_match and phrap algorithms developed by Phil Green's group at the University of Washington. Assembler integrates tightly into MacVector so that they appear as a single application. This means that when you create assembled contigs, you can immediately analyze the sequences using any of MacVector's DNA analysis or manipulation functions.

This chapter includes a tutorial that will guide you through the process of assembling sequences using MacVector Assembler. Read the quick start section to quickly get going with a simple assembly project. “Quick start” on page 341

Glossary

There are a few terms that are used in contig assembly that you may not be familiar with;

<table>
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<th>Term</th>
<th>Description</th>
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<tr>
<td>Base Call</td>
<td>The interpretation of the peaks in a trace file to identify the most likely DNA sequence.</td>
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<td>Chromatogram</td>
<td>The trace information from an automated sequencing machine. The terms “chromatogram files” and “trace files” are used interchangeably in this tutorial to describe the files generated by automated sequencing machines.</td>
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<td>Consensus</td>
<td>The most likely sequence of a contig, determined by taking all of the overlapping sequences into account.</td>
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<td>Contig</td>
<td>An assembly of two or more overlapping sequences.</td>
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<td>Quality Value</td>
<td>A value assigned to a base call to reflect the probability that the base call is in error. Uses a scale from 0 to 99.</td>
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<tr>
<td>Reads</td>
<td>A generic term used to describe the collection of DNA sequences that were generated during the sequencing project to be assembled into a contig. Typically these are trace files, but they can also be plain sequences.</td>
</tr>
<tr>
<td>Trace</td>
<td>The chromatogram data generated by an automated sequencing machine. The terms “chromatogram files” and “trace files” are used interchangeably in this chapter to describe the files generated by automated sequencing machines.</td>
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Using Assembler

Base calling using phred

Phred is an algorithm that takes chromatogram information from an automated sequencing run and re-evaluates the peaks to produce a "base call" that is usually significantly more accurate than the original call. In addition to recalculating the residues, phred also adds quality score information to each residue. This is a logarithmic value from 0 to 99 where a value of 10 indicates that there is a 1 in 10 chance that the call is in error, a score of 20 indicates in 1 in 100 chance the call is in error, a score of 30 indicates a 1 in 1,000 chance of an error etc. Assembler takes advantage of multi-CPU machines (such as the Intel Core Duo or multiple processor PowerPC machines) and splits up the phred jobs between the processors so that you see a speed up directly proportional to the number of processors. In addition, the phred executable is a Universal Binary, so it runs at full native speed on Intel or PowerPC processors. Once you have basecalled the imported sequences, you can view the phred base calls and the quality values by double-clicking on one of the sequences.
Trimming vector sequences using \texttt{cross\_match}

Many raw sequences from automated sequencing machines contain vector sequences at the beginning and/or end. Assembler lets you mask these out using the \texttt{cross\_match} algorithm. To use this you just need to supply the sequences of the vector(s) you used for the cloning - there is no need to indicate the cloning site you used as \texttt{cross\_match} can easily identify the exact position where the vector sequences terminate. Like \texttt{phred}, the \texttt{cross\_match} executable is a Universal Binary and MacVector Assembler splits up jobs between multiple CPUs if they are available. After processing by \texttt{cross\_match}, you can view the masked vector sequences in the trace editor window where they appear in grayed out italics.

Assembling sequences using \texttt{phrap}

Assembler assembles sequences using the \texttt{phrap} algorithm. \texttt{phrap} does not require the sequences to have been base called by \texttt{phred}, or to have had any vector sequences masked. However, using \texttt{phred} and \texttt{cross\_match} will improve the accuracy of \texttt{phrap} assemblies. \texttt{phrap} assembles sequences into contigs and creates a consensus
sequence with its own set of quality values, based on the quality and strandedness of the overlapping sequences. Contigs can be viewed and edited in a Contig editor that shows the aligned sequences along with the chromatograms in a lower pane. Clicking on a residue in the consensus sequence resets the chromatogram view so that they are all aligned to that base. This allows you to easily align the chromatograms so that you can resolve ambiguities in the consensus sequence.

Editing and analysis

You can edit the sequences in a contig and the consensus will be updated automatically. MacVector follows the phred/phrap quality value rule where edited residues are given a quality value of 99 to indicate they have been assigned by a user. These are shown in blue in the quality display. You can view a variety of statistics on the composition
of the contig in the annotations window. The annotations window contains a summary of the contig composition statistics

You can invoke any MacVector DNA analysis algorithm from the contig editor window, including online NCBI blast searches. Any gaps in the consensus are removed before the analysis is performed. This lets you scan the contig for restriction enzyme sites, then edit the consensus and rescan without having to export the consensus sequence or switch to a different module. The consensus sequence, without gaps, can be saved to disk as a single sequence at any time. The sequence can be saved in any format supported by MacVector and retains a list of the individual reads used to generate the consensus.

Algorithms

Phred uses simple Fourier methods to examine the four base traces in the region surrounding each point in the data set in order to predict a series of evenly spaced predicted locations. That is, it determines where the peaks would be centered if there were no compressions, dropouts, or other factors shifting the peaks from their "true" locations.

Next phred examines each trace to find the centers of the actual, or observed, peaks and the areas of these peaks relative to their neighbors. The peaks are detected independently along each of the four traces so many peaks overlap. A dynamic programming algorithm is used to match the observed peaks detected in the second step with the predicted peak locations found in the first step.

Phred evaluates the trace surrounding each called base using four or five quality value parameters to quantify the trace quality. It uses a
Quick start

quality value lookup table to assign the corresponding quality value. The quality value is related to the base call error probability by the formula

\[ QV = -10 \times \log_{10}(P_e) \]

where \( P_e \) is the probability that the base call is an error.

Phred uses data from a chemistry parameter file called 'phredpar.dat' in order to identify dye primer data. For dye primer data, phred identifies loop/stem sequence motifs that tend to result in CC and GG merged peak compressions. It reduces the quality values of potential merged peaks and splits those peaks that have certain trace characteristics indicative of merged CC and GG peaks. In addition, the chemistry and dye information are passed to phrap.

Quick start

To assemble a set of sequences follow these steps:

1. Chose **File | New | Assembly Project** to create a new empty project file.
2. Click the **Add Seqs** icon on the toolbar, then select the sequence files you wish to assemble and click on the **Open** button.
3. Click the **Phred** icon on the toolbar or choose **Analyze | Base Call (phred)** from the menu to run the phred algorithm on all of the sequences in the project.
4. Optionally, click the **CrossMatch** icon on the toolbar or choose **Analyze | Vector Trim (cross_match)** from the menu to mask vector sequences in the reads. You must import the vector sequences you used into the **Vectors** tab of the **Cross_match Paramters** dialog. The files must be in either MacVector or FastA format.
5. Click the **Phrap** icon on the toolbar or choose **Analyze | Assemble (phrap)** from the menu to assemble all of the sequences of the project.
6. After assembly, overlapping sequences are removed from the project and replaced by contigs. Double-click on a contig to open it in a Contig editor.
7. You can edit sequences in the Contig editor and also run any MacVector nucleic acid analysis function directly on the contig consensus sequence.
8. Finally you can save the consensus sequence in MacVector format by choosing **File | Save As...** from the Contig editor. You can also save the assembly project itself at any time and you will be prompted to save any changes when you close the project window.

**Tutorial**

**Sample files**

After installing MacVector Assembler, you will find example files for this tutorial in the folder:

**MacVector/Sample Files/SequenceConfirmation**

There are 32 trace files in SCF format along with two vectors (pSG933 and Tn1000) used in the sequencing experiment.

**Creating and Populating a Project**

The first step in the tutorial is to create a new project and add some sequences to it.

1. Select **File | New | Assembly Project**. An empty assembly project window will open.

2. Click the **Add Seqs** icon on the toolbar. In the dialog that opens, navigate to the **MacVector/Sample Files/SequenceConfirmation/TraceFiles/** folder.

3. Click on the first file (**A04a.scf**) to select it, then scroll to the end of the list, hold down the **<shift>** key and click on the last file (**ReversePrimer.scf**) to select all of the files in the folder.

4. Finally, click on the **Open** button to import the selected files into the project.

**Note.** The data in the files is copied into the project. If you subsequently edit the original files on disk, then the data in the project will be unaffected. Similarly, any edits you make to the project data will not affect the contents of the original files.

There are no limits to the numbers or sizes of the sequences that you import. However, you may run into performance problems with projects containing large numbers of sequences. We recommend that you use a computer that has at least 0.5 MB of physical RAM for each chromatogram file you import for optimum performance i.e. use at least 512MB for a 1,000 sequence assembly. If you do see a slowdown importing,
editing and saving large projects, adding more RAM to your computer is the most cost-effective way to improve performance.

The Project window

The Project window contains two tabbed views: Project and Properties.

The Project view

The Project view has a number of columns that display information about the individual sequences and contigs. Most of the columns can be sorted by clicking on the column header.

- **Name** – the name of the sequence. All sequences and contigs in a project MUST have a unique name. If you try to import sequences with duplicate names, you will be prompted to choose how they should be handled. The icon next to the name indicates if the object is a contig, a trace or a plain sequence. You can directly edit this field to change the name.

- **Status** – initially blank, the status field indicates if a sequence has been base called with phred ("P") or masked for vector sequences with cross_match ("X").

- **Length** – the length of the sequence or contig.

- **#** - for contigs, this field indicates the number of reads that have been assembled. For sequences in a contig, the field indicates orientation using “->” for forward reads and “<-“ for reverse reads.
Assembler

- **ClipL** – the first residue from the 5’ end that is not masked. Typically this will be “1”, although cross_match or phrap may change this.

- **ClipR** – the last valid residue at the 3’ end of a sequence. Initially, this is simply the last residue of the sequence, but cross_match and phrap may change this.

- **Start** – for sequences in a contig, the start location of the sequence within the contig.

- **Stop** – for sequences in a contig, the location of the last residue of the sequence within the contig.

- **Definition** – any descriptions associated with a sequence.

You can double-click on an item to open up the editor associated with the object, e.g. the trace editor or the contig editor. Note that in this version, you cannot directly edit plain sequences by double-clicking on them – you should complete any editing on these before adding them to the project.

The Project view also has the following toolbar buttons:

- **Add Seqs** – provides access to a dialog box which enables you to add additional sequences to the project. You can also use *Edit* | *Add Sequences From File*

- **Remove** – removes the selected sequences from the project. You can also use this button to dissolve selected contigs. You can also use the `<delete>` key or *Edit* | *Clear* to accomplish the same functions.

- **Prefs** – provides access to a dialog box which enables you to configure the default appearance of the Assembly editor and add vectors to the project.

- **Phred** – runs the **phred** algorithm on all of the sequences in the project.

- **CrossMatch** – masks vector sequences in the reads.

- **Phrap** – assembles all of the sequences of the project.

**The Properties view**

The Properties view displays various useful statistics about the project.
Saving and opening assembly projects

You can save assembly projects at any time. They are saved in an xml format, meaning that the file contents are text and can (potentially) be viewed in any standard text editor such as TextEdit or Microsoft Word. The file names are not given file-type extensions.

1. Choose File | Save.

If this is the first time you have saved the project, you will get prompted for a filename. Otherwise, the project will be saved with its current file-name.

The small tutorial project should save within a second or two. Large projects may take some time to save – approximately 15 seconds for every thousand trace sequences on an average machine. A progress dialog is displayed during the save. You can cancel this and your original file will not be affected.

2. Close the Project window.

You will be prompted to save if you have made any changes since the last save.

3. Click on the File menu.

A list of recent files is appended to the bottom of the menu.

4. Select the name you saved the project under.

The project will open. Again, a progress dialog is displayed during the load as large projects will take some time to open. If you have a very large project, it may take a few seconds before the progress dialog is displayed.

Base calling with phred

Phred is an algorithm developed by Phil Green’s group at the University of Washington. Phred re-evaluates the chromatogram peaks in a trace file, a process known as “base calling”. Not only is phred typically more accurate than the default base callers used by automated sequencing machines, but it also assigns “Quality Values” to each individual base call. Phred uses a statistically significant logarithmic scale from 0 to 99 where 10 means there is a 1 in 10 chance that the call is in error, 20 means there is a 1 in 100 chance the call is in error, 30 means there is a 1 in 1,000 chance of an error etc. The values 98 and 99 are reserved to indicate residues that have been edited by the user. A phred score of 20 or more is generally considered to be an acceptable
score. MacVector Assembler displays phred scores as a histogram above the sequence using colors to indicate the quality – scores below 20 are shown in red, scores of 20 or greater in green and edited residues (score 99) in blue.

Make sure you have no selections in the Project view. To toggle a selection off, click on the selection while holding down the command (⌘) key.

You can select a subset of sequences for analysis if you wish. However, if nothing is selected, all of the chromatogram sequences in the project will be submitted for analysis.

1. Click the Phred icon on the toolbar or choose Analyze | Base Call (phred) from the menu.

The Job Manager dialog box will appear. This allows you to follow the progress of the job.

When the job has completed, the project window will refresh to reflect the new base calls. The status of each entry changes to “P” to indicate you have run phred on the sequence.

**Viewing base calls**

Double-click on one of the phred-called sequences in the Project view to open up the Trace editor window.

![Trace editor window](image)

When you open the Trace editor window from an assembly project, two additional icons are displayed in the toolbar.

- **Basecalls** – toggle this button to show or hide the basecalls displayed immediately below the main sequence line.
- **Qualities** – toggle this button to show or hide the quality histogram displayed over the top of the sequence.
You can see that the original base call for this sequence was generated using a utility called “makeSCF”. The phred base call is shown directly underneath this. You cannot edit the base calls – they are read-only. You can edit the upper sequence if you wish – this is the “active” editable sequence that is used in all assemblies and analyses. If you subsequently re-run phred on a sequence, it will replace the phred base call and will also replace any edits you have made to the active sequence.

**Masking vector sequences with `cross_match`**

Typical sequencing projects use a directed or shotgun sub-cloning approach to generate short overlapping sequences that are then assembled into a single longer sequence. It is common for the reads to have vector sequences at the beginning and/or end which can interfere with the assembly. `cross_match` is an algorithm that can be used to mask out any vector sequences to prevent this interference. This is not an absolutely essential step as phrap (the assembly algorithm we will use) can often detect the vector sequences in a collection of similar sequences. However, using `cross_match` is highly recommended to reduce the likelihood of anomalous assemblies.

Make sure you have no selections in the Project view. To toggle a selection off, click on the selection while holding down the command (⌘) key.

1. Click the CrossMatch icon on the toolbar or choose Analyze | Vector Trim (cross_match) from the menu.
The **Cross_match Parameters** dialog box will appear. The algorithm needs to know which vectors were used in the sequencing experiments, so the dialog initially displays the empty **Vectors** tab.

2. Click **Add** to bring up the file selection dialog box.

3. Navigate to the **MacVector/Sample Files/Sequence-Confirmation/** folder and select the files `pSG933.txt` and `Tn1000.txt`.

4. Click **Open** to add the vector files to the **Vectors** tab.

5. The tab will refresh to reflect the new vectors that have been added.

6. Click on **Recent Vectors**.

The names of the files have been added to this menu. The menu remembers the last 20 vector files you added to any project, so you can use this as a shortcut to rapidly import common vectors into any new projects you create.
7. Click on the **Parameters** tab to view the other **cross_match** parameters.

For this tutorial, we will accept the default values.

8. Click **OK** to dismiss the dialog and run the algorithm.

The algorithm should complete within a few seconds. The Project view then updates with the new data.

In addition to the status of each sequence changing to “PX” to indicate that they have been trimmed with **cross_match**, many of the **ClipL** entries now show values other than “1” indicating that vector sequences were masked at the beginning. The sequence A04a.scf has a particularly short insert and you can see that its **ClipR** value is now only 186.

9. Double-click on the sequence A04a.scf to open up a Trace editor window.

The masked residues are shown in gray italics. If you scroll to the right, you will find additional masked residues from 186 onwards.

**Assembling sequences using phrap**

**phrap** is the assembly algorithm from the University of Washington that has been incorporated into Assembler. It is designed to work in concert with **phred** and **cross_match** – in particular it understands
quality values and will use them to make better assemblies, particularly in areas with repetitive sequences. \textit{phrap} also calculates quality values for each residue in the consensus sequence using the same scale as \textit{phred}. However, for assemblies, a value of 40 (1 error in 10,000) is considered an acceptable value.

\textit{phrap} is described in more detail in the \textit{phrap.pdf} document that can be found in the \textit{MacVector 9.5/Documentation} folder. This is the original documentation from the University of Washington. It is somewhat technical in places, but it describes the assembly algorithmic strategy and the effects of changing various parameters in great detail. Make sure you have no selections in the Project view. To toggle a selection off, click on the selection while holding down the command (\textasciitilde) key.

1. Click the \textbf{Phrap} icon on the toolbar or choose \textbf{Analyze} | \textbf{Assemble} (phrap).

The \textbf{Phrap Parameters} dialog box will appear. Not all of the parameters described in \textit{phrap.pdf} are available in the dialog. However, it is unlikely that you will ever need to adjust any parameters other than those displayed in the \textbf{Basic} tab.

2. Click \textbf{OK} to dismiss the dialog and run the algorithm using the default values.

\textit{phrap} is a remarkably fast algorithm and the assembly should be complete within a few seconds. Even with large (>1,000 reads) projects, assembly rarely takes more than a few minutes. As with \textit{phred} and \textit{cross_match}, \textit{phrap} has been compiled for MacVector as a Uni-
versal Binary, so the algorithm will run natively on an Intel-based Macintosh.

Once assembly is complete, the project window is updated to reflect the data change. In this case, all of the reads should be assembled into a single contig.

3. Click on the disclosure triangle next to the contig to reveal the contents of the contig.

The items within the contig are grayed out to indicate that you cannot open them individually. This is to prevent you from inadvertently changing the sequence of a trace that has been carefully aligned in a contig. However, you do have full editing control from within the Contig editor (see “Editing a contig” on page 351).

The #, Start and Stop columns have been updated to display additional information. The number of reads assembled in the contig is indicated on the top line, while the orientation of each read in the contig is indicated on the other lines. The start and stop locations of each read within the contig are also indicated in the appropriate columns.

Editing a contig

Although phrap does an excellent job of assembling reads and generating an accurate consensus sequence, there are likely to be times where
you need to edit the assembly, particularly if you have poor quality chromatograms, or areas of the contig that have low coverage.

1. Double-click on Contig_1 to open up the contig in the Contig editor.

The Contig editor is based on the Assembly editor used for Align to Reference, with a number of important differences:

- Base calls and quality values can now be displayed, controlled by the same toolbar buttons used in the trace editor.
- There is no “reference” sequence.
- The overlapping sequences can now be displayed in “tiled” or “untiled” mode.

The tiled/untiled mode requires additional explanation.

In “tiled” mode, each component sequence is given its own dedicated line in the upper panel. This is fine for relatively small assemblies (< 50 reads) but for large assemblies, the layout is impractical as there is too much white space and the user spends too much time scrolling to find the right sequences to edit.

In “untiled” mode, only those sequences that actually overlap the currently visible consensus sequence are shown on the screen. This minimizes the amount of white space and reduces the need for vertical
scrolling. The downside to this approach is that the reads may “move about” as you horizontally scroll through a contig.

In addition to the tiled/untiled mode, you can also choose to display the consensus sequence at the top of the panel, or in the center of the panel. This is controlled by the project preferences.

