

Primer

PREMIER

**Version 5.0 for Windows and Power
Macintosh**

Manual

PREMIER

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Content

CONTENT	I
GETTING STARTED.....	1
What's New in this Version.....	1
Navigational Basics	1
GENETANK.....	3
GeneTank Window.....	3
Viewing the Sequence.....	3
Open Sequence Menu	3
New Sequence Menu	4
Saving the Sequence	4
Editing Sequence	5
Search	5
Viewing different strands	5
Voice readback.....	6
Editing an Alignment	6
PASTE SEQUENCE WINDOW	7
Preferences Window.....	7
Rating Parameters	8
Aligning Sequences.....	9
Alignment Parameters.....	9
Translation.....	12
Edit Codon Table Window	13

PRIMER	14
Primer Premier Window	15
Direct Select Box.....	15
Direct Select with an Alignment	15
Table of Properties	15
Secondary Structures	16
Edit Primer Window	17
Search Criteria Window.....	18
Search Parameter Windows	19
Automatic Search	19
Manual Search (Science of Primer Design)	21
Search Results Window	26
Multiplex/Nested Primer Window	26
Database Window	28
Reports.....	29
Synthesis Order Form	30
Optical Activity	30
Degeneracy.....	31
Graphs.....	31
RESTRICTION ENZYME ANALYSIS	33
Restriction Enzyme Analysis Window.....	33
Restriction Sites Window.....	34
Edit Enzyme Window	34
Filter Window	35

MOTIF ANALYSIS	37
Motif Analysis Window.....	37
Motif Sites Window.....	37
Edit Motif Window.....	38
MENU COMMANDS.....	39
GeneTank Menu	39
File.....	39
Edit	39
View.....	39
Search	40
Function.....	40
Translate	40
Window.....	40
Help	40
Primer Menu	42
File.....	42
Edit	42
Function.....	42
Report.....	42
Graph.....	43
Window.....	43
Help	43
Enzyme Results Menu	44
File.....	44
Function.....	44
Window.....	44
Help	44
Motif Menu	45
File.....	45
Function.....	45
Window.....	45
Help	45

APPENDICES	46
APPENDIX A: FORMULAS AND ALGORITHMS	47
APPENDIX B: DEGENERATE CODE	50
APPENDIX C: AMINO ACID SYMBOLS	51
APPENDIX D: SOFTWARE LIMITS	52
APPENDIX E: REFERENCES	53
APPENDIX F: SYSTEM SPECIFICATIONS	54
Service and Support.....	54
Further Information.....	54

Getting Started

PRIMER PREMIER is an application ready to assist you in designing the best primers for your research needs. With its advanced features primer search, primer database, multiplex/nested primer design, primer editing and analysis, optimal primers can be designed to get higher efficiency amplifications. Designing primers for long PCR of sequences up to 50 kb is possible. It consists of four main functions:

GeneTank
Primer
Align
Enzyme
Motif

What's New in this Version

- Cross species primers - designs primers for amplifying sequences from multiple species
- Pathogenic detection primers - designs primers in highly conserved regions
- Allele specific primers - design primers that will exclusively amplify a single member of a set of related sequences

Navigational Basics

The navigation through *PRIMER PREMIER* is designed to be intuitive... not much needs to be said about it. Just a few points regarding menus and list boxes will suffice:

Each of the four main functions, GeneTank, Primer, Enzyme and Motif has its own specific menus, which are accessible only through windows within the function. The active window determines what menus you see. Here is a list of the main functions with their main functions:

GeneTank: File, Edit, View, Search, Function, Translate, Window, Help.

Primer: File, Edit, Function, Report, Graph, Window, Help.

Enzyme: File, Function, Window, Help.

Motif: File, Function, Window, Help.

Primer 5.0 Manual Reference

Using the function menu you can activate the main windows of each function. The window menu keeps track of all open windows and can be used to change the active window.

Most list boxes have multi-selection facilities. Holding **CTRL** key for Windows and Power Mac while clicking adds to the selection list. Holding **SHIFT** key down while clicking selects a range, everything between the last selection and the new SHIFT-click.

GeneTank

With the GeneTank function you can load DNA or Protein sequences. You can translate and back-translate in different frames. The standard codon table and several mitochondrial tables are provided for your convenience. You can also input additional mitochondrial codon tables if you wish. The sequence editor provides complete sequence editing capabilities including multi-media voice readback.