2. Click the Prefs icon on the toolbar to open up the Project Preferences dialog box.

3. Select the Use Tiled Mode checkbox and click OK.

Note. In tiled mode, the consensus is always placed at the top of the panel.

The Contig editor display will change to use tiled mode. For the remainder of the tutorial, you should set the display to your preferred configuration. The tutorial will use the default settings of non-tiled mode with the consensus in the center of the panel. You may also want to increase the size of the window so that you can see more data at one time. Similarly, you may also want to adjust the size of the upper panel by clicking and dragging on the vertical resize control in the right hand margin.

4. Click on any residue on the consensus line.

The residue highlights, but the display also resets so that all of the traces overlapping that residue become centered in the lower multiple trace panel.

You can use this feature to click on any dubious consensus residue and immediately see the overlapping traces aligned at that position. The consensus sequence is highlighted using your primary highlight color while the reads and traces are shown in a secondary highlight color.

5. Press the right arrow button on the keyboard. Continue to hold it down.
This will scroll the contig to the right. You could potentially slowly scroll through the entire contig this way. Whenever the consensus is highlighted, the traces are always aligned and centered at that position.

6. Click on one of the read sequences, either in the upper pane or in the lower mult-trace pane.

In this case the display does not reset to align the traces at the selected position. However, the primary highlight shifts to the selected residue (in both the upper and lower panes) to indicate which character will get changed if you press a valid key.

Select any residue in one of the reads and press a different DNA character key.

Note. You cannot directly edit the consensus sequence. It is always calculated indirectly from the overlapping reads. However, because of the way quality values are handled, this is not a significant limitation.

The residue changes to the chosen character and the quality value changes to 99, represented by the blue histogram. The value 99 is very important for consensus recalculation as it always overrides all other quality values. This has two important implications;

If you edit a residue to be a valid DNA character, the consensus will always change to match that character as it overrides all other considerations.

If you edit two residues at the same position but in different reads, and they do not agree, the consensus will be given an ambiguity character.

7. Choose Edit | Undo Typing from the menu.

As with most MacVector functions, there is just a single level of undo.

8. Close the Contig editor.

Changes to contigs in the Contig editor are considered to be changes to the project, so you do not get prompted to save those changes until you try to close the Project window, rather than the Contig editor.

9. Choose File | Save from the menu.

You will be prompted for a suitable filename.

Saving the consensus sequence

You can save the consensus sequence of a contig in MacVector format at any time.

1. Double-click on a contig in the Project view to open up the Contig editor for that contig.
2. Choose **File | Save**.
   
   You will be prompted for a suitable filename.
   
   The file will be saved in MacVector single sequence format. Any gaps in the consensus sequence will not be present in the saved file. The locations of the reads **ARE** written to the file as features, but this behavior may change in future.

**Analyzing contig sequences**

A Contig editor window is functionally equivalent to any normal MacVector single sequence window. You can run any nucleic acid sequence algorithm on the contig – in every case, it is the ungapped consensus sequence that is analyzed. Any gaps that appear in the contig editor consensus sequence are there only to maintain alignment with the overlapping reads. All analysis functions (along with save and copy functionality) strip out any gaps before analysis.

1. Double-click on the contig in the Project view to open the Contig editor, if it is not already open.

2. Choose **Analyze | NucleicAcid Analysis Toolbox**.

   The standard **Nucleic Acid Analysis** dialog box is displayed.

3. Select the **Open Reading Frames** checkbox, then click **OK** to initiate the analysis.

   In the graphic window that opens, select the first open reading frame in the “red” frame as shown below (outlined in a pale blue highlight).

   ![Contig editor window](image)

   With the selection still in place, click on the title bar of the **Contig_1** window (or anywhere in that window). The consensus sequence is selected, along with the reads that overlap that position.

4. Choose **Analyze | Translation**.
The **Translation Analysis** dialog box is displayed.

5. Make sure the **Create new protein** checkbox is selected, then click **OK**. A new protein Sequence window will be displayed. The protein sequence is a translation of the consensus sequence with all of the gaps removed. The same principle applies to all MacVector analysis functions – you can run any nucleic acid analysis (e.g. restriction enzyme analysis, or an online BLAST search) directly from the contig editor window and it is the ungapped consensus that gets analysed. This allows you to get instant feedback on edits affecting the consensus sequence without requiring clumsy export to a different analysis module.

**Dissolving contigs**

Make sure you have saved the assembly project you are working on. Most of the functions that dissolve or significantly modify contigs cannot be undone.

1. In the assembly Project window, select a contig and then click the **Dissolve** icon on the toolbar or press the `<delete>` key.

The contig will be dissolved into its constituent reads. The project window updates to indicate the fact that all of the reads that were in the contig have now been returned to the root of the project.

Any edits you made to the individual reads will be maintained, although all gaps will be removed. It is essential that the edits be retained as this allows you to edit sequences in a “bad” contig and then reassemble them taking your edits into account. This is particularly important when assembling sequences containing closely related repeats. *phrap* will not assemble overlapping sequences that have mismatched edited bases (i.e. that have quality values of 99) – you can use this to force misassembled repeats to split by editing the mismatched bases and re-assembling.

**Reassembling contigs**

1. In the assembly Project window, select a contig and then click the **Phrap** icon or choose **Analyze | Assemble (phrap)** from the menu. Accept the default parameters and click on the **OK** button.

You should see that the contig is dissolved and the assembled sequences appear in the project window. After assembly is complete, a new contig will appear in the project window.

The contig is dissolved prior to reassembly because there is no guarantee that the contig will reassemble with the same sequences as before. If
you have edited sequences in the contig, or chosen different phrap
parameters, one or more sequences may no longer assemble.
You can select any combination of contigs and sequences in the project
window to (re)assemble just those items.
The following chapters explain some of the theories and methods used by MacVector.

Chapter 17, “Understanding Protein and DNA Analysis”, explains some of the theories and methods used by MacVector for protein and DNA analysis.

Chapter 18, “Understanding Sequence Comparisons”, explains some of the theories and methods used by MacVector for sequence comparison, alignment, and phylogenetic analysis.
Overview

This chapter explains some of the theories and methods used by MacVector for protein and DNA analysis. Users are referred to the academic papers from which the methods are derived. This chapter provides greater detail only where significant changes have been made to published methods.
Refer to Appendix F, “References.”

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The protein analysis toolbox

One of the goals of molecular biology is to be able to determine the three-dimensional structure of a protein directly from its amino acid sequence—the so-called protein folding problem. As a first step toward solving this problem, researchers have developed algorithms for predicting the secondary structures of amino acid sequences and methods for predicting which regions of a protein might lie on the surface or might be buried in the protein’s interior. MacVector groups these algorithms together in the protein analysis toolbox.

When applying these methods to a protein of unknown structure, the researcher must always keep in mind certain limitations. Most standard methods are based on empirical data derived from proteins whose three-dimensional structure has been determined by X-ray crystallography. Therefore, the accuracy of the prediction will depend on how closely the protein of unknown structure resembles the set of known proteins used to derive the method. A method that performs well at predicting the structure of a cytochrome may fail when applied to an integral membrane protein; a method derived from a database of large globular proteins is probably inappropriate for deducing the structure of small peptides; and a method that is fairly accurate at predicting the structures of proteins in which alpha helix predominates may not work for proteins composed primarily of beta sheet.

Ideally, these predictive methods should be used in conjunction with biochemical and biophysical information about the protein of interest, e.g., sequence similarity with proteins of known structure, local similarities (such as patterns associated with known binding sites) with proteins of similar function, circular dichroism or Raman spectroscopy data, etc.

Secondary structure predictions

The Chou-Fasman method

This is the most popular and widely known method of protein secondary structure prediction. From proteins with known X-ray structures, Chou and Fasman compiled statistics on the tendency of an amino acid to appear in a given secondary structure, and used these statistics to assign the 20 amino acids into four classes: helix formers, helix breakers, sheet formers, and sheet breakers. They then devised a method to predict the secondary structure of a protein, by locating clusters of helix- or sheet-
forming residues in the sequence, and applying a set of heuristic rules to determine if these clusters are significant enough to nucleate a helix or beta sheet structure.

The method was originally intended to be performed by hand, which leads to its major drawback when it is adapted for computers. The same region of the protein often appears to be equally likely to nucleate a helix or sheet conformation, and the rules do not resolve this conflict by indicating the relative weight to place on each possible conformation at a given region.

By performing the method manually, the researcher resolves these conflicts by making subjective judgments (possibly biased by foreknowledge). But when the method is computerized, the programmer must explicitly assign the weights to be used to resolve such conflicts. Because each programmer may resolve them differently, one implementation of the Chou-Fasman method may yield different predictions from another implementation.

MacVector makes no attempt to resolve conflicting predictions at the same region. Instead, each prediction or conformation is treated independently of the others and each structure prediction is graphed separately. Thus, you may see a single region predicted to be in more than one conformational state. This form of presentation underscores the uncertainties of the method (or any current method for secondary structure prediction) and is a reminder that the predictions should not be over-interpreted.

The Robson-Garnier method

This is the other most popular method of protein secondary structure prediction. The Robson-Garnier method is based on information theory. Empirical studies show that an amino acid exerts a significant effect on the conformational state of residues up to eight residues distant; therefore, the information for the conformation of residue N can be based on the information contributions of the 16 nearest neighbors of N. Using a set of 25 proteins of known structure as a database, the directional information contributions of each of the 20 amino acids to a given conformational state for each of these 17 positions (N - 8 through N + 8) were derived. Using these information parameters, the likelihood of a given residue assuming each of the four possible conformations (alpha, beta, reverse turn, or coil) is calculated, and the conformation with the largest likelihood is assigned to the residue.
Unlike the Chou-Fasman method, the algorithm is clearly and unambiguously defined so that all computer implementations should yield the same result. The only choice open to the programmer involves the use of decision constants that can be assigned according to information known about the molecule’s secondary structure from other sources (such as circular dichroism). MacVector follows the most commonly used procedure and automatically calculates the decision constants based on estimates of alpha-helix and beta-sheet content from a first pass of the program itself.

**Combining the two methods**

By graphing the secondary structure predictions only where both the Chou-Fasman and Robson-Garnier methods agree, the researcher hopes to locate those regions that are most likely to be in the predicted conformation. However, we advise you that, at best, each method has about a 60 per cent probability of being correct and that the consensus of two possibly wrong predictions does not give a correct prediction.

**Protein profiles**

**Hydrophilicity**

This profile graphs the local hydrophilicity of a protein along its amino acid sequence. Each of the 20 amino acids is assigned a hydropathy value based on some experimental or empirical measure. A window of size N is run along the length of the protein; for each window, the hydropathy values of the N amino acids are summed and divided by N to obtain the average hydrophilicity per residue for the window. The value is then plotted on the graph at the center of the window. Values above the axis denote hydrophilic regions which may be exposed on the outside of the molecule; values below the axis indicate hydrophobic regions which tend to be buried inside the molecule or inside other hydrophobic environments such as membranes.

Various amino acid hydropathy scales have been developed for hydrophilicity profiles. Three commonly used scales are Kyte and Doolittle (1982), Hopp and Woods (1981), and Engelman, Steitz and Goldman (1986) (GES). The Kyte-Doolittle and GES scales were originally used for hydrophobicity profiles. We have reversed the signs of the values so that hydrophilicity is plotted instead.

The Kyte-Doolittle scale is the most commonly used hydropathy scale. Its values are assigned using a combination of the water-vapor transfer
Protein profiles

free energies for amino acid side chains and the preference of amino acid side chains for interior or exterior environments, with small adjustments made to the final values (based on the experience of the authors). The Hopp-Woods scale was designed to predict the locations of antigenic determinants in a protein, assuming that the antigenic determinants would be exposed on the surface of the protein and thus would be located in hydrophilic regions. Its values are derived from the transfer free energies for amino acid side chains between ethanol and water.

The GES scale was developed in order to identify possible transmembrane helices in a protein. The scale values are the sums of hydrophobic and hydrophilic components for each amino acid. Hydrophobic components are derived from the free energy of water-oil transfer for the side chains; hydrophilic components take into consideration the free energy for inserting charged groups into a bilayer and the free-energy contributions of hydrogen-bonding with water and with backbone carbonyl groups (if the residues can form such bonds when participating in a helical structure).

For most globular proteins, window sizes between seven and eleven are appropriate. (Hopp and Woods recommend a window size of six when using their scale.) In general, smaller window sizes yield noisier profiles, while larger window sizes tend to miss small hydrophilic or hydrophobic regions. Window sizes of five to seven can be useful in locating small hydrophilic regions that may protrude from the protein surface and thus be antigenic sites. Window sizes of 19 to 21 are often useful in locating possible membrane-spanning regions in integral membrane proteins.

Surface probability

This profile was designed to predict which regions of a protein are most likely to lie on the protein’s surface, based on knowledge of which amino acids are more likely to be found on the surface of proteins of known structure.

Janin et al. (1978) examined 28 proteins whose atomic coordinates were known and determined the solvent-accessible surface area of each residue. Residues were classified as “buried” if their accessible surface area was smaller than 20 Å and as “exposed” if the accessible surface area was larger than 60 Å. For each of the 20 amino acids, information was compiled on what percentage of the time the amino acid was found in an
exposed position and what percentage of the time it was found in a buried position in these 28 proteins (Table 1 of Janin et al. (1978)).

Emini et al. (1965) used these percentages to calculate a fractional surface probability for each amino acid (\(\frac{\%\text{exposed}}{\%\text{exposed} + \%\text{buried}}\)). The fractional surface probabilities were used to compute a surface "probability" profile for hexapeptide windows along a protein sequence by multiplying together the six fractional probabilities in the window, then multiplying this product by \((.037)^{-6}\).

Because this formula permits the "probability" to exceed 1.0, MacVector uses a different formula. MacVector sums the six fractional probabilities of the amino acids in the window and divides by six to yield a running average of the fractional surface probability along the length of the protein. Thus, a value of 1.0 at any point (which will never occur) would mean that the hexapeptide centered about that point is definitely exposed at the surface of the protein and a value of 0.0 (which also will never occur) means that the hexapeptide is definitely buried in the interior of the protein.

**Flexibility**

Because segmental flexibility appears to correlate with known antigenic determinants, Karplus and Schulz (1985) devised a method to predict potential antigenic sites by locating regions of the protein chain that might be relatively flexible.

Using a set of 31 proteins of known three-dimensional structure as a database, the 20 amino acids were assigned to one of two groups - rigid or flexible - depending on the normalized crystallographic temperature factors (B-values) of their alpha-carbon atoms. Three sets of B-norm values were then calculated for each amino acid, one set for each of the possible combinations of nearest neighbors: no rigid neighbors, one rigid neighbor, and two rigid neighbors. The predicted relative flexibility at a given residue is the weighted sum of the neighbor-correlated B-norm values (B) for the given residue and the six residues flanking it:

\[
\text{flex}[i] = 0.25(B[i-3]) + 0.5(B[i-2]) + 0.75(B[i-1]) + 1.0(B[i]) + 0.75(B[i+1]) + 0.5(B[i+2]) + 0.25(B[i+3])
\]

Using this method, the average flexibility of a protein is 1.0. Regions with values greater than 1.0 are predicted to be more flexible than aver-
Protein profiles

age, while values below 1.0 indicate regions predicted to be less flexible than average.

Antigenic index

This profile is designed to locate possible exposed surface peaks of a protein, i.e., peaks that might be antigenic sites. Rather than use a single type of analysis, this method combines information from hydrophilicity, surface probability, and backbone flexibility predictions along with the secondary structure predictions of Chou-Fasman and Robson-Garnier in order to produce a composite prediction of the surface contour of a protein.

The method used in MacVector differs in one respect from that of B.A. Jameson and H. Wolf (1988): scores for each of the analyses are normalized to a value between -1.0 and +1.0 instead of using the values in Table I of the reference. The score for each analysis at each residue is multiplied by an empirically-determined weighting factor for that analysis and the weighted scores for each of the analyses are summed to yield the antigenic index, as in the reference.

\[
\text{Antigenic index} = \Sigma \{ 0.3\text{hydro}[i] + 0.15\text{surf\_prob}[i] + 0.15\text{flex}[i] + 0.2\text{chou\_fas}[i] + 0.2\text{rob\_garn}[i] \}
\]

Regions that plot above the graph axis are predicted to be exposed at the protein’s surface.

Amphiphilicity

Amphiphilicity profiles are used to detect regions of a protein that may form amphiphilic (or amphipathic) structures—structures that tend to be polar on one side and apolar on the other. Such regions are often found at protein-solvent or membrane-protein interfaces.

One way of looking for such structures is to examine the periodicity of the protein’s hydrophobicity (its hydrophobic moment). An amphiphilic alpha helix will exhibit a periodicity of about 100 degrees; for a strand of beta sheet, the periodicity theoretically would be 180 degrees, but because beta strands tend to twist, the angle varies in practice from about 160 to 180 degrees. As a compromise, MacVector uses 170 degrees when calculating beta strand amphiphilicity.
To compute the amphiphilicity profile, a window of size \( N \) is moved along the entire protein sequence. For each of these windows, the hydrophobic moment is calculated according to the formula:

\[
\text{moment} = \left( \frac{\sum H(i) \sin(Ai)}{\sum H(i) \cos(Ai)} \right)^{1/2}
\]

where:
- \( H(i) \) is the hydrophobicity (according to the Eisenberg et al. \( 1984 \)) normalized consensus scale) of the \( i^{th} \) residue of the window
- \( A \) is the size of the angle (in radians) at which successive side chains emerge from the protein backbone
- the index \( i \) takes on values from 1 to \( N \).

The hydrophobic moment for the window is divided by \( N \) to yield an average moment per residue, and this value is plotted at the center of the window. Values greater than about 0.4 indicate amphiphilic regions.

The window size should at least be the size of one “unit” of the structure (three residues for alpha helix, two residues for beta strands) and ordinarily is chosen to equal the typical length for that structure type. Eisenberg et al. \( 1984a \) suggest a window size of 11; however, von Heijne (1986) finds that the optimal window size for surface-seeking peptides ranges from 17 to 26, and for mitochondrial targeting sequences from 12 to 26.

Estimating the pI

The pI of a protein is the pH where the protein has a net charge of zero. It is calculated using the Henderson-Hasselbach equation to figure the percentage disassociation of each amino acid in the protein at various pHs. This calculation can only be an estimate, however, because it assumes that the percentage dissociation of an amino acid in a protein is the same as that of a free amino acid. This assumption is known to be false: there are effects on the ionization constants from neighboring amino acids, and these effects may extend over a considerable range of the protein.

The estimate for the pI in MacVector is based on the pKa values for the amino acid side chains and the amino and carboxy ends of the protein published in “Biochemistry, a Problems Approach”, by Wood, Wilson, Benbow, and Hood. The values are:
These values were tested by comparing the experimentally determined values of the pIs of several proteins with estimated pIs of related proteins from the NBRF PIR database. Because the published experimental values of the pI do not state which species or exact sequence was used, the representatives chosen from the database may not correspond exactly to the proteins used in the experimental determinations. This may result in some of the discrepancy seen below between the experimental and estimated pIs:

<table>
<thead>
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<th>Protein</th>
<th>NBRF name</th>
<th>experimental pI</th>
<th>estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg Albumin</td>
<td>OACH</td>
<td>4.6</td>
<td>5.02</td>
</tr>
<tr>
<td>Serum Albumin</td>
<td>ABBOS</td>
<td>4.9</td>
<td>5.83</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>HBBOB</td>
<td>6.8</td>
<td>7.38</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>MYBO</td>
<td>7.0</td>
<td>7.54</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>CCHU</td>
<td>10.7</td>
<td>9.59</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>LZHU</td>
<td>11.0</td>
<td>10.20</td>
</tr>
</tbody>
</table>

**Primers and probes**

**Primer design**

MacVector incorporates primer design using Primer3.

**Note.** The following is extracted from the Primer3 readme file, which is supplied in full in the MacVector application folder.

Primer3 picks primers for PCR reactions, considering as criteria:

- oligonucleotide melting temperature, size, GC content and primer-dimer possibilities
- PCR product size
- positional constraints within the source (template) sequence
- possibilities for ectopic priming (amplifying the wrong sequence)
- many other constraints
All of these criteria are user-specifiable as constraints, and some are specifiable as terms in an objective function that characterizes an optimal primer pair.

This section provides a cross-reference between the Primer3 constraints and the equivalent MacVector parameters. It is organized by MacVector user interface component.

**Primer Sequences panel**

- **PRIMER_LEFT_INPUT** (nucleotide sequence, default empty)
  The sequence of a left primer to check and around which to design right primers and optional internal oligos. Must be a substring of SEQUENCE.

- **PRIMER_RIGHT_INPUT** (nucleotide sequence, default empty)
  The sequence of a right primer to check and around which to design left primers and optional internal oligos. Must be a substring of the reverse strand of SEQUENCE.

**Main function panel**

- **PRIMER_PRODUCT_SIZE_RANGE** (size range list, default 100-300)
  The associated values specify the lengths of the product that the user wants the primers to create, and is a space separated list of elements of the form <x>-<y> where an <x>-<y> pair is a legal range of lengths for the product. For example, if one wants PCR products to be between 100 to 150 bases (inclusive) then one would set this parameter to 100-150. If one desires PCR products in either the range from 100 to 150 bases or in the range from 200 to 250 bases then one would set this parameter to 100-150 200-250.

Primer3 favors ranges to the left side of the parameter string. Primer3 will return legal primers pairs in the first range regardless the value of the objective function for these pairs. Only if there are an insufficient number of primers in the first range will Primer3 return primers in a subsequent range. For those with primarily a computational background, the PCR product size is size (in base pairs) of the DNA fragment that would be produced by the PCR reaction on the given sequence template. This would, of course, include the primers themselves.

**Amplify Feature/Region - Region to Scan - Flanking Regions panel**

- **PRIMER_TASK** (string, default pick_pcr_primers)
  Tell primer3 what task to perform. Legal values are pick_pcr_primers, pick_pcr_primers_and_hyb_probe, pick_left_only, pick_right_only,
pick_hyb_probe_only. The tasks should be self-explanatory, except that we note that pick_pcr_primers_and_hyb_probe is equivalent to the setting PRIMER_PICK_INTERNAL_Oligo to a non-zero value and setting PRIMER_TASK to pick_pcr_primers.

Advanced options - Primer Binding

Primer vs Primer

- PRIMER_SELF_ANY (decimal, 9999.99, default 8.00)

The maximum allowable local alignment score when testing a single primer for (local) self-complementarity and the maximum allowable local alignment score when testing for complementarity between left and right primers. Local self-complementarity is taken to predict the tendency of primers to anneal to each other without necessarily causing self-priming in the PCR. The scoring system gives 1.00 for complementary bases, -0.25 for a match of any base (or N) with an N, -1.00 for a mismatch, and -2.00 for a gap. Only single-base-pair gaps are allowed. For example, the alignment

5' ATCGNA 3'
\[\text{| | |}\]
3' TA-CGT 5'

is allowed (and yields a score of 1.75), but the alignment

5' ATCCGNA 3'
\[\text{| | |}\]
3' TA--CGT 5'

is not considered. Scores are non-negative, and a score of 0.00 indicates that there is no reasonable local alignment between two oligos.

3' end vs 3' end

- PRIMER_SELF_END (decimal 9999.99, default 3.00)

The maximum allowable 3'-anchored global alignment score when testing a single primer for self-complementarity, and the maximum allowable 3'-anchored global alignment score when testing for complementarity between left and right primers. The 3'-anchored global alignment score is taken to predict the likelihood of PCR-priming primer-dimers, for example

5' ATGCCCTAGCTTCCGGATG 3'
\[\text{| | | | | | |}\]
3' AAGTCCTACATTAGCCTAGT 5'
or

5' AGGCTATGGGCCTCGGA 3'

3' AGCGCTCCGGGTATCGGA 5'

The scoring system is as for the Maximum Complementarity argument. In the examples above the scores are 7.00 and 6.00 respectively. Scores are non-negative, and a score of 0.00 indicates that there is no reasonable 3'-anchored global alignment between two oligos. In order to estimate 3'-anchored global alignments for candidate primers and primer pairs, Primer assumes that the sequence from which to choose primers is presented 5'->3'. It is nonsensical to provide a larger value for this parameter than for the Maximum (local) Complementarity parameter because the score of a local alignment will always be at least as great as the score of a global alignment.

**Primer vs Product**

- PRIMER_MAX_TEMPLATE_MISPRIMING (decimal, 9999.99, default -1.00)

The maximum allowed similarity to ectopic sites in the template. A negative value means do not check. The scoring system is the same as used for PRIMER_MAX_MISPRIMING, except that an ambiguity code in the template is never treated as a consensus (see PRIMER_LIB_AMBIGUITY_CODES_CONSENSUS).

**Allowed Ns in primers**

- PRIMER_NUM_NS_ACCEPTED (int, default 0)

Maximum number of unknown bases (N) allowable in any primer.

**Allow ambiguous residues**

- PRIMER_LIBERAL_BASE (boolean, default 0)

This parameter provides a quick-and-dirty way to get primer3 to accept IUB / IUPAC codes for ambiguous bases (i.e. by changing all unrecognized bases to N). If you wish to include an ambiguous base in an oligo, you must set PRIMER_NUM_NS_ACCEPTED to a non-0 value. Perhaps '-' and '*' should be squeezed out rather than changed to 'N', but currently they simply get converted to N's. The authors invite user comments.

**Unused Primer3 constraints**

- PRIMER_PAIR_MAX_TEMPLATE_MISPRIMING (decimal, 9999.99, default -1.00)
Primers and probes

The maximum allowed summed similarity of both primers to ectopic sites in the template. A negative value means do not check. The scoring system is the same as used for PRIMER_PAIR_MAX_MISPRIMING, except that an ambiguity code in the template is never treated as a consensus (see PRIMER_LIB_AMBIGUITY_CODES_CONSENSUS). Primer3 does not check the similarity of hybridization oligos (internal oligos) to locations outside of the amplicon.

Advanced options - Characteristics

Length

- PRIMER_OPT_SIZE (int, default 20)
  Optimum length (in bases) of a primer oligo. Primer3 will attempt to pick primers close to this length.
- PRIMER_MIN_SIZE (int, default 18)
  Minimum acceptable length of a primer. Must be greater than 0 and less than or equal to PRIMER_MAX_SIZE.
- PRIMER_MAX_SIZE (int, default 27)
  Maximum acceptable length (in bases) of a primer. Currently this parameter cannot be larger than 35. This limit is governed by maximum oligo size for which primer3’s melting-temperature is valid.

Percent G+C

- PRIMER_MIN_GC (float, default 20.0%)
  Minimum allowable percentage of Gs and Cs in any primer.
- PRIMER_OPT_GC_PERCENT (float, default 50.0%)
  Optimum GC percent. This parameter influences primer selection only if PRIMER_WT_GC_PERCENT_GT or PRIMER_WT_GC_PERCENT_LT are non-0.
- PRIMER_MAX_GC (float, default 80.0%)
  Maximum allowable percentage of Gs and Cs in any primer generated by Primer.

Tm (C)

- PRIMER_OPT_TM (float, default 60.0C)
  Optimum melting temperature (Celsius) for a primer oligo. Primer3 will try to pick primers with melting temperatures are close to this temperature. The oligo melting temperature formula used can be specified by user. Please see PRIMER_TM_SANTALUCIA for more information.
- PRIMER_MIN_TM (float, default 57.0C)
Minimum acceptable melting temperature (Celsius) for a primer oligo.
- PRIMER_MAX_TM (float, default 63.0°C)

Maximum acceptable melting temperature (Celsius) for a primer oligo.

**GC Clamp**
- PRIMER_GC_CLAMP (int, default 0)

Require the specified number of consecutive Gs and Cs at the 3' end of both the left and right primer. (This parameter has no effect on the internal oligo if one is requested.)

**Maximum difference in Tm between primers**
- PRIMER_MAX_DIFF_TM (float, default 100.0°C)

Maximum acceptable (unsigned) difference between the melting temperatures of the left and right primers.

**Maximum Poly-X**
- PRIMER_MAX_POLY_X (int, default 5)

The maximum allowable length of a mononucleotide repeat, for example AAAAAA.

**Unused Primer3 constraints**
- PRIMER_PRODUCT_MAX_TM (float, default 1000000.0)


\[
Tm = 81.5 + 16.6(\log_{10}(Na^+)) + .41*(\%GC) - 600/\text{length}
\]

Where \([Na^+]\) is the molar sodium concentration, \((\%GC)\) is the percent of Gs and Cs in the sequence, and length is the length of the sequence.

A similar formula is used by the primer selection program in GCG (http://www.gcg.com), which instead uses 675.0 / length in the last term (after F. Baldino, Jr, M.-F. Chesselet, and M.E. Lewis, Methods in Enzymology 168:766 (1989) eqn (1) on page 766 without the mismatch and formamide terms). The formulas here and in Baldino et al. assume Na+ rather than K+. According to J.G. Wetmur, Critical Reviews in BioChem. and Mol. Bio. 26:227 (1991) 50 mM K+ should be equivalent in these formulae to .2 M Na+. Primer3 uses the same salt concentration value for calculating both the primer melting temperature and the oligo melting temperature. If you are planning to use the PCR product for hybridization later this behavior will not give you the Tm under hybridization conditions.
Primers and probes

- PRIMER_PRODUCT_MIN_TM (float, default -1000000.0)
  The minimum allowed melting temperature of the amplicon. Please see the documentation on the maximum melting temperature of the product for details.

Advanced options - Reaction Conditions

Total initial primer concentration (nM)

- PRIMER_DNA_CONC (float, default 50.0 nM)
  The nanomolar concentration of annealing oligos in the PCR. Primer3 uses this argument to calculate oligo melting temperatures. The default (50nM) works well with the standard protocol used at the Whitehead/MIT Center for Genome Research - 0.5 microliters of 20 micromolar concentration for each primer oligo in a 20 microliter reaction with 10 nanograms template, 0.025 units/microliter Taq polymerase in 0.1 mM each dNTP, 1.5mM MgCl2, 50mM KCl, 10mM Tris-HCL (pH 9.3) using 35 cycles with an annealing temperature of 56 degrees Celsius. This parameter corresponds to 'c' in equation (ii) of the paper [Rychlik W, Spencer WJ and Rhoads RE (1990) "Optimization of the annealing temperature for DNA amplification in vitro", Nucleic Acids Res 18:6409-12 http://www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pubmed&pubmedid=2243783], where a suitable value (for a lower initial concentration of template) is "empirically determined". The value of this parameter is less than the actual concentration of oligos in the reaction because it is the concentration of annealing oligos, which in turn depends on the amount of template (including PCR product) in a given cycle. This concentration increases a great deal during a PCR; fortunately PCR seems quite robust for a variety of oligo melting temperatures. See “Advice for picking primers” on page 383.

Monovalent cation concentration (mM)

- PRIMER_DIVALENT_CONC (float, default 0.0 mM)
  The millimolar concentration of divalent salt cations (usually MgCl\(^2+\)) in the PCR. (New in v. 1.1.0, added by Maito Remm and Triinu Koressaar) Primer3 converts concentration of divalent cations to concentration of monovalent cations using formula suggested in the paper [Ahsen von N, Wittwer CT, Schutz E (2001) "Oligonucleotide Melting Temperatures under PCR Conditions: Nearest-Neighbor Corrections for Mg\(^2+\), Deoxynucleotide Triphosphate, and Dimethyl Sulfoxide Concentrations with Comparision to Alternative Empirical Formulas", Clinical Chemistry 47:1956-61 http://www.clinchem.org/
[Monovalent cations] = [Monovalent cations] + 120*([divalent cations] - [dNTP])^0.5 According to the formula concentration of desoxynucleotide triphosphate [dNTP] must be smaller than concentration of divalent cations. If the specified concentration of dNTPs is larger than specified concentration of divalent cations then the effect of divalent cations is not considered. The concentration of dNTPs is included to the formula because of some magnesium is bound by the dNTP. Attained concentration of monovalent cations is used to calculate oligo/primer melting temperature. Use tag PRIMER_DNTP_CONC to specify the concentration of dNTPs.

**Unused Primer3 constraints**

- **PRIMER_DNTP_CONC** (float, default 0.0 mM)
  The millimolar concentration of deoxyribonucleotide triphosphate. This argument is considered only if PRIMER_DIVALENT_CONC is specified. See PRIMER_DIVALENT_CONC.

- **PRIMER_SALT_CORRECTIONS** (int, default 0)
  Specifies the salt correction formula for the melting temperature calculation. (New in v. 1.1.0, added by Maito Remm and Triinu Koressaar)

**Advanced options - Hybridization Primer**

Because the laboratory detection step using internal oligos is independent of the PCR amplification procedure, internal oligo tags have defaults that are independent of the parameters that govern the selection of PCR primers. For example, the melting temperature of an oligo used
Primers and probes

for hybridization might be considerably lower than that used as a PCR primer.

These tags are analogous to the global input tags (those governing primer oligos) discussed above.

The exception is PRIMER_INTERNAL_OLG0_SELF_END which is meaningless when applied to internal oligos used for hybridization-based detection, since primer-dimer will not occur. We recommend that PRIMER_Internal_OlG0_SELF_EnD be set at least as high as PRIMER_INTERNAL_Oligo_SELF_Any.

- PRIMER_INTERNAL_OlG0_OPT_SIZE (int, default 20)
- PRIMER_INTERNAL_OlG0_MIN_SIZE (int, default 18)
- PRIMER_INTERNAL_OlG0_MAX_SIZE (int, default 27)
- PRIMER_INTERNAL_OlG0_OPT_TM (float, default 60.0 degrees C)
- PRIMER_INTERNAL_OlG0_OPT_GC_PERCENT (float, default 50.0%)
- PRIMER_INTERNAL_OlG0_MIN_TM (float, default 57.0 degrees C)
- PRIMER_INTERNAL_OlG0_MAX_TM (float, default 63.0 degrees C)
- PRIMER_INTERNAL_OlG0_MIN_GC (float, default 20.0%)
- PRIMER_INTERNAL_OlG0_MAX_GC (float, default 80.0%)
- PRIMER_INTERNAL_OlG0_DNA_CONC (float, default 50.0 nM)
- PRIMER_INTERNAL_OlG0_MIN_POLY_X (decimal 9999.99, default 12.00)
- PRIMER_INTERNAL_OlG0_MAX_POLY_X (int, default 5)

Unused Primer3 constraints

- PRIMER_INTERNAL_OlG0_SALT_CONC (float, default 50.0 mM)
- PRIMER_INTERNAL_OlG0_SELF_END (decimal 9999.99, default 12.00)
- PRIMER_INTERNAL_OlG0_DIVALENT_CONC (float, default 0.0 mM)
- PRIMER_INTERNAL_OlG0_DNTP_CONC (float, default 0.0 mM)

Advanced options - Misc.

- PRIMER_NUM_RETURN (int, default 5)
The maximum number of primer pairs to return. Primer pairs returned are sorted by their "quality", in other words by the value of the objective function (where a lower number indicates a better primer pair). Caution: setting this parameter to a large value will increase running time.

Constraints not used by MacVector or used only at their default values

- **PRIMER_PICK_ANYWAY** (boolean, default 0)
  If true pick a primer pair even if PRIMER_LEFT_INPUT, PRIMER_RIGHT_INPUT, or PRIMER_INTERNAL_Oligo_INPUT violates specific constraints.

- **PRIMER_PRODUCT_OPT_TM** (float, default 0.0)
  The optimum melting temperature for the PCR product. 0 indicates that there is no optimum temperature.

- **PRIMER_PRODUCT_OPT_SIZE** (int, default 0)
  The optimum size for the PCR product. 0 indicates that there is no optimum product size. This parameter influences primer pair selection only if PRIMER_PAIR_WT_PRODUCT_SIZE_GT or PRIMER_PAIR_WT_PRODUCT_SIZE_LT is non-0.

- **PRIMER_EXPLAIN_FLAG** (boolean, default 0)
  If this flag is non-0, produce PRIMER_LEFT_EXPLAIN,

- **PRIMER_WT_TM_GT** (float, default 1.0)
  Penalty weight for primers with Tm over PRIMER_OPT_TM.

- **PRIMER_WT_TM_LT** (float, default 1.0)
  Penalty weight for primers with Tm under PRIMER_OPT_TM.

- **PRIMER_WT_SIZE_LT** (float, default 1.0)
  Penalty weight for primers shorter than PRIMER_OPT_SIZE.

- **PRIMER_WT_SIZE_GT** (float, default 1.0)
  Penalty weight for primers longer than PRIMER_OPT_SIZE.

- **PRIMER_WT_GC_PERCENT_LT** (float, default 1.0)
  Penalty weight for primers with GC percent greater than PRIMER_OPT_GC_PERCENT.

- **PRIMER_WT_GC_PERCENT_GT** (float, default 1.0)
  Penalty weight for primers with GC percent greater than PRIMER_OPT_GC_PERCENT.

- **PRIMER_WT_COMPL_ANY** (float, default 0.0)
Primers and probes

- PRIMER_WT_COMPL_END (float, default 0.0)
- PRIMER_WT_NUM_NS (float, default 0.0)
- PRIMER_WT_REP_SIM (float, default 0.0)
- PRIMER_WT_SEQ_QUAL (float, default 0.0)
- PRIMER_WT_END_QUAL (float, default 0.0)
- PRIMER_WT_POS_PENALTY (float, default 0.0)
- PRIMER_WT_END_STABILITY (float, default 0.0)
- PRIMER_WT_TEMPLATE_MISPRIMING (float, default 0.0)
- PRIMER_PAIR_WT_PR_PENALTY (float, default 1.0)
- PRIMER_PAIR_WT_IO_PENALTY (float, default 0.0)
- PRIMER_PAIR_WT_DIFF_TM (float, default 0.0)
- PRIMER_PAIR_WT_COMPL_ANY (float, default 0.0)
- PRIMER_PAIR_WT_COMPL_END (float, default 0.0)
- PRIMER_PAIR_WT_PRODUCT_TM_LT (float, default 0.0)
- PRIMER_PAIR_WT_PRODUCT_TM_GT (float, default 0.0)
- PRIMER_PAIR_WT_PRODUCT_SIZE_GT (float, default 0.0)
- PRIMER_PAIR_WT_PRODUCT_SIZE_LT (float, default 0.0)
- PRIMER_PAIR_WT_REP_SIM (float, default 0.0)
- PRIMER_PAIR_WT_TEMPLATE_MISPRIMING (float, default 0.0)
- PRIMER_IO_WT_TM_GT (float, default 1.0)
- PRIMER_IO_WT_TM_LT (float, default 1.0)
- PRIMER_IO_WT_GC_PERCENT_GT (float, default 1.0)
- PRIMER_IO_WT_GC_PERCENT_LT (float, default 1.0)
- PRIMER_IO_WT_SIZE_LT (float, default 1.0)
- PRIMER_IO_WT_SIZE_GT (float, default 1.0)
- PRIMER_IO_WT_COMPL_ANY (float, default 0.0)
- PRIMER_IO_WT_COMPL_END (float, default 0.0)
- PRIMER_IO_WT_NUM_NS (float, default 0.0)
- PRIMER_IO_WT_REP_SIM (float, default 0.0)
- PRIMER_IO_WT_SEQ_QUAL (float, default 0.0)
- PRIMER_IO_WT_END_QUAL (float, default 0.0)
Primer3 output file format

In debug mode, MacVector can produce a raw Primer3 output file. This section explains the syntax of this file.