The function has the following sections:

GeneTank Window to load, view, edit and translate the sequence.
Translation to translate loaded sequence into other type and back.
Edit Codon Table Window to edit codon table for translation.

GeneTank Window

The main GeneTank window helps you view the sequence and its header information. The sequence can be viewed in 3 or 10 base groups. You can translate a loaded sequence into another type and back. You can edit the sequence you are working on, using standard Cut, Copy and Paste. It provides access to the Primer, Align, Enzyme and Motif functions.

Viewing the Sequence

You can access the header, 5' end sequence number and group into 3 or 10 base groups by clicking the buttons or using the View menu. The header is the text upstream of the first line of nucleotides. Display the header by clicking on the header button or selecting it from the menu. If you change the 5' sequence number, the numbering of whole sequence gets updated starting from the number you have given to the 5' end.

Alternate Access:

View > Show Header

View > Grouping

View > 5' Seq No

Open Sequence Menu

This is where you load sequences you want to work on. You can choose the file containing the desired sequence by using the file

selection dialog.

Note for Windows: The default selection assumes that you store your DNA sequences with SEQ suffix and protein sequences with PRO suffix. Of course, you may use any convention you wish.

If the sequence is in standard GenBank format *PRIMER PREMIER* recognizes the start of the sequence and loads it. Or if you have saved the sequence previously using *PRIMER PREMIER File > Save* menu, the sequence is in *PRIMER PREMIER* format and is loaded automatically.

Otherwise, the **Import Sequence** window opens with the entire sequence visible in the scrolling box. Double click on first line of nucleotides where the sequence begins. Everything above this line is the "header". *PRIMER PREMIER* filters out illegal characters and loads the sequence in the **GeneTank** window. Double clicking the first line of nucleotides closes the **Import Sequence** window and returns you to the **GeneTank** window with the sequence loaded.

Insider Info: If the file you chose is wrong and does not contain the sequence you wish to load, simply close the **Import Sequence** window and start all over.

New Sequence Menu

This function is intended primarily for you to type in a sequence that you want to enter manually.

You can type in any sequence i.e., DNA or Protein and save it in your favorite directory. Once the sequence is entered you can perform all of the functions on it as if it was loaded from GenBank or any other format.

Saving the Sequence

If you save the sequence, you can reopen it easily as *PRIMER PREMIER* loads it correctly without asking you to identify the first line of nucleotides with a double click. You should save the sequence if:

- You want to revisit the same sequence frequently and want to be able to load it quickly.
- You have edited a sequence and want to save the results.
- You have typed in a new sequence.

Editing Sequence

Now you can edit DNA or protein sequences directly in the GeneTank without going into any other window. Editing original translated sequences can be done effectively. Changes in any one type are reflected in the other, as it should be. For example, if you delete an amino acid of a protein sequence (whether translated or original), the corresponding codon will be deleted from the DNA sequence. If you make a change in the translated sequences then the corresponding changes are reflected only in the other translated sequences and not in the original sequence. You can type in the sequence or import it through the clipboard.

The usual cut, copy and paste functions are available to facilitate editing. The keyboard short cuts are CTRL-X, CTRL-C and CTRL-V.

Grouping of 3 nucleotides is available to simplify working with proteins.

When entering DNA you can use A, C, G, T or any of the common ambiguous bases listed in **Appendix B**. When entering proteins you can use the single letter abbreviation of the amino acids listed in **Appendix C**.

To import a sequence through the clipboard, copy it onto the clipboard using any editor such as Notepad and then paste it in. Save the sequence after you finish editing.

Search

You can search for a particular pattern within the sequence starting from the current position of the cursor, using the Find button on the GeneTank Window. You can continue the search for the occurrence of same pattern in the remaining document using Find Next button. You can place the cursor at a particular position by selecting Go To Position from the Search Menu.

Alternate Access:

Search > Find

Search > Find Next

Search > Go To Position

Viewing different strands

To view the other strand of the loaded sequence i.e., anti-sense strand, you just click on A button on the GeneTank Window and the anti-sense strand will be displayed. To revert back to the original strand click on the S button. You can also view both strands by clicking on ds DNA button.



Alternate Access:

View > Sense Strand

View > Anti-sense Strand

View > ds DNA

Voice readback

Once the sequence is loaded in the GeneTank, you can get voice readback of sequence by pressing the  button. The sequence will be read starting from the current position of the cursor. Pressing the button again will terminate voice readback. To get voice readback while typing in the sequence press the  button and then type. Pressing the same button will terminate the process.