For each boulderio record passed into Primer3 via stdin, exactly one boulderio record comes out of Primer3 on stdout. These output records contain everything that the input record contains, plus a subset of the following tag/value pairs. Unless noted by (*), each tag appears for each primer pair returned.

The first version is
PRIMER_{LEFT,RIGHT,INTERNAL_OLIGO,PAIR}_<tag_name>.

Tags of additional primers chosen are of the form
PRIMER_{LEFT,RIGHT,INTERNAL_OLIGO,PAIR}_<j>_<tag_name>, where <j> is an integer from 1 to n, where n is at most the value of PRIMER_NUM_RETURN.

In the descriptions below, 'i,n' represents a start/length pair, 's' represents a string, 'x' represents an arbitrary integer, and 'f' represents a float.

**PRIMER_ERROR=s (*)**
s describes user-correctible errors detected in the input (separated by semicolons). This tag is absent if there are no errors.

**PRIMER_LEFT=i,n**
The selected left primer (the primer to the left in the input sequence). i is the 0-based index of the start base of the primer, and n is its length.

**PRIMER_RIGHT=i,n**
The selected right primer (the primer to the right in the input sequence). i is the 0-based index of the last base of the primer, and n is its length.

**PRIMER_INTERNAL_OLIGO=i,n**
The selected internal oligo. Primer3 outputs this tag if PRIMER_PICK_INTERNAL_OLIGO was non-0. If primer3 fails to pick a middle oligo upon request, this tag will not be output. i is the 0-based index of start base of the internal oligo, and n is its length.

**PRIMER_PRODUCT_SIZE=x**
x is the product size of the PCR product.

**PRIMER_{LEFT,RIGHT,INTERNAL_OLIGO}_EXPLAIN=s (*)**
s is a (more or less) self-documenting string containing statistics on the possibilities that primer3 considered in selecting a single oligo. For
Primers and probes

example PRIMER_LEFT_EXPLAIN=considered 62, too many Ns 53, ok 9
PRIMER_RIGHT_EXPLAIN=considered 62, too many Ns 53, ok 9
PRIMER_INTERNAL_Oligo_EXPLAIN=considered 87, too many Ns 39, overlap excluded region 40, ok 8. All the categories are exclusive, except the 'considered' category.

PRIMER_PAIR_EXPLAIN=s (*)
s is a self-documenting string containing statistics on picking a primer pair (plus internal oligo if requested). For example
PRIMER_PAIR_EXPLAIN=considered 81, unacceptable product size 49, no internal oligo 32, ok 0

All the categories are exclusive, except the 'considered' category. In some cases primer3 will examine a primer pair before it discovers that one of the primers in the pair violates specified constraints. In this case PRIMER_PAIR_EXPLAIN might have a non-0 number 'considered', even though one or more of PRIMER_LEFT_EXPLAIN,
PRIMER_RIGHT_EXPLAIN,
orPRIMER_INTERNAL_Oligo_EXPLAIN has 'ok 0'.
PRIMER_PAIR_PENALTY=f

The value of the objective function for this pair (lower is better).

PRIMER_{LEFT,RIGHT,INTERNAL_Oligo}_PENALTY=f

The contribution of this individual primer or oligo to the objective function.

PRIMER_{LEFT,RIGHT,INTERNAL_Oligo}_SEQUENCE=s

The actual sequence of the oligo. The sequence of left primer and internal oligo is presented 5' -> 3' on the same strand as the input SEQUENCE (which must be presented 5' -> 3'). The sequence of the right primer is presented 5' -> 3' on the opposite strand from the input SEQUENCE.

PRIMER_{LEFT,RIGHT,INTERNAL_Oligo}_TM=f

The melting TM for the selected oligo.

PRIMER_{LEFT,RIGHT,INTERNAL_Oligo}_GC_PERCENT=f

The percent GC for the selected oligo (denominator is the number of non-ambiguous bases).

PRIMER_{LEFT,RIGHT,INTERNAL_Oligo}_SELF_ANY=f
PRIMER_{LEFT,RIGHT,INTERNAL_Oligo}_SELF_END=f
The self-complementarity measures for the selected oligo.

**PRIMER_PAIR_COMPL_ANY=f**

**PRIMER_PAIR_COMPL_END=f**

The inter-pair complementarity measures for the selected left and right primer

**PRIMER_WARNING=s (*)**

s lists warnings generated by primer (separated by semicolons); this tag is absent if there are no warnings

**PRIMER_{LEFT,RIGHT,PAIR}_MISPRIMING_SCORE=f, s**

f is the maximum mispriming score for the right primer against any sequence in the given PRIMER_MISPRIMING_LIBRARY; s is the id of corresponding library sequence.

**PRIMER_PAIR_MISPRIMING_SCORE**

the maximum sum of mispriming scores in any single library sequence (perhaps a more reasonable estimator of the likelihood of mispriming).

**PRIMER_{LEFT,RIGHT,PAIR}_TEMPLATE_MISPRIMING=f**

Analogous to PRIMER_{LEFT,RIGHT,PAIR}_MISPRIMING_SCORE, except that these output tags apply to mispriming within the template sequence. This often arises, for example, in genes with repeated exons. For backward compatibility, these tags only appear if the corresponding input tags have defined values.

**PRIMER_PRODUCT_TM=f**


**PRIMER_PRODUCT_TM_OLIGO_TM_DIFF=f**

f is the difference between the melting temperature of the product and the melting temperature of the less stable primer. Printed only if PRIMER_MAX_PRODUCT_TM or PRIMER_MIN_PRODUCT_TM is specified.

**PRIMER_PAIR_T_OPT_A=f**
Primers and probes


PRIMER_INTERNAL_OOLIGO_MISHYB_SCORE=f, s

f is the maximum mishybridization score for the right primer against any sequence in the given
PRIMER_INTERNAL_OOLIGO_MISHYB_LIBRARY; s is the id of corresponding library sequence.

PRIMER_{LEFT,RIGHT,INTERNAL_OLAGO}_MIN_SEQ_QUALITY=i

i is the minimum _sequence_ quality within the primer or oligo (not to be confused with the PRIMER_PAIR_QUALITY output tag, which is really the value of the objective function.)

PRIMER_{LEFT,RIGHT}_END_STABILITY=f

f is the delta G of disruption of the five 3' bases of the primer.

PRIMER_STOP_CODON_POSITION=i

i is the position of the first base of the stop codon, if primer3 found one, or -1 if primer3 did not. Printed only if the input tag
PRIMER_START_CODON_POSITION with a non-default value is supplied.

Advice for picking primers


Some of the most important issues in primer picking can be addressed only before using Primer3. These are sequence quality (including making sure the sequence is not vector and not chimeric) and avoiding repetitive elements.

Techniques for avoiding problems include a thorough understanding of possible vector contaminants and cloning artifacts coupled with database searches using BLAST, FASTA, or other similarity searching program to screen for vector contaminants and possible repeats. Repbase (J. Jurka, A.F.A. Smit, C. Pethiyagoda, and others, 1995-1996, ftp://
ncbi.nlm.nih.gov/repository/repbase) is an excellent source of repeat sequences and pointers to the literature. (The Repbase files need to be converted to FASTA format before they can be used by Primer3.) Primer3 now allows you to screen candidate oligos against a Mispriming Library (or a Mishyb Library in the case of internal oligos).

Sequence quality can be controlled by manual trace viewing and quality clipping or automatic quality clipping programs. Low quality bases should be changed to N's or can be made part of Excluded Regions. The beginning of a sequencing read is often problematic because of primer peaks, and the end of the read often contains many low-quality or even meaningless called bases. Therefore when picking primers from single-pass sequence it is often best to use the INCLUDED_REGION parameter to ensure that Primer3 chooses primers in the high quality region of the read. In addition, Primer3 takes as input a Sequence Quality list for use with those base calling programs (e.g. Phred, Bass/Grace, Trout) that output this information.

What to do if Primer3 cannot find any primers?

Try relaxing various parameters, including the self-complementarity parameters and max and min oligo melting temperatures. For example, for very A-T-rich regions you might have to increase maximum primer size or decrease minimum melting temperature. It is usually unwise to reduce the minimum primer size if your template is complex (e.g. a mammalian genome), since small primers are more likely to be non-specific. Make sure that there are adequate stretches of non-Ns in the regions in which you wish to pick primers. If necessary you can also allow an N in your primer and use an oligo mixture containing all four bases at that position.

Try setting the PRIMER_EXPLAIN_FLAG input tag.

Primer and probe screening analysis

MacVector provides three screens for likely primers and probes:

- screening a nucleic acid sequence for likely PCR primer pairs
- screening a nucleic acid sequence for likely sequencing primers or hybridization probes
- screening a protein sequence for the least degenerate oligos that can serve as hybridization probes.

The first two work in a similar way and will be discussed together.
Screening a nucleic acid for primers or probes

There are several characteristics to consider when choosing an oligonucleotide to use as a sequencing primer, PCR primer, or hybridization probe:

- it should form a stable duplex with the target sequence under the conditions to be used in the reaction (temperature, salt concentration, and the oligo concentration)
- it should be specific for the intended target sequence, and not bind to other regions within the sequence
- it should not anneal to itself, to another copy of itself, or, in the case of PCR primer pairs, to a copy of its sister primer (hairpin or dimer formation).

Hybridization probes should be checked for possible competing binding activity; with PCR primer pairs or sequencing primers, it is more important to check the 3’ end of the primer.

A computerized method can greatly reduce the amount of work needed to select a primer or probe from a nucleic acid sequence. There are two basic approaches. The first is user-oriented—the user selects a possible primer, inputs it to the computer program, and receives information about the primer that help to decide whether or not to use the primer. This can be time-consuming and demands a certain amount of knowledge from the user, but is a good method for a researcher with special needs. MacVector also offers a second approach - an automatic screening method. The user tells the program the criteria that constitute a bad primer, and the program scans the sequence, eliminating “bad” primers and displaying a list of the primers that pass this screening process. While it does not allow the degree of control that the first method provides, it works well in most cases, demands less of the user, and is faster.

MacVector’s analyses for finding PCR primer pairs and single primers for sequencing (or hybridization probes) have most of their parameters in common. You control the following:

- whether to scan an entire nucleic acid sequence or designate a shorter region (for sequencing primers / probes you can also restrict the scan to a given strand)
- the range of melting or dissociation temperatures that the oligo-sequence duplexes should have
• the required G+C content of the oligo
• the required range of oligo lengths
• the sequence of an oligo at the 3' end (a G or C “anchor,” for example).

Both analyses use the same parameters for eliminating oligos that have too much secondary structure. You control the maximum number of consecutive bonds an oligo has when it forms either a hairpin with itself or a duplex with another oligo. You can set separate limits on the following bond types:
• any bond
• G-C bonds only
• bonds only between the 3' ends of oligos.

The default values for the first two were suggested by Lowe et al. (1990), for the third by Rychlik and Rhoads (1989).

You can also eliminate oligos that may bind to other sites on the sequence. For PCR primer pairs, the 3' end of each primer of a pair is compared with the product that the pair amplifies. For sequencing primers / probes, the 3' end of the oligo or the entire oligo is compared against the entire sequence, or a specified region of the sequence. As with the scan region, you can limit this comparison step to a single strand.

The final set of parameters relate to the reaction conditions. The monovalent salt concentration (Na and K salts) and the oligo concentration are needed in order to compute the melting temperatures.

What if no primers or probes are found? Use the output statistics as a clue, and see what the most common reasons for oligo rejection were. Try expanding your criteria. The first two parameters that are tested by MacVector are the 3' dinucleotide and the primer length, so these are the first two that you should adjust. If the dinucleotide was set to NS, set it to NN to increase the number of oligos that will pass this first criterion. If you are using a narrow range of primer lengths or if they are skewed toward the longer sizes, widen the range and include shorter lengths. Sometimes you may have difficulty finding compatible PCR primer pairs because of widely different G+C content (and thus different Tm values) along the sequence. For example, many coding regions contain an A+T-rich region just upstream of the start of the coding region, while the coding region itself and the sequence downstream may be G+C-rich.
Primers and probes

If you try to find one primer upstream of the coding region and the other downstream, you may need to expand the range of G+C content and the range of temperatures.

Screening a protein to find a hybridization probe

If you want to find an oligo to use as a hybridization probe for a gene whose exact DNA sequence is not known, but whose amino acid sequence is known, you could reverse-translate the amino acid sequence and then scan the resulting DNA sequence to find a region that was not very degenerate to minimize the number of oligonucleotide probes that would have to be synthesized. This is easier to do with a computer than by hand, and is a feature provided by MacVector as part of the reverse translation analysis. For each probe length that you specify, MacVector scans the sequence, finds the oligos of that length that are least degenerate, and outputs a list of their sequences, dissociation temperatures, G+C content, and the number of permutations (the number of oligos that would have to be synthesized to cover all possible sequences represented by the degenerate oligo). No further analysis of the probes, such as checking for secondary structure or alternate binding sites, is performed.

You can reduce the degree of degeneracy by modifying the genetic code that is used to perform the reverse translation. For example, serine has six codons in the universal genetic code, four of the form TCN and two of the form AGY. These two forms reduce to the highly degenerate codon WSN.

If you know that your organism uses primarily the AGY codons, you can create a new genetic code that is missing the TCN codons, so that serines will be reversed translated as TCN instead of WSN. See “Modifying genetic codes” on page 240, for further details.

Computing the Tm and Td

While the terms Tm (melting temperature) and Td (dissociation temperature) are often used interchangeably, they are not quite the same. The melting temperature is used to describe the separation of a duplex nucleic acid molecule in solution solely because of changes in temperature of the solution. The dissociation temperature is used to describe the temperature at which a duplex bound to a substrate (as in a filter hybridization) dissociates because of temperature and washing effects. Rychlik and Rhoads (1989) estimate that Td is approximately 7.6° C lower than the Tm.
Because of its simplicity, a rule-of-thumb equation is often used to calculate the Td of an oligonucleotide duplex: \(4 \times (\text{number of G+C bases}) + 2 \times (\text{number of A+T bases})\). This equation provides a rough estimate for oligonucleotides between ten and twenty bases long. A more accurate determination of Tm or Td is obtained using nearest-neighbor methods, which provide valid estimates over a wider range of oligo lengths (Rychlik and Rhoads (1989), Rychlik et al. (1990), based on values published by Breslauer et al. (1986) for DNA and Freier et al. (1986) for RNA). In computing Tm and Td values, MacVector uses nearest-neighbor methods for oligos up to thirty bases long. For longer oligos, or when the effects of formamide are taken into account, the equation of Baldino et al. (1989) is used for DNA-DNA hybrids, Bodkin and Knudsen (1985) for RNA-RNA hybrids, and Casey and Davidson (1977) for DNA-RNA hybrids.

One factor that complicates Tm calculations for PCR primers is that the primers are consumed during the course of the reaction, leading to a wide variation in their actual concentration. Rychlik et al. (1990) examined this experimentally and suggested an adjustment factor that could be used to calculate an effective Tm which would be more applicable to real-world PCR reaction conditions. MacVector uses this factor in PCR primer calculations, yielding adjusted values that are typically 2°C to 10°C lower than the values obtained for sequencing primers.

**Coding regions**

After you determine the sequence of a piece of DNA, one of the first things you would like to know is whether it codes for a protein. If you are lucky, your sequence will contain at least one long open reading frame—a reading frame of at least 50 to 100 codons that contains no stop codons. You can translate the open reading frame and search the NBRF Protein Identification Resource database or the GenBank nucleic acid database to see if there is a match with a known protein. If you find a match, you will have answered your question.

If there is no match, you need some other method of determining the biological significance of the open reading frame. It may code for a previously unsequenced protein, or it may have no biological significance whatsoever - after all, not all open reading frames are protein coding regions.

MacVector provides a range of analyses to help you make this decision. In addition to open reading frame analysis, the program provides vari-
Coding regions

Various methods use that base or codon composition to help you determine if your open reading frame has the characteristics of a protein coding region. In conjunction with these methods, you can use nucleic acid subsequence analysis to look for motifs in your sequence, such as ribosome binding sites or intron-exon splice sites, that may help define the exact boundaries of coding regions.

Open reading frame analysis

Unlike many other sequence analysis programs, MacVector enables you to define which codons are to be used as start and stop codons. For prokaryotic sequences, you would normally set ATG as a start codon, and possibly also GTG and TTG. The termination codons TAA, TAG, and TGA should be assigned as stop codons, unless your sequence contains suppressor mutations.

In eukaryotic sequences, there may be exons present that do not start with ATG. Therefore, in addition to using ATG as a start codon, you could also assign the stop codons (TAA, TAG and TGA) as start codons, in which case MacVector will treat the codon immediately after a stop codon as a start codon.

You can also designate the beginning and end of the sequence to act as ORF starts or stops. This can be useful if you suspect that the open reading frame for your sequence extends beyond the ends of the sequence.

Base composition methods

MacVector provides four methods that use base composition to predict protein-coding regions. These are based on bias in the overall nucleotide base composition, or on the tendencies for bases to occur at particular codon positions. These methods do not use organism-specific information such as codon bias tables.

G+C % composition

Many organisms have a skewed composition of G+C bases, which results in a strongly biased codon composition. In such cases, non-coding regions tend to reflect the overall G+C percentage, but coding regions, under the constraints of natural selection, show a different pattern. Because of redundancy in the genetic code, there is less selection on the third codon position, so that its G+C % shows a strong bias. The second position shows no bias, as there is no degeneracy in the code. The first position has some bias, due to amino acids such as Leu that can be coded by six different codons. The overall effect in an organism such
as *Streptomyces coelicolor* is that coding regions tend to have ~70% G+C at the first position, ~50% at the second position and > 95% at the third. In non-coding regions, all three plots will tend to be around the average G+C% for the DNA.

The algorithm for calculating the percentages is simple. Starting at base 1 and sampling every third base in a window of \( t \) codons (i.e. base 1, 4, 7, 10, ... ), count the number of G and C bases and plot their combined percentage. Slide the window one codon to the right, and plot again; repeat until the end of the sequence. Now repeat the procedure, this time starting at base 2 (i.e. sampling 2, 5, 8, 11, ...), and plotting the % points in a second color. Finally, repeat once more, starting at base 3 (i.e. sampling 3, 6, 9, 12, ...) and plotting the % points in a third color.

**Fickett's TESTCODE algorithm for predicting coding regions**

As DNA sequences accumulated in databases, researchers examined known coding and non-coding regions and discovered that there were characteristic differences in their base compositions. First of all, because of selection pressure at the amino acid level, the triplet frequencies differ. Some amino acids are more common than others and thus their codons are repeated more often within coding regions than within non-coding regions. As a consequence, nucleotides tend to be repeated with a periodicity of three in coding regions. In addition, coding regions tend to have a slightly higher G+C content than non-coding regions.

Starting from these observations, Fickett (1982) examined coding regions of sequences from the GenBank database and developed a statistical method that could be used to predict coding regions in DNA sequences of at least 200 base pairs. This method uses eight parameters that are based on the periodic properties of each of the four nucleotides within the sequence.

The first four parameters are called position parameters. A count is made of the number of times A appears in codon position 1 (A1), the number of times it appears in codon position 2 (A2), and the number of times it appears in codon position 3 (A3) in the sequence. The first parameter, A-Position, is then defined as:

\[
A\text{-Position} = \frac{\max(A_1,A_2,A_3)}{\min(A_1,A_2,A_3)}
\]

The position parameters for the other three bases are computed similarly. The last four parameters are the content parameters. These are just...
the percentage composition of the sequence for each of the four bases (%A, %C, %G, %T).

Fickett calculated each of the eight parameters for a number of known coding and non-coding regions. He assigned weights (W) to each of the parameters according to the percentage of the time that a coding region could be correctly predicted using the value of that parameter alone. He also derived a “probability of coding” (P) for ten intervals of each of the parameter values. For example, he looked at all regions whose A-Position parameter value fell between 0.0 and 1.1. Twenty-two per cent of these were coding regions, so for his interval, P1 = 0.22. Regions with an A-Position parameter value between 1.6 and 1.7 were distributed as 93% coding and 7% non-coding, so for this interval, P1 = 0.93. The weights and probabilities for each of the eight parameters were combined to define a single TESTCODE indicator value:

\[
\text{Indicator} = P1W1 + \ldots + P8W8
\]

Fickett next examined the distribution of the indicator values of 321 coding and 249 non-coding sequence fragments. Only 29% of the regions with indicator values below 0.74 were coding regions, while 92% of regions with indicator values above 0.95 were coding regions.

In the borderline areas were regions scoring between 0.74 and 0.84 (40% were coding) and regions scoring between 0.84 and 0.95 (77% were coding regions).

As it was originally defined, Fickett’s method assigns a region with a coding probability below 0.29 as a “non-coding” region and a region with a probability of coding above 0.92 as a “coding” region. Regions with probabilities between these values are tagged “no opinion.”

Fickett’s method has two limitations. As we mentioned above, it may not give valid results for sequences shorter than 200 bases (67 codons). Moreover, it cannot distinguish the correct reading frame - or indeed the correct strand.

If you have more than one open reading frame in a region that is assigned as coding, you must use another method to determine which reading frame is the correct one.

As an interesting aside, Fickett notes that many of the known coding regions that this method mis-classifies as non-coding are areas of the sequence where some specialized use is made of the DNA. The mecha-
nisms responsible for ensuring this specialized use are apparently stronger than the forces that cause the difference in coding frequencies. Some examples are viruses with overlapping genes and the variable regions of immunoglobulin genes.

**Uneven positional base frequencies**

In protein coding regions, the four nucleotide bases do not occur equally frequently in the three codon positions. This algorithm, developed by Staden (1984), measures the deviation from an even distribution across codon positions. It does not require prior knowledge of the organism’s codon usage. A single plot is generated, showing the likelihood that a region of the sequence is protein coding. The method cannot distinguish which frame might be the actual coding frame.

In a sliding window of $t$ codons, the algorithm calculates how many times each base $i$ occurs in position $j$ of a codon, to give an array $N[i,j]$. It then calculates the expected non-coding value for each base in each codon position for that window, i.e.:

$$E[i, 1] = E[i, 2] = E[i, 3] = \frac{(N[i, 1] + N[i, 2] + N[i, 3] + N[i, 3])}{3}$$

Finally, it computes the divergence from an even distribution across codon positions, by summing the absolute differences between observed and expected values for each of the 12 $i,j$ values (4 bases X 3 positions):

$$D = \Sigma |E[i,j] - N[i,j]|$$

The window is moved along the sequence one base at a time, and the calculation of $D$ repeated. The results are displayed as a single plot. Seventy-six per cent of coding regions have a $D$ score higher than 0.78, and 76% of non-coding regions score lower than 0.78.

**Positional base preference**

Like the uneven positional base frequencies algorithm, this was also developed by Staden (1984). However, this method can show the likely protein coding frame.

It is based on the observation that, across a large sample set of protein coding regions from different species, the first two codon positions
Coding regions

show marked base specificity. It estimates how closely a potential open reading frame matches this base composition.

A window of \( t \) codons is moved along the length of the sequence in increments of 3 bases. Within each window, each codon is scored by adding up three values from this table:

<table>
<thead>
<tr>
<th>Frame</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>27.67</td>
<td>30.97</td>
<td>23.96</td>
</tr>
<tr>
<td>C</td>
<td>21.08</td>
<td>23.78</td>
<td>25.06</td>
</tr>
<tr>
<td>G</td>
<td>33.57</td>
<td>18.18</td>
<td>25.92</td>
</tr>
<tr>
<td>T</td>
<td>17.68</td>
<td>27.07</td>
<td>25.06</td>
</tr>
</tbody>
</table>

For instance, A at position 1 scores 27.67, and if the next base is T, then 27.07 is added. The score for each window is simply the sum for each codon in the window. Three scores are calculated for each window, starting from positions 1, 2 and 3 in the window. Finally, the user can choose to view the values as absolute or relative. In absolute mode, the score for a window is divided by the window size to get a normalized score. In relative mode, the score for each position is divided by the sum of the scores for all three positions - this accentuates differences between the frames, at the cost of some increased noise.

Codon preference and codon bias tables

There is another way that codon usage in protein coding regions may differ from that in non-coding regions. Most organisms exhibit a preferential use of codons according to the abundance of the corresponding tRNA species. In such organisms, non-coding regions exhibit no codon preference. Regions that code for proteins having a low level of expression tend to have a codon distribution that parallels the abundance of the corresponding tRNA species. Regions that code for highly-expressed proteins are biased even further—they preferentially use those codons that correspond to the most abundant tRNA species for that amino acid. For these organisms, the codon preference can be used as a basis for determining the likelihood that a given region of DNA codes for a protein. MacVector offers three algorithms for identifying coding regions using codon preference.

To use codon preference methods, you must first determine empirically the degree of codon bias that the organism possesses, by creating a codon bias table for that organism. Because codon usage in mitochondrial DNA, for example, is typically different from that of chromosomal
DNA, you should choose only sequences from the same genome source.
A sequence database is searched for all sequences belonging to a given
organism that contain coding regions. (MacVector uses regions of the
sequence that are listed as “pept” in the features table.) The number of
occurrences of each of the 64 codons in these regions is added up and
divided by the total number of codons in the search to obtain a codon
frequency \( f_{abc} \) for each codon \( abc \). The frequency of each codon family
(all codons that encode the same amino acid) is then calculated by sum-
ming the frequencies for each codon in the family. For example, the fre-
quency of the leucine family would be:

\[
F_{\text{leu}} = f_{\text{CTT}} + f_{\text{CTC}} + f_{\text{CTA}} + f_{\text{CTG}} + f_{\text{TTA}} + f_{\text{TTG}}
\]

Lastly, the relative frequency of each codon within the family is
obtained by dividing that codon’s frequency by the codon family fre-
quency. The relative frequencies are stored in the codon bias table.
Codon preference methods are not always applicable. Some organisms
do not exhibit a codon bias. For other organisms, there are not enough
highly-expressed gene sequences in the database to derive a useful
codon bias table. Even if a codon preference is present, it may only be
useful for locating genes of highly-expressed or moderately-expressed
proteins. Weakly-expressed genes usually have little codon bias, and
plots of these sequences can be difficult to interpret.

**Gribskov codon preference**

This algorithm is used in the Wisconsin package. It uses a 64-codon bias
table, and identifies coding regions by measuring the relative occur-
rence of codons that are alternatives for specifying the same amino acid.
The Gribskov method normalizes for the base composition of the
sequence. The algorithm calculates a preference parameter \( P_{abc} \) for each
codon \( abc \), as follows:

Given a target sequence of length \( N \), the base composition is first calcu-
lated, generating the four probabilities \( N_A, N_C, N_G \) and \( N_T \).
The next step is to determine \( f_{abc} \), the absolute frequency of the codon in
the supplied usage table. The frequency of its codon family (i.e. all the
codons that code for the same amino acid as \( abc \)), \( F_{abc} \), is then calcu-
lated as the sum of the frequencies of the family members. Next, the
Coding regions

“random” probability of \(abc\) in the target sequence, \(r_{abc}\), is calculated as:

\[ r_{abc} = \frac{N_aN_bN_c}{N^3} \]

and the random probability of the \(abc\) codon family, \(R_{abc}\), is obtained as the sum of the \(r_{abc}\) values in the family. The preference parameter for each codon can then be calculated as:

\[ P_{abc} = \frac{f_{abc}/F_{abc}}{(r_{abc}/R_{abc})} \]

For a window of length \(w\), the \(P_{abc}\) values for each codon in the window are multiplied together, and the preference statistic is the \(w\)th root of this product. To simplify calculations, this is computed using logarithms.

Where a codon in the supplied table has a zero probability, it is assigned a probability equal to the reciprocal of the sum of codons in its codon family. If it has no family, the reciprocal of the total number of codons in the table is used.

**MacVector codon preference**

The MacVector codon preference analysis is a variant of the Gribskov algorithm that is not normalized for base composition. It is included primarily to allow comparisons with results from earlier versions of MacVector.

**Staden codon preference**

In its basic form, the algorithm of Staden & McLachlan (1982) uses the “raw” codon probabilities contained in codon usage tables such as those generated by MacVector. The table contains 64 entries, one for each codon; the sum of their probabilities is 1.0. The algorithm proceeds as follows:

A window of \(t\) codons is moved along the sequence in increments of 3 bases. For each window, three scores are calculated for \(t\) codons starting at positions 1, 2, and 3. The coding probability for each position is calculated by multiplying together the probabilities of each codon. The final score for each position is then divided by the sum of the probabilities in all three positions to produce a relative coding probability.
A variation of the method uses the coding probabilities normalized to amino acid composition. In this case the probabilities of all six Leucine codons would add up to 1.0, and the single Tryptophan codon (TGG) would always have a probability of 1.0.

Interpreting coding preference plots

The main use of the coding preference plots is to help identify potential protein coding open reading frames in a piece of DNA. Interpretation of the plots will depend to a large extent upon the source of your DNA, and in particular whether or not introns are likely to be present. It can be reasonably straightforward for sequences where introns are absent or rare, e.g. prokaryotic sequences, cDNA clones, many mitochondrial genomes, and simple eukaryotes like *S. cerevisiae*. It can also be complicated by sequencing errors, particularly insertions or deletions, which may cause frame shifts in potential coding regions.

The following tips and suggestions may help you to get the best results from your coding preference analyses.

Sequence length

The length of the analyzed sequence affects how easy it is to interpret the graphs. As most protein-coding open reading frames are less than 3kb in length, it is easier to visualize the plots if the visible on-screen range is less than 15kb. You can easily zoom in on longer sequences by selecting a region with the mouse or using the arrow keys.

Window size

The window size used for each of the algorithms is a trade-off between ease of visualization and the identification of short protein-coding regions. Longer windows give smoother plots with less noise. However, they may miss short open reading frames and may also make it difficult to determine the most likely start codon, where several alternatives exist.

Separate vs. combined mode

Algorithms that generate three plots can be viewed in separate or combined modes. The combined mode, where all three plots appear on the same graph, is very useful for accentuating differences between the frames. However, using this mode, it is not possible to display the frame-specific starts and stops with the plot.
Coding regions

One way to overcome this problem is to turn on just the ORF plot and the algorithm of interest. The two plots will then appear next to each other in the output window permitting color-coded visual comparison between the codons in the ORF plot and the algorithm plots. The combined mode is also very useful for identifying the ends of open reading frames, as the three plots typically cross each other at this point. You can even zoom in to the residue level to identify the exact base at which the crossovers occur.

The separate mode is useful for a tighter integration of the codons and the frame-specific plots. For most algorithms, the start and stop of a protein-coding region often corresponds to the point at which the frame-specific plot crosses the midpoint. By turning on the start and stop codon displays with the center location, the most likely start codon and corresponding stop codon can be clearly seen.

Open reading frames

In searching for coding regions, ORF plots are often a good starting point. In many genomes, the G+C% is less than or equal to 50, and so the standard stop codons (TAG, TAA, TGA) appear quite frequently. As a result of this, ORFs longer than 50 amino acids rarely occur, and are a good indication of protein coding. However, in DNA with a high G+C%, longer ORFs occur frequently by chance, since the A/T-containing stop codons are rare. In such cases the ORF plots are less informative.

Another drawback of using ORF plots is that they are very susceptible to frameshift sequencing errors.

If you are dealing with bacterial sequences, you may want to use the **Universal + GUG start** genetic code to see more potential ORFs. (In *E. coli* over 10% of protein-coding ORFs start with this codon).

Fickett’s TestCode and Uneven Positional Base Frequencies

These methods are both useful as a general guide for identifying regions of sequence that exhibit a biased base composition typical of protein-coding regions. Neither method can identify the likely frame. TestCode scores of greater than 0.92 are considered highly likely to represent protein coding regions. Similarly, Uneven Positional Base Frequency scores greater than 0.78 are also highly likely to encode proteins. Select the **use fixed scaling** option to view the plots in an optimal way - this will truncate scores above or below the optimal ranges. Turning this off pre-
vents truncation, allowing you to see regions with exceptionally high or low scores.

**Positional Base Preference**

This plot is useful if you need to confirm the frame used by a coding region, but do not have additional codon usage information. Plots with values greater than 1.0 indicate a bias towards coding, whereas plots below 1.0 indicate a bias towards non-coding. Typical coding regions will have one plot significantly above 1.0, with the other two plots considerably below. Non-coding regions have all three plots close to 1.0. Plotting the scores in relative mode can help to accentuate the differences between the frames although it can introduce some noise. The fixed scaling mode displays the plots with an optimal range for identifying coding regions, although there will be truncation of high and low scores.

**Codon Preference Plots**

If you have access to codon usage tables for your organism or genome, the three Codon Preference Plots offer the most sensitive way of identifying coding regions. The MacVector and Gribskov plots are essentially the same, except that the Gribskov algorithm normalizes for the G+C% composition of the DNA. For 50% G+C DNA, they produce essentially identical results. For skewed G+C% DNA, the MacVector algorithm may be overly optimistic in assigning likely coding regions. All three plots allow you either to view the entire range, or to scale to twice the standard deviation. When most of the DNA is thought to encode protein, scaling to 2x Std. Dev. helps to distinguish between the different open reading frames. However eukaryotic genomes, where coding regions are relatively rare, are best viewed at full scale: otherwise the background noise becomes amplified and interferes with the visualization.

**G+C%**

The G+C% plot is extremely useful for analyzing genomes that diverge significantly from 50% G+C, although it can still be useful for other organisms, as many species exhibit a codon-specific G+C% bias in coding regions. The plot is harder to interpret than the other algorithms. Consider a genome with an overall G+C composition of 75%. In non-coding regions of DNA, there will be no codon-specific G+C bias, and the three plots will be poorly separated, all showing G+C percentages close to the average for the test DNA. However, where proteins are encoded, the plots will diverge so that the first plot averages 70%, the
Coding regions

second 50% and the third 95%. This means that although the third plot lies nearest to the top of the graph, it is actually the middle line that represents the likely frame of the coding region. For high A+T% DNA, the converse is true (the third position will be particularly AT rich), although the middle line still represents the frame.

The situation becomes more complex when we consider issues of strandedness. The MacVector color defaults are: frame 1 = blue, frame 2 = green, and frame 3 = red. For coding regions on the top (or forward) strand, if the blue frame (position 1) represents the first position and is the middle line, then the green line should be the lower line and the red line should be uppermost. Similarly, if the red line represents the first position, then blue should be the lower line (position 2) and green the upper line (position 3). For coding regions on the opposite strand, however, the order of the second and third lines is swapped, so that if position 1 is blue, position 2 will be red, and position 3 green. If you are having difficulty, copy the sequence to a separate document, reverse complement it, and repeat the analysis.

Effect of introns

Intron-containing genomic DNA can be analyzed in much the same way, except that you should be aware that:

- internal exons may not have start or stop codons, and
- many coding exons may be less than 50 amino acids in length.

Ideally, you should combine the analysis with the results of searches for potential splice-site donor and acceptor sequences. As you may find large regions of non-coding DNA, it is useful to use the zoom functionality to home in on potential coding regions.

Sequencing errors

Sequencing errors can interfere with this analysis, particularly if they introduce frameshifts into the sequence. These often show up as areas where the plots appear to indicate that the frame has changed, but there is no corresponding start/stop codon. Use the zoom function to home in on the area where the plots cross, to identify the likely region containing the error. Re-evaluation of the original sequencing data will often reveal the source of the error.

You can use coding preference plots as a method for detecting these sequencing errors.
Overview

This chapter explains some of the theories and methods used by MacVector for sequence comparison. Users are referred to the academic papers from which the methods are derived. This chapter provides greater detail only where significant changes have been made to the methods.

Refer to Appendix F, “References”.

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Understanding Sequence Comparisons

Pustell matrix analysis

When looking for similarities between two sequences, a matrix comparison is the method of first choice. A matrix analysis is unsurpassed for getting an overall picture of how sequences are related, and it can detect features that other methods may miss.

By displaying results graphically, a matrix method takes advantage of the superb pattern recognition capabilities of the human eye to allow detection of even weak similarities between two sequences. Computational alignment methods usually report only one “best” alignment between two sequences; the matrix method will reveal any significant alternative alignments that may exist, the presence of weaker regions of similarity, such as duplications, and the existence of rearrangements. Computational region-finding methods present the researcher with a list of matching regions; the matrix method displays matching regions in the context of the sequence as a whole, making it easy to determine if the regions are repeats or inverted repeats, for example.

The simplest form of matrix analysis (also called a dot plot or dot matrix analysis) can be done by hand for short sequences. The residues of one sequence are written along the X-axis of a two-dimensional graph and the residues of the other are written along the Y-axis. A dot is placed at each x,y coordinate where the residues of the two sequences are identical. Regions of similarity stand out as diagonal dotted lines against an amorphous cloud of dots.

The patterns of the diagonals in the plot can tell you a great deal about the sequences. A long unbroken diagonal from the upper left corner to the lower right corner indicates that the sequences are identical (this is called the identity diagonal). If the diagonal runs from the lower left corner to the upper right corner, one sequence is the reverse complement of the other. Short diagonals that parallel the identity diagonal represent repeats; diagonals paralleling the reverse diagonal represent reverse repeats. Breaks appearing within a diagonal represent mismatching regions; a gap is seen as a diagonal that stops, then continues at a different level. In the Sample Files supplied with MacVector are a number of sequences that demonstrate these patterns. If you compare SV4CG with itself and zoom in to view the region between bases 1-200, you will see examples of repeats and reverse repeats. Comparing the protein sequences CVJB and FVVFB shows an example of a rearrangement.
MacVector’s Pustell matrix analyses are more sophisticated variations on the simple dot-plot method. They offer a number of parameters to customize the analysis—more than most implementations do. In general, leaving the parameters set at their default values will result in a usable matrix plot. Once you know more about your sequence, for example, if it contains coding regions, a known transcriptional control region, and so on, the various parameters will become more useful.

The parameters are:

- the scoring matrix
- hash size
- window size
- minimum percent score
- jump parameter.