Alternate Access:

Function > Speaker

Editing an Alignment

After performing a multiple sequence alignment, the alignment results can be edited to produce a more accurate match. Usually, ClustalW produces an accurate alignment so manual editing is not necessary. But in case of difficult sequences or specialized research needs, the ability to edit an alignment is provided. Place the cursor where you wish to edit by clicking on the base. To insert a gap, press the space bar. To remove a gap press the delete key. As the alignment is edited the majority and minority consensus automatically update.

Paste Sequence Window

This window enables an oligo to be pasted in four different ways.



The oligo can be pasted as it is by selecting As Is, reversed, complemented, or reverse complemented. This makes it easy to paste in oligos from another program regardless of the sense or direction in which it was stored.

Preferences Window

The Preferences window sets up the default values for various parameters that are used in the product. It also allows you to access the Rating Parameters window where weights for secondary structures of primers, used in primer rating calculation can be set.

Default Primer Length - Default value = 25.

This value determines the length of the primer in the Direct Select function of the Primer Premier Window. It also determines the length of default primers when *PRIMER PREMIER* window is initially launched. The default primer length can be set between 1 and 70 bases..

Maximum Number of Pairs Searched - Default value = 100.

When searching for primer pairs, the number of pairs found can be very large. Since we search the pairs based on how good they are, the primers that pass the search criteria, but are rated very low may not be useful. Thus an arbitrary limit of 100 has been selected by default. You can change it to any number between 10 and 1000.

Nucleic acid concentration - Default value = 250 pM.

According to the formulation of Freier *et al.* (7), this is the total molar concentration of the annealing oligos, when oligos are not self-complementary. It is neither the primer concentration nor the initial template concentration. It is based on the PCR product concentration. Since the concentration of the template changes dramatically during the course of PCR, it is difficult to define its value. It has been empirically determined that setting its value to 250 pM, as suggested by Rychlik, W. *et al.* (11), gives good results in a wide variety of PCR and sequencing reactions. This value is used for calculating the melting temperature of the primer.

Monovalent ion concentration - Default value = 50 mM.

This value determines the concentration of all the monovalent ions present in the reaction mixture.

Free [Mg²⁺] ion concentration - Default value = 1.5 mM.

This value determines the concentration of Mg²⁺ ions, used as binders, in a reaction mixture.

Total [Na⁺] equivalent -

This value is calculated using the value of Monovalent Ion Concentration and Free [Mg²⁺] ion concentration. The formula for calculating Total [Na⁺] equivalent is as follows:

Total [Na⁺] equivalent = Monovalent ion concentration + 4 * Sqrt (Free [Mg²⁺] ion concentration * 1000)

This value is used for calculating melting temperature of primer.

Temperature for Free Energy Calculation - Default value = 25 °C.

This is the value of temperature that will be used to calculate the ΔG values. ΔG is calculated using the formula $\Delta G = \Delta H - T * \Delta S$.

Rating Parameters

Rating parameters window is used to set weights for secondary structures to obtain primer rating. The more stable are the secondary structures, the lesser will be the rating. If the secondary structure is a

Paste Sequence Window

3' end secondary structure, it reduces the PCR amplification efficiency more than the one that occurs internally. To take that into account we subtract 1 from the ΔG value of the 3' end secondary structure. This makes it more stable than the normal one by a difference of 1 in ΔG .

The factor values are based on unpublished experimental results. You can change them to suit your specific applications.

The values used for ΔG are of the most stable secondary structure of all. If any of the secondary structures from amongst the all is not formed, a 0 value is assigned to the ΔG of that secondary structure.

Aligning Sequences

The following section applies only to the Windows version of Primer Premier. For the Macintosh version, alignments are done using the web and then loading the aligned sequence. Complete information for doing this is contained in the Primer Tour.

The Align window allows you to select which of the currently opened sequences to use in the alignment. The Selected Sequences box displays all of the sequences that will be aligned. Use the Add and Delete buttons to add or remove sequences.

Use the Options button to open the Alignment Parameters Window.

Alignment Parameters

The alignment Parameters Window allows you to set all of the parameters used by ClustalW in optimizing the alignment. The alignment can be done in 2 modes, slow/accurate and fast/approximate. As the names imply the slow/accurate is a more algorithmically intensive alignment that is more accurate but takes longer. Depending on which mode is selected, a different set of pairwise alignment parameters are available.

SLOW/ACCURATE alignment parameters

These parameters do not have any affect on the speed of the

alignments. They are used to give initial alignments which are then rescored to give percent identity scores. These % scores are the ones which are displayed on the screen. The scores are converted to distances for the trees.

- 1) Gap Open Penalty: the penalty for opening a gap in the alignment.
- 2) Gap extension penalty: the penalty for extending a gap by 1 residue.
- 3) Protein weight matrix: the scoring table which describes the similarity of each amino acid to each other.
- 4) DNA weight matrix: the scores assigned to matches and mismatches (including IUB ambiguity codes).

FAST/APPROXIMATE alignment parameters

These similarity scores are calculated from fast, approximate, global alignments, which are controlled by 4 parameters. 2 techniques are used to make these alignments very fast: 1) only exactly matching fragments (k-tuples) are considered; 2) only the 'best' diagonals (the ones with most k-tuple matches) are used.

K-TUPLE SIZE: This is the size of exactly matching fragment that is used. INCREASE for speed (max= 2 for proteins; 4 for DNA), DECREASE for sensitivity. For longer sequences (e.g. >1000 residues) you may need to increase the default.

GAP PENALTY: This is a penalty for each gap in the fast alignments. It has little affect on the speed or sensitivity except for extreme values.

TOP DIAGONALS: The number of k-tuple matches on each diagonal (in an imaginary dot-matrix plot) is calculated. Only the best ones (with most matches) are used in the alignment. This parameter specifies how many. Decrease for speed; increase for sensitivity.

WINDOW SIZE: This is the number of diagonals around each of the 'best' diagonals that will be used. Decrease for speed; increase for sensitivity.

Multiple Alignment Parameters

These parameters control the final multiple alignment. Each step in

Paste Sequence Window

the final multiple alignment consists of aligning two alignments or sequences. This is done progressively, following the branching order in the GUIDE TREE. The basic parameters to control this are two gap penalties and the scores for various identical/nonidentical residues.

1) and 2) The GAP PENALTIES are set by menu items 1 and 2. These control the cost of opening up every new gap and the cost of every item in a gap. Increasing the gap opening penalty will make gaps less frequent. Increasing the gap extension penalty will make gaps shorter. Terminal gaps are not penalized.

3) The DELAY DIVERGENT SEQUENCES switch, delays the alignment of the most distantly related sequences until after the most closely related sequences have been aligned. The setting shows the percent identity level required to delay the addition of a sequence; sequences that are less identical than this level to any other sequences will be aligned later.

4) The TRANSITION WEIGHT gives transitions (A <--> G or C <--> T i.e. purine-purine or pyrimidine-pyrimidine substitutions) a weight between 0 and 1; a weight of zero means that the transitions are scored as mismatches, while a weight of 1 gives the transitions the match score. For distantly related DNA sequences, the weight should be near to zero; for closely related sequences it can be useful to assign a higher score.

5) PROTEIN WEIGHT MATRIX leads to a new menu where you are offered a choice of weight matrices. The default for proteins is the BLOSUM series of matrices by Jorja and Steven Henikoff. Note, a series is used! The actual matrix that is used depends on how similar the sequences to be aligned at this alignment step are. Different matrices work differently at each evolutionary distance.

6) DNA WEIGHT MATRIX leads to a new menu where a single matrix (not a series) can be selected. The default is the matrix used by BESTFIT for comparison of nucleic acid sequences.

7) In the weight matrices, you can use negative as well as positive values if you wish, although the matrix will be automatically adjusted to all positive scores, unless the NEGATIVE MATRIX option is selected.

8) PROTEIN GAP PARAMETERS displays a menu allowing you to set some Gap Penalty options which are only used in protein alignments.

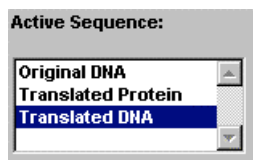
Further Information

For more information on setting parameters and the ClustalW algorithm, complete documentation is available from the author at:

<http://www-igbmc.u-strasbg.fr/BioInfo/ClustalW/clustalw.html>

Translation

Active Sequence



The list box on the GeneTank window contains the original sequence and the results of any translations performed on the original sequence.

Mechanisms to generate translations are as follows:

Translated DNA: You translate an Original Protein into DNA. You translate Original DNA into Protein then back-translate it to DNA.