The values in the scoring matrix are the weights given to different kinds of matches. If you care only about perfect matches (A-A, G-G, etc.), you would use an identity matrix—one that scores exact matches as +1 and mismatches as 0. But under some situations for nucleic acids and, more frequently, for amino acids, you may wish to consider some mismatches to be partial matches according to criteria such as hydrophobicity, charge, or structure, and to consider some mismatches to be worse than others. By altering the values in the scoring matrix, you can control the matching criteria.

The hash size is also known as the k-tuple size or the word size. When the analysis initially scans the sequences for matches, it uses a very fast technique known as hashing. When the hash size is set to six, the program will only consider exact matches of six residues to be a “hit.” If it is set to one, it will consider a single residue match to be a hit, and so on. Using larger hash sizes will result in a less sensitive but faster search.

The jump parameter is used in conjunction with the hash size. When the jump parameter is set to one, the “word” used in the hash step is formed from consecutive residues. So if the hash size is six, and the jump is one, a word consists of six consecutive residues. If the jump parameter is set to three, the word is composed of every third residue. Therefore if the hash size is six and the jump is three, the word consists of the first residues of six consecutive triplets.

When a hit is found, the program begins the second step of the comparison, the scoring step. In this step, the scoring matrix, window size
parameter, and minimum percent score come into play. The hit is placed in the center of a “window” whose length is the window size set by the user. The window is then scored, using the values for the residue pairs that are found in the scoring matrix. If the score meets or exceeds the minimum percent score set by the user, the location of the window is saved by the program.

As with hash size, larger values for the window size result in less “noise” in the matrix, but the sensitivity of the search is reduced. Experience has shown that for two random sequences, the minimum percent score should be set no lower than 65% if an identity scoring matrix is used.

Let us now illustrate these concepts with an example, matching a short sequence against itself:

AGCCTCGATCGATTCGATCGGCGCTAGCTAGGCTAG

Hashing both copies of the sequence at a hash size of three will make the program look at the following three-letter “words” in both sequences to see if there are any matches:

Sequence A words:
1 AGC CTC GAT CGA TTC GAT CGG CGC TAG
CTA GGC TAG
Sequence B words:
1 AGC CTC GAT CGA TTC GAT CGG CGC TAG
CTA GGC TAG

Since these two sequences are identical, there is obviously a match starting with the first base of each sequence. But other shorter matches may exist. Note that there are two GAT words in each sequence, which means a region of similarity may also exist centered at residue 7 in A and 16 in B. Let’s assume the window size is 12. The 65% match criterion means that at least 7 of the 12 residues in a window must match. Obviously, the window containing the first GAT word in both sequences will be a valid window since 12 out of 12 residues match.

But what about the regions of the two sequences where the first instance of GAT in A matches the second instance of GAT in B?

1 AGC CTC GAT CGA TTC GAT CGG CGC TAG
CTA ...
* ** *** **
10 CGA TTC GAT CGG CGC TAG CTA GGC TAG

There are eight matches in the 12-base window surrounding the matching word GAT, so this window is also a valid match. After checking the
windows that surround each of the matching words, the program shifts one base in the second sequence, rehashes the sequence in this phase, and compares the resulting set of words for sequence B with the original set of words for sequence A:

```
1  AGC  CTC  GAT  CGA  TTC  GAT  CGG  CGC  TAG  CTA  ...
   *        *
2  GCC  TCG  ATC  GAT  TCG  ATC  GGC  GCT  AGC  TAG  ...
```

Again, the program checks the windows surrounding each matching word to locate valid windows. Again, it shifts one base over in the second sequence and repeats the hashing and scoring process for the third time.

Now we’ll consider the effect of the jump parameter. A jump setting of one with a hash size of three applied to our test sequence gives the words you have already seen:

```
AGC  CTC  GAT  CGA  TTC  GAT  CGG  CGC  TAG  CTA  GGC  TAG
```

But with a jump setting of three and a hash size of three, we get a different set of words:

Original sequence:
```
AGCC TCG AT CGAT TCG ATCGC GCTAG CTA GGC TAG
```

After the jump is applied, the sequence is:
```
ACG  CTG  CCT  CGT
```

The words obtained from hashing the processed sequence are:
```
ACG  CTG  CCT  CGT
```

Notice that we are still comparing words of size three, but they are derived from different “samples” of the same sequence.

The jump parameter is useful when we compare nucleic acid sequences that code for proteins. Imagine comparing two distantly related protein-coding regions using a hash level of three and a jump setting of one. This means that no windows are scored unless the program finds three bases in a row that match. Because the triplet code’s redundancy allows much variation in the third base, these sequences could code for identical proteins, yet still have no matching triplets because of different codon usage. In such a case, the matrix analysis would show no diagonals! If we used a jump setting of three, we would be comparing every third base. We would get strong signals when we coincided the first and
second bases of the codons, even though there would be no signal for the third bases. Now the matching coding regions should show up as obvious diagonals in the matrix display.

**Lipman-Pearson DNA and Wilbur-Lipman protein library searches**

These high-speed sequence library search routines have been a boon to molecular biologists. A full local alignment of a query sequence against a whole library of sequences would take an enormous amount of time, even for a supercomputer. By using an initial quick hashing step to screen the sequences, as is done in the matrix comparison discussed in “Pustell matrix analysis” on page 402, the time-consuming alignment process is only performed on sequences that are shown to have a certain degree of similarity. MacVector uses these routines to compare a query sequence to a folder containing one or more sequences.

There are a number of differences between these analyses and the matrix comparisons. While the matrix comparison will display all matching regions that it finds, these library search methods will only show the one “best” alignment between the matching regions of two sequences.

Both methods use the hashing technique as the first step in comparing the sequences. However, the library search methods do not allow you to change the jump parameter: it is permanently set at one. As with the matrix analysis, when a hit is found, the second step of the analysis is a scoring step using the values in the scoring matrix. Unlike the matrix analysis, there is no fixed window size for the scoring step and no percent score is taken into account—the scoring starts with the hit and continues until a significant region of mismatch is encountered. The score obtained from this step is called the initial score.

Unlike the matrix analysis, there is a third step to the comparison: a full local alignment. Three additional parameters are involved:

- the cut-off score
- the deletion penalty (also called the gap-opening penalty)
- the gap penalty (also called the gap-extension penalty).

These parameters are accessed by opening the scoring matrix file and clicking the Tweak button (labeled with an icon that looks like a mouse) to open a dialog box in which the values of these parameters can be changed. The cut-off score is determined by the four parameters p1, p2,
Customizing similarity searches

p3, and p4; ordinarily you would only change these if the query sequence is very short.

The third step, the time-consuming alignment step, is performed only if the initial score exceeds the cut-off score. This full local alignment inserts gaps in the alignment as necessary to improve the score. The score resulting from this step of the analysis is called the optimized score. The optimized score is the score of the best local alignment of the matched region of the library sequence and the query sequence. In the case of perfect identity, the initial and optimized scores will be identical and will be four times the length of the matching region (if you haven’t changed any of the scoring matrix parameters with “Tweak” or changed the scoring matrix itself). So, for a 50-base-long perfect match, both scores will be 200.

It is necessary to balance the speed of the procedure (via parameter choices) with the accuracy of the result. For example, an incorrect choice of match, mismatch, gap-opening, and gap-extension parameters can produce alignments which favor gaps over mismatches, a generally unlikely occurrence. It may even be possible to create a parameter set which will miss near-optimal, but biologically significant, alignments.

As in the similarity matrix procedures, the hash level is an important parameter. The larger the hash level value, the faster the search, and the less sensitive the search. If the hash level is set to one and the other parameters are handled appropriately, the library search routine becomes a full metric alignment procedure. This is of significant advantage for evolutionary comparisons of genes within a gene cluster. It is the most rigorous search routine available in MacVector.

For your first pass, the best hash setting to use for amino acid sequences is two and for nucleotide sequences is six. As matching regions are identified, it is frequently useful to modify the search parameters through the “Tweak” option or the scoring matrix.

Customizing similarity searches

Tweak

At the beginning of the sequence library search, the program calculates a cut-off score. Matching regions whose scores fall below the cut-off score are not aligned and therefore not saved. The parameters used by MacVector to calculate the score can be changed.
The parameters p1, p2, p3, and p4 should normally be left at their default settings. The parameters that are useful to modify are the deletion penalty (penalty for a single residue insertion or deletion, called an indel) and the gap penalty (penalty for a continuing indel). The higher the value of the deletion penalty, the less frequently the program will introduce an indel in preference to allowing a mismatch. The higher the gap penalty, the more costly it is to extend that indel once it is opened. By decreasing the deletion penalty, you can make it easier to introduce an indel. This can be desirable in AT-rich regions, or up- and downstream control regions of a gene cluster. Similarly, for protein sequences, changing these penalties from their default values can increase the likelihood that insertions and deletions, such as have occurred in the cytochrome family, will be permitted in the sequence comparison.

**Scoring matrix**

In any search procedure, there are values assigned both for a match and for a mismatch. These values can be found in MacVector’s scoring matrix files.

For example, in DNA database matrix, the score assigned for a match is +4, while the score assigned to a mismatch is -2. It is six times more costly to introduce an indel (insertion or deletion) than it is to tolerate a mismatch (the default deletion penalty is -12) and twice as costly to extend an existing indel than to tolerate a mismatch (the default gap penalty is -4 per base).

These default settings were assigned based on general experience with DNA sequences. For specific applications, it may be useful to alter the match and mismatch scores. You can edit the existing scoring matrix files, or create new scoring matrix files within MacVector. In setting these values, it is the ratio between the match and mismatch, and between the match and deletion penalty values, that are important, not the actual magnitude of the scores. If you report similarity scores for sequences in a scientific presentation, you should always include the values of all four parameters (match score, mismatch score, deletion penalty, and gap penalty).

What are the circumstances that might lead you to alter a scoring matrix? It is often useful to try different scoring schemes for comparing proteins. The scoring matrices pam250 and pam250S are based on Dayhoff’s log-odds scoring matrix, whose values were assigned empirically.
Customizing similarity searches

on the basis of observed amino acid replacements. This matrix has been shown to be more effective than other scoring matrix schemes in verifying distant relationships between homologous proteins (Feng, et al. 1985). However, you may wish to assign matrix scores based on other criteria when you search for protein similarities.

The simplest scheme is an identity matrix which scores an exact match as +1 and a mismatch as 0 (the important cysteine-cysteine match is often assigned a score of +2). Another matrix scheme assigns scores based on the genetic code—higher scores are assigned to amino acid pairs whose codons differ by two or three nucleotides. It is also possible to assign scores by taking into account the structural similarities of the amino acid side chains, for example, assigning a higher score for a leucine-isoleucine pair than for a leucine-lycine pair.

In addition to allowing you to change the match / mismatch values themselves, MacVector makes it easy for you to treat groups of amino acids as if they were identical. This is done by assigning them the same hash (or group) number. To see how this works, compare the scoring matrix pam250 with its simplified version, pam250S, shown in Appendix C, “Reference Tables”. Notice that these two matrices contain identical match / mismatch values. If you look at the tweak values table, you will see that each non-ambiguous amino acid is assigned a unique hash number in pam250. The matrix pam250S, on the other hand, assigns several different amino acids to the same hash number. All amino acids that share the same hash number are considered to be identical according to the properties of their side chains.

Additional hash schemes are certainly possible. For instance, when searching protein sequences, it may be useful to use Kyte and Doolittle classification parameters. You might develop your own classification for amphipathic helices and NTP binding sites, among others. For nucleic acid sequences in extremely AT- or GC-rich segments, it may well be useful to use a pyrimidine-pyrimidine and purine-purine scoring scheme instead.

Protein scoring

Because of the way MacVector computes scores when matching protein sequences, it is possible for a region to exceed 100 percent match. Another artifact of the scoring method is that a region of the two
sequences that is an exact match may have a score of less than 100 per-
cent. How does this arise, and is it something to worry about?

The pam250 scoring matrix assigns scores from 1 to 17 for matches and
scores from -1 to -8 for mismatches. The magnitude of the score reflects
the likelihood that an amino acid in one sequence could have been
replaced by the corresponding amino acid in the other sequence. For
example, cysteines tend to be highly conserved, so a C-C match
between two sequences gets the score 12. A replacement of a cysteine
by a serine (or vice versa) is less likely, so a C-S pairing gets the score
0. The likelihood that a cysteine will be replaced by a tryptophan (or
vice versa) by mutation is fairly low, so this pairing gets the score -8.

Before comparing the two sequences, MacVector computes the total
score (using the scoring matrix) that would occur if the sequence on the
X-axis were compared with itself. The total score is then divided by
the number of amino acids in the sequence to yield the average contribution
per amino acid to the total score. The amino acid score is multiplied by
the window size to give the theoretical maximum score for a 100 per-
cent match for a window of that size. This theoretical maximum score
may be less than the score attained by a small region of the sequence, or
it may exceed the score attained by a perfect match in a small region of
the sequence. An example may clarify matters:

The peptide:

```
G D V E K G K K I F I M K C S Q C H T V
```

has scores of:

```
5 4 4 4 5 5 5 5 5 9 5 6 5 12 2 4 12 6 3 4
```

when it is compared with itself. The total score is 110, or an average of
5.5 per residue, which MacVector truncates to 5. If the window size is 8,
the theoretical score for 100 percent match is 5*8, or 40. Let’s now
compare the sequence with itself window-by-window as the program
does. The score for the first 8-residue window is 37. This becomes 37 /
40 = 0.93, so the algorithm has calculated a 93 percent match, even
though the match is perfect. The score for the second window is also 37
(0.93), the score for the third window is 42 (42 / 40 = 1.05), and the
score for the fourth window is 43 (43 / 40 =1.08).

Why don’t we calculate the maximum score per window on-the-fly,
using the scores for the amino acids that are actually present instead of
the theoretical maximum score? This doesn’t solve the problem, since
the maximum score (and hence the percent match) for a window could
depend on which of the two sequences was used to calculate the maxi-
If the X-axis sequence within a window is DIMCYWRH and the
Corresponding Y-axis sequence within the window is EVICFFKQ, the
Maximum score would be either 66 or 52, depending on which sequence
Was compared with itself to calculate the maximum score. The score
Obtained by comparing the two windows with each other using pam250
Is 34, so the match would be either 52 percent or 65 percent, depending
On which maximum score was used. In addition, this method is slower
Because of the added computation.

The important thing to remember is that a matrix analysis is really a
Qualitative analysis method (even though our assigning letters every two
Percentage points makes it look very quantitative). Using the line display mode, you can easily locate regions of two sequences that are similar. By switching to the character display mode, you can instantly get a rough idea of the significance of matches that all look the same in the line display mode: a diagonal consisting of mostly Cs and Ds obviously represents a better match than one consisting of mostly Zs and As.

Finding subsequences and motifs

DNA and protein subsequence analyses allow you to locate small consensus sequences or motifs within a sequence. The search sequences are stored in a special subsequence file, see “Subsequence files” on page 73.

MacVector gives you a great deal of flexibility on how to describe the motif. Many of the subsequences you want to search for are simple, consisting of a single short motif. You can specify that you will allow a certain number of mismatches, and also indicate which residues in the motif must match exactly.

MacVector also allows you to search for motifs that are more complex. If the sequence has more than one part, you have two choices on how the parts should be combined: you can use AND logic or OR logic. Combining parts using OR logic means that only one of the specified parts needs to be present for a match to occur (either Part A OR Part B must be present). Combining parts using AND logic means that all parts must be present in order for a match to occur (both Part A AND Part B must be present).

An example using OR logic: site-directed mutagenesis

Site-directed mutagenesis is a powerful technique for introducing a mutation into a DNA sequence at a single known location. A synthetic
oligonucleotide is made whose sequence matches the sequence of a region of the parent or wild type DNA except for a specific nucleotide. This oligo is then substituted into the parent DNA. To make sure this technique is in fact site-specific, you need to verify that the oligonucleotide will not match regions of the parent DNA other than the one specific region where you want to insert the mutation. You can do this by performing a subsequence analysis of the parent DNA using the oligonucleotide as the subsequence. If your oligo is truly site-specific, the program will report only one match.

You could use the whole oligo as a single simple subsequence, just as we did in the section on simple subsequence analyses. But a problem may arise if your oligo is very long.

Suppose, for example, that your oligo is 21 bases long, with the base to be mutated in the center. It might be possible for a short region at one end of the oligo (about seven bases or so) to match several sites in the DNA, which means your mutation would be inserted in more than one place. If you searched for the whole oligo as a single subsequence, the non-matching portion of the oligo would score more heavily than the small amount of match at the end, and the program would not report the extra matches, lulling you into a false sense of security as to the specificity of your oligo.

A way to deal with this situation is to analyze long oligos in parts. For this example, we could enter bases 1 through 10 and bases 12 through 21 into a subsequence file as two separate subsequences with separate names. Or we could enter them into the subsequence file as two parts of one subsequence, using OR logic.

An example using AND logic: cell division motif

A cell division motif is shared by the SV40 large-T antigen, adenovirus E1A protein, papilloma virus E7 protein, the v- and c-myc oncogene products, and the CDC25 gene product (yeast mitotic regulator). This motif has two parts which are separated by a gap which varies in size from one to eight amino acids:

Part 1. (ND)XXCX(STE) (beta turn)
Part 2. (DE)(DEST)(DE)XXX (alpha helix)

By entering these into the subsequence file as two parts of a single subsequence using AND logic and setting the gap range from 1 to 8, we can direct the program to look for this motif.
Phylogenetic analysis

The main method that molecular biologists use to elucidate gene function is to perform experiments with cloned DNA sequences. However, comparative analysis is an increasingly important alternative. For example, one of the first tasks of a researcher, when characterizing a newly cloned sequence, is to perform a BLAST search against a reference library such as one of the NCBI databases. If there are significant matches between query and library sequences, this may indicate functional similarities. Such findings often provide the starting point for further studies of the cloned sequence.

MacVector provides a suite of methods for undertaking sequence comparisons, including Pustell matrix analysis, DNA and protein library searches, and subsequence analysis. For the most part these methods are concerned with identifying regions of similarity between pairs of sequences. None of the methods make any assumptions about the underlying mechanisms by which sequences diverge from one another during evolution.

Phylogenetic analysis provides an alternative approach to comparative sequence analysis. Instead of concentrating on the relationships between a sequence and one or more reference sequences, phylogenetic analysis provides a means of simultaneously describing the relationships between all sequences in a given sample. Phylogenetic methods allow the researcher to look for patterns of similarity within an alignment, without having to restrict attention to a specific sequence. At the same time, phylogenetics provides a rigorous framework in which researchers can make quantitative statements about sequence similarities, and make better informed judgements as to whether a given match reflects true homology rather than chance similarity. Li (1997) and Swofford et al. (1996) provide overviews of phylogenetic analysis.

Methods for calculating phylogenies

Molecular phylogenetics offers a diverse array of methods for analyzing sequence alignments (Li 1997). A common way to categorize these approaches is to distinguish three groups of methods:

- **Distance matrix** methods are derived from statistical methods for performing cluster analysis. A pairwise distance matrix is constructed for all sequences in the alignment, and this is used to reconstruct a tree.
• **Parsimony** methods use a hypothetico-deductive approach to analyze sequence alignments. The alignment positions are considered one at a time, and the tree that requires the least number of changes is chosen.

• **Maximum likelihood** methods require an explicit model of sequence evolution. By applying the statistical approach of maximum likelihood to a quantitative model and a data set, the optimal tree can be identified for a given set of conditions.

The choice of method depends to some extent on what kind of sequence alignment is being analyzed. Most sequence alignments that show a consistent pattern of variation can be successfully analyzed using any method. In contrast, alignments that contain very diverged sequences may require particular methods of analysis. In choosing which method to use, researchers must usually make a trade-off between reliability and speed of computation.

MacVector offers two methods, both of them distance matrix approaches. Distance matrix methods are among the fastest reconstruction methods, and they are known to be reliable for a variety of sequence alignments. There are two steps to the process:

1. Estimating the evolutionary distance between every pair of sequences in the alignment
2. Reconstructing the phylogeny, using clustering methods to add the sequences one by one.

MacVector provides a variety of metrics for estimating the evolutionary distance between sequences, and two methods of phylogenetic reconstruction.

**Estimating evolutionary distance**

To understand distance methods it is necessary is to appreciate the underlying evolutionary process. If we compare a homologous nucleotide position in two DNA sequences there are two possibilities: identity or non-identity. If the two sequences are very closely related, it may be reasonable to assume that identity indicates that no substitutions have occurred since the two evolutionary lineages diverged. However, if the two sequences are less closely related, and have diverged over longer periods of time, it becomes increasingly likely that recent evolutionary events have masked earlier events.
Phylogenetic analysis

For example, if we see an A at the same position in two sequences, it is possible that in one lineage A occurred in the ancestral sequence, but was then replaced by G, which was again replaced by A at some later time. Likewise, if the two sequences show a difference at a given position, this does not give us enough information to reconstruct the full series of evolutionary events since the two lineages diverged. A consequence of this is that calculations of evolutionary distance between very diverged sequences will tend to underestimate the true amount of evolution that has occurred.

Evolutionary biologists attempt to correct for unseen evolutionary events, using models of nucleotide and amino acid substitution. Nucleotide substitution models are generally the more sophisticated, largely because the process of DNA evolution is much simpler to describe than protein evolution. Consequently, a greater variety of methods for estimating DNA distances have been developed, and this is reflected in the choice of distances provided in MacVector.

Invalid distances

In some cases no valid distance can be calculated for a pair of sequences, because the selected algorithm generates an illegal operation, e.g. division by zero, or the logarithm of a negative number. This is likely to happen if the sequences are highly divergent, or if they have not been properly aligned.

MacVector warns the user when any invalid distances are found. If you choose to continue with the analysis, MacVector will use the largest value calculated in the matrix to substitute for any failed calculations - interpret with care!

Nucleotide distances

Absolute number of differences

This is calculated as the total number of nucleotide differences between two DNA sequences. This method should generally be avoided as the basis for phylogenetic reconstruction, but may occasionally be useful if the user wishes to visualize the total number of differences between sequences in an alignment.

$p$-distance

The $p$-distance is calculated as the proportion of differences between two sequences. In general, if the sequences being analyzed have $p$-distances less than 0.1, this method is suitable as the basis for phylogenetic reconstruction.
reconstruction. However, when sequences have significantly diverged from one another, the $p$-distance is likely to underestimate the true amount of evolutionary divergence and produce misleading results.

**Jukes-Cantor**

Jukes & Cantor’s (1969) model of nucleotide substitution makes the assumption that the rate of nucleotide substitution is the same between all pairs of nucleotides, so that transitions (e.g. purine replaced by purine) and transversions (e.g. purine replaced by pyrimidine) are equally likely. For moderately diverged sequences this method provides more robust estimates than the $p$-distance, but when sequences are more diverged it may be misleading.

**Kimura 2-Parameter**

The method of Kimura (1980) does not assume that transition and transversion rates are equal. Since an excess of transitional substitutions is a general feature of diverging DNA sequences, the Kimura 2-parameter distance is often more robust than the Jukes-Cantor method.

**Tajima-Nei**

The method of Tajima & Nei (1984) is appropriate when the base composition of nucleotides is unequal. Many sequences deviate from nucleotide frequencies of 0.25, and the Tajima-Nei method corrects for this bias. However, this method should be used with caution, since it assumes that transition and transversion rates are equal.

**Tamura-Nei**

The method of Tamura & Nei (1993) is the most general of the methods provided by MacVector. This method corrects not only for differences between transitions and transversions, but also for the different types of transitions (i.e. A <> G, and C <> T). This method is particularly appropriate for very diverged sequences with biased base compositions.

**LogDet**

The LogDet method for estimating nucleotide distance is useful in sequences where there are extreme differences in the base compositions of sequences in an analysis (Lockhart et al., 1994). For example, in some bacterial genomes G+C contents may be close to 90%. If we include sequences from G+C rich genomes in an analysis, they are likely to be grouped in subsequent reconstruction, even if they are actu-
Phylogenetic analysis

ally quite unrelated. The LogDet method is very powerful for resolving such situations, but is not appropriate as a general method.

Protein distances

Methods for estimating distances between amino acid sequences are currently not as sophisticated as those for nucleotide sequences. As a result MacVector offers only three methods:

**Absolute number of differences**

This is calculated as the total number of amino acid differences between two protein sequences.

**p-distance**

The $p$-distance is calculated as the proportion of differences between two sequences. In general, if the sequences being analyzed have $p$-distances less than 0.1, this method is suitable as the basis for phylogenetic reconstruction. However, when sequences have significantly diverged from one another, the $p$-distance is likely to underestimate the true amount of evolutionary divergence.

**Poisson-correction**

This method assumes that the number of substitutions occurring at each site follows a Poisson distribution. This method may be the most appropriate method to use when protein sequences are significantly diverged (e.g., average $p$-distance $> 0.1$).

Phylogenetic reconstruction methods

MacVector provides two distance matrix methods for reconstructing phylogenies, *neighbor joining* and *UPGMA* (Unweighted Pair-Group Method with Arithmetic mean). Both methods are similar in their implementation and are defined by well specified clustering algorithms. Both proceed by scanning the pairwise distance matrix and identifying the two most similar sequences. These two sequences are joined in the tree, and the underlying matrix is then recalculated, treating the newly joined sequences as a new phylogenetic unit. The process continues until all sequences have been added to the tree. Neighbor joining and UPGMA differ in the way that the matrix is transformed as each sequence is added. The important points to note are as follows:

- The UPGMA method is useful if we assume that the rate of evolution is constant in different lineages. If, in some group of organisms, a particular protein attains greater functional significance
and starts to evolve at a faster rate, that assumption will be violated. This is believed to be a common situation for many genes. In such cases the UPGMA method is likely to generate misleading results.

- Neighbor-joining (Saitou & Nei 1987) makes no assumption about constant evolutionary rates, so the method is more robust than UPGMA.
- UPGMA generates rooted trees. This gives some indication of the polarity of evolution in a phylogeny. By contrast, neighbor-joining generates an unrooted tree, and the only way of making inferences about the direction of change is to use one of the supplied rooting methods.

For details of the algorithms, see Swofford et al. (1996). This article also describes several more sophisticated phylogenetic methods. However, when the appropriate distance and phylogenetic reconstruction methods are used in combination, MacVector will provide robust estimates of phylogeny.

### Analyzing phylogenies

In addition to providing methods for generating phylogenetic trees, MacVector also provides methods for analyzing them. One approach is to use the set of tools provided in the Tree Viewer window. These enable users to format trees in different ways, to investigate the effect of rooting the tree using different methods, and to examine the effect of excluding sequences from an analysis (see “Editing Trees” on page 295). A second approach is to test the reliability of the phylogeny using bootstrap analysis (Felsenstein 1985).

#### Bootstrapping

If there is variation among the sequences in an alignment, the methods provided by MacVector are guaranteed to provide a single best tree. However, in many circumstances the resulting phylogeny may be meaningless. For example, if we generate an alignment of completely unrelated DNA sequences, the relationships suggested by the phylogeny are likely to be positively misleading. The question of how much confidence can be associated with a phylogeny is a long-standing problem in phylogenetics which has attracted much attention.

The most general method available for assessing the confidence associated with a phylogeny is *bootstrapping*. This is one of a well known
group of statistical techniques called resampling methods. The essence of the method is to generate a series of random subsamples of the data set and repeat the phylogenetic reconstruction for each subsample, recording how often a particular grouping in the tree is recovered. Ideally, the same tree will always be recovered from the data set, no matter what random sample is used for the reconstruction. If, however, the data set contains no consistent phylogenetic signal, completely different trees could be recovered each time a different sample of nucleotide positions was analyzed.

To summarize the results of a bootstrap analysis, a **consensus bootstrap tree** is made of the phylogenies reconstructed from the various bootstrap data samples. The consensus bootstrap tree summarizes the shared features of the set of resampling trees. Branching points (internal nodes) are retained only if they occur in a minimum percentage of resampling trees; all other nodes are collapsed.

If the phylogeny is strongly supported, the consensus bootstrap tree will include the majority of nodes resolved as bifurcations, like this:

![Best tree and Bootstrap tree diagrams](image.png)
If there is little or no resolution, the tree will show a small number of multifurcations, with multiple branches originating from the same ancestral nodes, like this:

**Best tree**

**Bootstrap tree**
A Setting up NCBI's *Entrez* and BLAST Services

Overview

This appendix contains details of how to access the *Entrez* Internet database and the BLAST search service.
Setting up NCBI's Entrez and BLAST Services

NCBI's Entrez database

The Entrez database, produced by the National Center for Biotechnology Information (NCBI), provides access to DNA and protein sequences and related bibliographic information. The sequence data include the complete nucleotide and protein sequence data from the Genbank, EMBL, DDBJ, PIR, PRF, PDB, SWISS-PROT, dbEST and dbSTS databases, as well as data from U.S. and European patents. Entrez also contains a subset of the MEDLINE database, including references and abstracts which are cited in the sequence databases and other related MEDLINE records. The data are updated on a daily basis.

NCBI's BLAST search service

The NCBI BLAST search system provides a way of searching all the Entrez sequence databases by similarity. The Internet configuration for accessing BLAST is the same as for other Entrez searches.

Accessing the NCBI services

MacVector can directly browse the Entrez molecular sequence database over the Internet, and search it using BLAST. With MacVector, databases residing at NCBI are accessible by users at remote locations over the Internet.

MacVector has been designed to make accessing Entrez and BLAST as effortless as possible. You do not need to be an Internet expert to use MacVector. After an Internet connection is established, MacVector will handle all the communications with NCBI’s databases.

To set up your MacVector package to access Entrez and BLAST, you can use either a permanent network connection or a dial-up connection. Your Internet connection must be of the kind which can support a Web browser.

Firewalls

MacVector 8.0 and later uses a communication protocol that is substantially different from that used in earlier versions of MacVector. It connects through http instead of a custom IP protocol. This simplifies the firewall set-up for network managers. MacVector 8 and above use the identical protocol to a browser for both Entrez and BLAST. MacVector now posts requests directly to the NCBI home page using the http protocol on port 80. The data that is sent back is actually the identical NCBI web page that you would get if you were using a browser. Embedded in
the pages are non-displayed XML which is the "machine readable" data that we parse out using tools provided by the NCBI.

Simply speaking if you can connect with a browser to the NCBI, you should be able to connect with MacVector. If a proxy server setting is required then this will need to be done for ALL internet applications on OSX, and is done in the Network control panel for the entire machine and all applications automatically take advantage of those settings. For example you can see this in Safari - if you click on the advanced proxy settings, it opens up the system network preferences.

Prior to MacVector 8.0 anything other than a straightforward configuration would need a special configuration file called `ncbi.cnf` in the System Folder | Preferences folder. However, this file is NOT needed for MacVector 8.0 and above, and if it exists it will just be ignored.
Using the Job Manager

Overview

This appendix describes how to use the Job Manager to control background jobs started by MacVector.
About the Job Manager

The MacVector Job Manager was introduced with MacVector 8.0 as a way to allow NCBI BLAST searches to be run in the background so that the user can continue to work within MacVector while waiting for the search to complete. The Job Manager also allows multiple BLAST searches to be submitted without waiting for the first job to complete, and allows submitted searches to be cancelled through a simple list-based interface.

With MacVector 9.5 this functionality was extended to include ClustalW alignment jobs. Future versions will see additional long-running analyses migrated to the control of the Job Manager. Additionally, all Assembler analysis functions are submitted as jobs to the Job Manager. The **phred** base calling algorithm and the **cross_match** vector masking algorithm are automatically split across as many cores/CPUs as the target machine possesses.

When all of the cores/CPUs are busy, the Job Manager queues additional jobs and starts them automatically when a core/CPU becomes available. You can monitor the status of all currently submitted, running and queued jobs using the Job Manager window, invoked by selecting the **Windows | Show Job Manager** menu item.

Using the job manager

You can open the Job Manager window at any time by either choosing **Windows | Show Job Manager**, or pressing `<command> - B`

![Job Manager Window](image)

**View**

This button is only enabled if a job has completed, but the results have not yet been displayed. Currently this only applies to ClustalW and
Using the job manager

Blast jobs that completed at a time when the user was busy with other windows. If you start either of these jobs, dismiss the progress dialog and then switch to another window, the results are not automatically displayed when the job completes. This is to prevent a result dialog from being displayed and distracting you while you are busy with some other task. When you are ready, you can see the completed job results by selecting the job in the Job Manager window and then clicking View. Usually, phred, cross_match and phrap jobs are always removed from the Job Manager window when they complete because they do not need to display a completion dialog. In those cases, the Assembly Project window is simply updated with the results.

Note. If a job fails for any reason, it will remain in the Job Manager list with a status of Failed. You can then view the job to learn more about the failure.

Stop

By highlighting a running job and then clicking the Stop button, the job will be stopped and removed from the Job Manager window.

Preferences

With some older multiple CPU machines, stability problems have occasionally been seen when MacVector uses both CPUs. If you believe you are seeing problems that might be related to this, you can disable the use of more than one CPU using the Preferences button. Please note that these problems have not been seen on newer Intel multi core machines.

Status

The Status column can show a number of words to indicate the state of a job:

- **Complete** indicates a job that has finished and can show some results.
- **Running** indicates active jobs
- **Queued** indicates a job has been submitted and is waiting for a free processor.
- **Reading** is specific to Blasts jobs and indicates that the process is receiving data (results) from the NCBI servers.
- **Failed** indicates a job failed to complete for some reason. Additional messages will typically be displayed giving an explanation of the problem.
Using the Job Manager

Job progress dialog

When a job is first submitted to the Job Manager, a dialog appears that indicates the status of the job. This dialog can be closed at any time by selecting the **Close** button. This does not stop the job and you can continue to monitor the status of the job from the main Job Manager window. You can also terminate the job at any time by selecting **Stop**. This dialog does not appear for Assembler related jobs.

With BLAST jobs, information related to the estimated completion time is also displayed.
Overview

This appendix contains reference tables for the matrix analysis methods, including letter codes and scoring matrix values.
### IUPAC-IUB codes for nucleotides and amino acids

#### Nucleic Acids

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<th>Description</th>
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<td>Adenine</td>
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</tr>
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<td>Guanine</td>
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#### Amino acids

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<td>Asx</td>
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<tr>
<td>C</td>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>D</td>
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<tr>
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### IUPAC-IUB codes for nucleotides and amino acids

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<td>Trp</td>
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<td>Xxx</td>
<td>Any amino acid, unknown</td>
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<tr>
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<td>Tyr</td>
<td>Tyrosine</td>
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<tr>
<td>Z</td>
<td>Glx</td>
<td>Glutamine or glutamic acid</td>
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<sup>a</sup> Proposed symbol for selenocysteine. You cannot use it in a MacVector sequence. When files are imported, MacVector converts U residues to X.
### Letter codes for matrix analyses

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@>100 percent (protein matrix)
Hash codes and “tweak” values for scoring matrices supplied with MacVector

pam250 protein scoring matrix

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<td>-- 19 Phe 5 Leu 10 Arg 15 Tyr 20 p1 27</td>
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<td>Ala 1 Gky 6 Met 11 Ser 16 Asx 3 p2 200</td>
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<td>Asp 3 Ile 8 Pro 13 Val 18 Xxx 19 p4 50</td>
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<tr>
<td>Glu 4 Lys 9 Gln 14 Trp 19 End 19</td>
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deletion penalty (single residue indel) = 12
gap penalty (continuing indel, per residue) = 6

pam250S scoring matrix

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<td>Glu 1 Lys 2 Gln 1 Trp 4 End 4</td>
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deletion penalty (single residue indel) = 12
gap penalty (continuing indel, per residue) = 6

DNA matrix nucleic acid scoring matrix

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deletion penalty (single residue indel) = 12
gap penalty (continuing indel, per residue) = 4

**DNA database matrix nucleic acid scoring matrix:**
**match / mismatch scores**

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-   A  C  M  G  R  S  V  T  W  Y  H  K  D  B  N
### pam250 and pam250S protein matrix: match / mismatch scores

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**X**

$$\text{ACDEFGHIJKLMNOPQRSTUVWXYZ}$$

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_MacVector User Guide_ 435
## Residue-specific gap modification factors for ClustalW sequence alignment

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Overview

This Appendix provides some examples of formatting the MacVector output display windows.
Formatting the annotated display

Example 1

To display restriction enzyme site results with translated protein coding (CDS) and binding sites from the features table, use the settings shown below from Options | Format Annotated Display. The Show panel labels the binding sites and translations from the features table. The Translate panel automatically translates each CDS feature and shows the sequence blocked to the phase of the translation.

Resulting Output
Example 2

To display restriction enzyme site results with only a translated protein, use the settings shown below from Options | Format Annotated Display. The Translate panel automatically translates each peptide entered on the features table and the protein is not labeled because the checkbox for peptide in the Show panel is not chosen.

Resulting output

![Image of annotated sequence with restriction sites and translation]

Restriction Site

Translation
Formatting Examples

Formatting the aligned display

Example 1

This aligned display has the default settings from Options | Format Aligned Display. The settings for the query sequence, shown double-stranded, are set on Options | Format Annotated Display.

Resulting output

![Image of aligned display with annotations]
Formatting the aligned display

Example 2

In this display, the score line is a "|", the Query Line is turned on, and the horizontal and vertical characters are turned off.

Resulting output
Example 3

This shows the effect of annotated formatting on the aligned sequence display. The annotation (Show panel) for translation is checked, and both CDS and block to phase (Translate panel) are checked. The query sequence is shown double-stranded.

Resulting output
### Formatting the aligned display

#### Example 4

To display results simply, turn off all settings on **Options | Format Aligned Display** and **Options | Format Annotated Display**.
Formatting Examples

Resulting output
Formatting the aligned display

Database: UserFolder: barnes

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1. pHBG
   | 1630 | 1640 | 1650 | 1660 | 1670 |
   |      |      |      |      |      |
   [ 3432 ]

2. Oda...[9803]
   | 570  | 560  | 550  | 540  |
   |      |      |      |      |
   [ 794 ]

   (gk-yTdggTs --na-Gtynt dgsTykrrnC rdTaTh-gnA syTaGky
   t)

3. hshbb
   | 0    | 1290 | 1300 | 1310 | 1320 |
   |      |      |      |      |      |
   [ 406 ]

   TTA-aA AgaaA-TAA-C AG-AGAgCgC CcAgCcCcG- GcTgGacA-A Tggaaac
GenBank Feature Tables

Overview

This appendix contains details of the GenBank feature tables used by MacVector.
**GenBank feature format**

GenBank is the primary US repository of DNA and protein sequences, curated by the National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH) in Bethesda, Maryland.

Sequences maintained by GenBank have two main types of metadata, which we arbitrarily split up into features - which we define as annotations have a defined location on the sequence (such as a gene or site) - and annotations - which are general data associated with a sequence (such as accession number, publication or authors).

GenBank features have defined types (such as CDS, mRNA, promotor, etc.) and only a limited set of types are allowed. The tables below list the feature types allowed for both protein and nucleic acid sequences in MacVector.

Further information about the GenBank file format can be found at: http://www.ncbi.nlm.nih.gov/collab/FT/

**Protein feature keywords**

The following GenBank feature keywords are supported in MacVector for proteins:

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## GenBank feature format

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### Nucleic acid feature keywords and qualifiers

The following GenBank keywords and associated qualifiers are supported in MacVector for set nucleic acids.

**Note.** Some keywords have mandatory qualifiers, as well as optional ones. These are shown in bold text in the table below.

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### GenBank feature format

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</tbody>
</table>
References

Overview

This section contains a full listing of references quoted in the User Guide, plus selected academic papers for background reading. There is also a short list of recommended reading about the use of computers in sequence analysis.
References

BLAST algorithms

Issues in searching molecular sequence databases.

Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.

Methods for assessing statistical significance of molecular sequence features by using general scoring schemes.

Applications and statistics for multiple high-scoring segments in molecular sequences.

IUPAC codes for nucleotides and amino acids

Nomenclature for incompletely specified bases in nucleic acid sequences recommendations 1984.

A one-letter notation for amino acid sequences. Tentative rules.

Protein secondary structure prediction

Chou-Fasman method

Conformational parameters for amino acids in helical, beta-sheet, and random coil regions calculated from proteins.

Prediction of protein conformation.

Empirical predictions of protein conformations.

Prediction of the secondary structure of proteins from their amino acid sequence.

General
References


**Robson-Garnier method**


**Protein flexibility**


**Protein profiles**

**Amphiphilicity**


**Antigenicity**

References

The antigenic index A novel algorithm, for predicting antigenic determinants.


Hydropilicity

Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins.

Prediction of protein antigenic determinants from amino acid sequences.

A simple method for displaying the hydropathic character of a protein.

Hydrophobicity


Surface probability

Conformation of amino acid side-chains in proteins.
References

Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide.

Transmembrane


Primer and probe screening analysis


Predicting DNA duplex stability from the base sequence.


Improved free-energy parameters for predictions of RNA duplex stability.


OSP a computer program for choosing PCR and DNA sequencing primers.


A computer program for selection of oligonucleotide primers for polymerase chain reactions.


A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and in vitro amplification of DNA.


Optimization of the annealing temperature for DNA amplification *in vitro.*
References

Coding regions

Recognition of protein coding regions in DNA sequences.

The codon preference plot: graphic analysis of protein coding sequences and prediction of gene expression.

Measurement of the effects that coding for a protein has on a DNA sequence and their use in finding genes.

Codon preference and its use in identifying protein coding regions in long DNA sequences.

Sequence comparisons

Sequence similarity (‘homology’) searching for molecular biologists.

A non-metric sequence alignment program.

Aligning amino acid sequences comparison of commonly used methods.

Multiple-alphabet amino acid sequence comparisons of the immunoglobulin kappa-chain constant domain.

Rapid and sensitive protein similarity searches.

Improved tools for biological sequence comparisons.

Rapid and sensitive sequence comparison with FASTP and FASTA.

Interactive molecular biology computing.
References

A high speed, high capacity homology matrix zooming through SV40 and polyoma.

A convenient and adaptable package of computer programs for DNA and protein sequence management, analysis, and homology determination.

A multiple sequence alignment program.

Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice.

General methods of sequence comparison.

Rapid similarity searches of nucleic acid and protein databanks.

Phylogenetic trees

General


Distance methods


A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences.

Recovering evolutionary trees under a more realistic model of sequence evolution.
References


Phylogenetic reconstruction methods


Bootstrapping


Sequence Assembly

Phred


Further reading

If you are not familiar with using computers to analyze DNA and protein sequences, the following books are good general references:


Overview

This section contains a full listing of all the sequence file formats supported by MacVector. It also provides details of the file extensions that are used by MacVector for output files.
MacVector has the ability to read and write sequence files in a wide variety of formats. In addition to the standard file Open and Save/Save As commands, MacVector reads sequences files through a number of other functions:

- **Multiple Sequence Alignment file import.** This lets you add sequences from a file(s) to a nucleic acid or protein alignment.
- **Align to Reference file import.** This lets you add sequences to the Align to Reference window ready for a sequence confirmation or cDNA assembly.
- **Align to Folder.** When you select a folder to align a sample sequence to, MacVector will automatically read all sequences of any supported format in the folder.
- **Assembler file import.** If you are using Assembler, you can import files in any format into the Assembler Project window.
- **cross_match vector trimming.** (Assembler only). cross_match requires a list of sequences to mask out any vector from input Reads.

Prior to MacVector 10.5, these other functions could only read a subset of the full range of MacVector supported file formats. However, now all of the functions can read any supported file format from the list below.

**Note.** MacVector automatically detects the format of the file(s) you have selected, so there is never a need to define that ahead of time. Accordingly, unlike many other sequence analysis applications, MacVector has no general import functions, the File | Open menu item is all that is required.
## Supported file formats

### Single nucleic acid sequences

<table>
<thead>
<tr>
<th>Format</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MacVector</td>
<td>MacVector 10.5 is forwards and backwards compatible with all versions of MacVector back to 7.0. MacVector 10.5 does save some additional information to the files that will be ignored by earlier versions, but all the sequence, features, annotations and feature appearance information will be maintained.</td>
</tr>
<tr>
<td>BSML</td>
<td>This is an XML format promoted by LabBook and a collaboration of various companies. The original site at <a href="http://www.bsml.org">www.bsml.org</a> is now defunct, but the format lives on with the Accelrys Gene product from Accelrys. See <a href="http://xml.coverpages.org/bsml.html">http://xml.coverpages.org/bsml.html</a>.</td>
</tr>
<tr>
<td>GenBank</td>
<td>This is the preferred format for US based researchers wishing to share annotated nucleic acid sequence information with the general community. MacVector fully supports all of the GenBank feature types and qualifiers, so this is the preferred format if you wish to send sequence files to collaborators who do not use MacVector. The disadvantage is that the GenBank format cannot handle &quot;meta data&quot; such as colors and fonts that you may have used to graphically enhance the map of your sequence. The GenBank format is maintained by the National Center for Biotechnology Information (NCBI), a US Government agency that also provides the popular <em>Entrez</em> and BLAST online database search capabilities. GenBank format files can contain multiple sequences. See ftp://ftp.ncbi.nih.gov/genbank/gbrel.txt.</td>
</tr>
<tr>
<td>EMBL</td>
<td>The EMBL format is particularly popular in Europe where it is maintained by the European Bioinformatics Institute. Like GenBank, it has excellent support for features and annotations, but cannot be used to remember graphical appearance information. EMBL format files can contain multiple sequences. See <a href="http://www.ebi.ac.uk/embl/Documentation/User_manual/usrman.html">http://www.ebi.ac.uk/embl/Documentation/User_manual/usrman.html</a>.</td>
</tr>
<tr>
<td>GCG RSF</td>
<td>This is the &quot;Rich Sequence Format&quot; from the Genetics Computer Group &quot;Wisconsin Package&quot; set of programs. GCG was acquired by Accelrys and the product has been discontinued, although many scientists worldwide continue to use the product. RSF files can contain multiple sequences.</td>
</tr>
<tr>
<td>GCG SSF</td>
<td>This is the older &quot;Single Sequence Format&quot; from GCG.</td>
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### Supported file formats and file extensions

<table>
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<tr>
<th>Format</th>
<th>Description</th>
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</thead>
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<td>FastA</td>
<td>FastA files have a very simple format - each sequence entry starts with a &quot;&gt;&quot; character, followed by the name of the sequence. Any text on that line after the first &lt;space&gt; character is considered to be the definition of the sequence. The actual sequence characters (upper or lower case) start on the next line and can continue on subsequent lines until the end of the file or the next &quot;&gt;&quot; is encountered. For readability purposes, the sequence lines are typically wrapped every 80 characters or so.</td>
</tr>
<tr>
<td>ASCII/Plain</td>
<td>MacVector can read and write plain sequence files where the only sequence characters are present in the file. These are similar to FastA files but without the sequence name/definition line and are also typically wrapped at 80 characters for readability purposes.</td>
</tr>
<tr>
<td>DNAStar</td>
<td>The standard single sequence &quot;.seq&quot; LaserGene format is basically the GCG SSF format with the characteristic &quot;..&quot; characters introducing the sequence replaced with &quot;^^&quot;. These are the only DNAStar format files MacVector can read, it cannot read the binary LaserGene &quot;Map&quot; files.</td>
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<tr>
<td>Vector NTI</td>
<td>To read sequences from Vector NTI you must first export the sequences from the Vector NTI database, choosing GenBank format. MacVector can read these files, skipping over any non-GenBank-standard entries that Vector NTI may write to the file.</td>
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<tr>
<td>GeneWorks</td>
<td>GeneWorks is an old Macintosh application last developed by Oxford Molecular. It was discontinued in ~2000, but MacVector can read the single sequence files created by GeneWorks.</td>
</tr>
<tr>
<td>DNAStrider</td>
<td>DNA Strider was a popular low-end sequence analysis program from the late 1990s and early 2000s. MacVector can read the DNA, NA and Protein files produced by versions 1.1 and 1.2</td>
</tr>
<tr>
<td>IG_Suite</td>
<td>Intelligenetics was one of the first bioinformatics companies. It was acquired by Oxford Molecular in the mid 1990s. The file format was popular at the time but is rarely encountered nowadays.</td>
</tr>
<tr>
<td>Staden</td>
<td>This is the format of the Staden suite of biological analysis software. See <a href="http://staden.sourceforge.net/overview.html">http://staden.sourceforge.net/overview.html</a>.</td>
</tr>
<tr>
<td>ABI</td>
<td>These are the chromatogram files produced by the ABI series of automated sequencing machines. MacVector can read files produced by the 310, 373, 377, 3130, 3700 and 3730 machines.</td>
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### Supported file formats

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<th>Format</th>
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<td>SCF</td>
<td>This is the &quot;Staden Chromatogram Format&quot;, a standard format for chromatogram data. This is the only chromatogram format that MacVector can write.</td>
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<tr>
<td>ALF</td>
<td>The file format produced by the Pharmacia Automated Laser Fluorescence (ALF) DNA sequencer</td>
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### Single protein sequences

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<tr>
<td>GenPept</td>
<td>This is the preferred format for US based researchers wishing to share annotated protein sequence information with the general community. MacVector fully supports all of the GenPept feature types and qualifiers, so this is the preferred format if you wish to send sequence files to collaborators who do not use MacVector. The disadvantage is that the GenPept format cannot handle &quot;meta data&quot; such as colors and fonts that you may have used to graphically enhance the map of your sequence. The GenPept format is maintained by the National Center for Biotechnology Information (NCBI), a US Government agency that also provides the popular <em>Entrez</em> and BLAST online database search capabilities. GenPept format files can contain multiple sequences.</td>
</tr>
<tr>
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<td>GCG SSF</td>
<td>This is the older &quot;Single Sequence Format&quot; from GCG.</td>
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<tr>
<td>UniProt</td>
<td>UniProt is the collaborative amino acid database of EBI, SIB and PIR.</td>
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Supported file formats

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<tr>
<td>PIR</td>
<td>Protein information resource (PIR) is an annotated, non-redundant and cross-referenced database of protein sequences at the NBRF.</td>
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<tr>
<td>trEMBL</td>
<td>trEMBL is a large protein database, in Swiss-Prot format, generated by computer translation of the genetic information from the EMBL Nucleotide Sequence Database database. It is the protein equivalent of the EMBL DNA format.</td>
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<tr>
<td>Swiss-Prot</td>
<td>Swiss-Prot is a manually curated biological database of protein sequences. Swiss-Prot was developed by the Swiss Institute of Bioinformatics and the European Bioinformatics Institute. It provides reliable protein sequences associated with a high level of annotation (such as the description of the function of a protein, its domains structure, post-translational modifications, variants, etc.), a minimal level of redundancy and high level of integration with other databases.</td>
</tr>
<tr>
<td>DNAStrider</td>
<td>DNA Strider was a popular sequence analysis program from the late 1990s and early 2000s. MacVector can read the DNA, NA and Protein files produced by versions 1.1 and 1.2</td>
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Multiple sequences

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</thead>
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<tr>
<td>MacVector</td>
<td>MacVector 10.5 is forwards and backwards compatible with all versions of MacVector back to 7.0.</td>
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Supported file formats and file extensions

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<tr>
<th>Format</th>
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<tr>
<td>GenBank/GenPept</td>
<td>This is the preferred format for US based researchers wishing to share annotated sequence information with the general community. MacVector fully supports all of the GenBank feature types and qualifiers, so this is the preferred format if you wish to send sequence files to collaborators who do not use MacVector. The disadvantage is that the GenBank format cannot handle &quot;meta data&quot; such as colors and fonts that you may have used to graphically enhance the map of your sequence. The GenBank format is maintained by the National Center for Biotechnology Information (NCBI), a US Government agency that also provides the popular Entrez and BLAST online database search capabilities. See ftp://ftp.ncbi.nih.gov/genbank/gbrel.txt.</td>
</tr>
<tr>
<td>EMBL/trEMBL</td>
<td>The EMBL format is particularly popular in Europe where it is maintained by the European Bioinformatics Institute. Like GenBank, it has excellent support for features and annotations, but cannot be used to remember graphical appearance information. See <a href="http://www.ebi.ac.uk/embl/Documentation/User_manual/usrman.html">http://www.ebi.ac.uk/embl/Documentation/User_manual/usrman.html</a>.</td>
</tr>
<tr>
<td>GCG RSF</td>
<td>This is the &quot;Rich Sequence Format&quot; from the Genetics Computer Group &quot;Wisconsin Package&quot; set of programs. GCG was acquired by Accelrys and the product has been discontinued, although many scientists worldwide continue to use the product. RSF files can contain multiple sequences.</td>
</tr>
<tr>
<td>GCG MSF</td>
<td>This is the older &quot;Multiple Sequence Format&quot; from GCG. No feature information is kept in these files.</td>
</tr>
<tr>
<td>FastA</td>
<td>FastA files have a very simple format - each sequence entry starts with a &quot;-&gt;&quot; character, followed by the name of the sequence. Any text on that line after the first &lt;space&gt; character is consider to be the definition of the sequence. The actual sequence characters (upper or lower case) start on the next line and can continue on subsequent lines until the end of the file or the next &quot;-&gt;&quot; is encountered. For readability purposes, the sequence lines are typically wrapped every 80 characters or so.</td>
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## Supported file formats

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<td>NEXUS</td>
<td>The NEXUS file format was introduced by Maddison <em>et al.</em> (1997) and is used by a number of popular phylogeny programs. See <a href="http://www.ncbi.nlm.nih.gov/pubmed/11975335?dopt=Abstract">http://www.ncbi.nlm.nih.gov/pubmed/11975335?dopt=Abstract</a>. This is a popular format for use in dedicated phylogenetic reconstruction applications such as PAUP.</td>
</tr>
<tr>
<td>PHYLIP</td>
<td>This is the format of Joe Felsenstein's Phylogeny Inference Package. See <a href="http://cmgm.stanford.edu/phylip/">http://cmgm.stanford.edu/phylip/</a>. MacVector defaults to a sequence title width of 10 characters, the PHYLIP default.</td>
</tr>
</tbody>
</table>
MacVector now uses Uniform Type Identifiers (UTIs) to uniquely identify file formats that the application either reads or creates. See Apple's description of UTIs on their documentation pages:

- http://developer.apple.com/documentation/Carbon/Conceptual/understanding_utis/understand_utis_intro/chapter_1_section_1.html#/apple_ref/doc/uid/TP40001319-CH201-SW1

The table below is a list of the file types MacVector will read and create. MacVector exports those UTIs that are unique to MacVector: matrix, bias, binary sequence files, etc. In addition MacVector imports a number of chemical MIME type or text sequence files. These UTIs are all registered with Mac OS X and Launch Services uses the information to associate files with MacVector.

The UTI brings together type identification from multiple methodologies. The files saved on Mac OS, where OSType has been a common means of identifying files with earlier systems, can now work with other systems (Windows, Linux) that might have used file extensions. When downloading files MIME types are employed to identify data formats so that browsers and other tools can locate appropriate applications to handle the data. You should be able to open all supported file formats whether created on a Macintosh or transferred from another system.
### MacVector file extensions

#### Third party formats

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<td>public.plain-text</td>
<td>ABIF</td>
<td>abi</td>
<td>application/x-dna</td>
</tr>
<tr>
<td>public.bsml</td>
<td>BSML File</td>
<td>public.plain-text</td>
<td></td>
<td>bsml</td>
<td></td>
</tr>
<tr>
<td>public.emb</td>
<td>EMBL Nucleotide File</td>
<td>public.plain-text</td>
<td></td>
<td>emb, embl</td>
<td>chemical/x-embel-dl-nucleotide</td>
</tr>
<tr>
<td>public.fa</td>
<td>FASTA Sequence File</td>
<td>public.plain-text</td>
<td></td>
<td>fa, fna, fasta, mpfa</td>
<td>chemical/x-fasta, chemical/seq-aa-fasta, chemical/seq-na-fasta</td>
</tr>
<tr>
<td>public.gb</td>
<td>GenBank Flat File</td>
<td>public.plain-text</td>
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<td>gb, gen</td>
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<tr>
<td>public.gp</td>
<td>GenPept Flat File</td>
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<tr>
<td>public.msf</td>
<td>Multiple Sequence File</td>
<td>public.plain-text</td>
<td></td>
<td>msf</td>
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<tr>
<td>public.nex</td>
<td>Nexus File</td>
<td>public.plain-text</td>
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<td>public.phy</td>
<td>Phylip File</td>
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<td>public.plain-text</td>
<td>Text Document</td>
<td>public.plain-text</td>
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<tr>
<td>public.rcf</td>
<td>Rich Sequence File</td>
<td>public.plain-text</td>
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<tr>
<td>public.scf</td>
<td>DNA Sequence Chromatogram File</td>
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<td>public.sqn</td>
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<td>public.tbl</td>
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<td>axml</td>
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<td>com.macvector.basis</td>
<td>MacVector Codon Bias File</td>
<td>BIAS</td>
<td></td>
<td>bias</td>
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<tr>
<td>com.macvector.library</td>
<td>MacVector Library File</td>
<td>MVTF</td>
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</tr>
<tr>
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<td>MacVector NA Alignment File</td>
<td>MSAN</td>
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<tr>
<td>com.macvector.msap</td>
<td>MacVector AA Alignment File</td>
<td>MSAP</td>
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<tr>
<td>com.macvector.nmat</td>
<td>MacVector NA Matrix File</td>
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<tr>
<td>com.macvector.nsub</td>
<td>MacVector NA Subsequence File</td>
<td>NSUB</td>
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<td>nsub</td>
</tr>
<tr>
<td>com.macvector.nucleotide</td>
<td>MacVector NA Sequence File</td>
<td>NUCL</td>
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<td>nucl</td>
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<tr>
<td>com.macvector.penz</td>
<td>MacVector AA Enzyme File</td>
<td>PENZ</td>
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<td>penz</td>
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<td>com.macvector.pmat</td>
<td>MacVector AA Matrix File</td>
<td>PMAT</td>
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<td>MacVector AA Sequence File</td>
<td>PROT</td>
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<td>MacVector AA Subsequence File</td>
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<td>MacVector NA Enzyme File</td>
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