Note: If you translate a sequence more than once, only the last translation will be kept. Prior translations will be discarded.

Translated Protein: You translate an original DNA into protein. You translate Original Protein into DNA then back-translate it to Protein.

DNA Translation Button

This button is not available if the selected sequence is Original or Translated DNA. If it is a protein, **Codon Table Selection** window opens. You can select a codon table and reading frame.

Common degenerate bases listed in **Appendix B** of this manual are used when the translation is ambiguous.

Paste Sequence Window

Alternate Access:

Translate > To DNA

Protein Translation Button

The button is not available if the selected sequence is Original or Translated Protein. If it is DNA, codon tables selection window opens. You can select a codon table and reading frame.

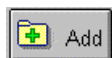
If a base triplet does not translate into an amino acid due to ambiguity in the bases, the character * is used to indicate the indeterminate translation.

Alternate Access:

Translate > To Protein

Edit Codon Table Window

With this window, you can create new codon tables, delete codon tables or modify existing codon tables. *PRIMER PREMIER* supplies the standard (universal) table, which cannot be modified or deleted.



button creates a codon new codon table using the standard codon table with the name <New Organism: some number>.

You can change the name by using the **Edit Codon Table Name**



box. button deletes the selected codon table. To change the position of a codon, simply grab it and drag it to the desired place. On grabbing the codon the cursor changes its shape to indicate that you have grabbed it. When you drop the codon on to its new home, the consensus codon changes in both rows, the row that lost it and the row that gained it.

Primer

Primer function contains the main search engine that designs primers. The powerful automatic search algorithm is designed to yield the very best primers with just a few mouse clicks. If you have unique experimental needs, *PRIMER PREMIER* yields the complete control of the search engine to you so that you can drive it manually, using your own criteria. The resultant primers can be analyzed with comprehensive on-line analysis facilities that dynamically calculate several primer properties and analyze secondary structures. Critical properties such as T_m , GC % and ΔG can be displayed as graphs. There is another window added to show the activity (in nmoles/OD and $\mu\text{g}/\text{OD}$) and Molecular weight of current primer pair. Selected primers can be saved to the database, printed or ordered with the synthesis order form.

To design primers for site-directed mutagenesis, the function provides primer editing facilities with instant analysis and update of restriction enzyme analysis. You can change the primers by entering bases or amino acids.

To check for a number of primers in a reaction i.e., for a multiplex/nested PCR, you can analyze as many primers as you want, using Multiplex/Nested function. You can select different primers from an earlier search and can also add primer sequences manually.

Facilities to store primers of interest in the database are provided. A function to fill out a form for ordering synthesis of selected primers which automatically fills-in the primer sequence and other pertinent information is provided.

The function has the following sections:

- Preferences Window
- Primer Premier Window
- Edit Primer Window
- Search Criteria Window
- Search Parameter Windows
- Search Results Window
- Multiplex/Nested Primer Window
- Database Window
- Synthesis Order Form
- Reports

Graphs

Primer Premier Window

This window is your key to the analysis of primers. The three major sections of the window; direct select, table of properties and secondary structures are described below:

Direct Select Box

When you move your cursor over the box, the cursor changes its shape from an arrow to a pencil. Clicking anywhere in the box selects a primer with sequence number closest to the place where you clicked and with the strand selected using the **Strand** box. All the properties are instantly updated. The Primer is displayed on the sequence along with the ends of primer marked as 5' and 3'. This helps in easy identification of the direction of amplification.

If direct select is used on an aligned sequence, the sequence of the primer is taken from the majority consensus. This allows easy visualization of where the primer may have mismatches with any particular sequence.

Direct Select with an Alignment

When doing highly conserved sequence primer design, Primer Premier displays the alignment on the Primer window to allow easy visualization of mismatches between the primer and each template. Clicking anywhere on the sequence creates and analyzes a primer that matches the Majority Consensus. The primer can then be edited using edit primer to match any specific sequence. This allows easy creation of allele specific primers.

The view of the Majority or Minority Consensus can be toggled from the View menu. The Minority Consensus allows visualization of which nucleotides do not have a perfect match among all of the aligned sequences.

Table of Properties

The table shows the properties of the currently selected pair. You can select a pair using one of the following three ways:

- **Direct Select Box:** Select a primer as described above,

change the strand and select again to select a pair.

- Choose a pair in the **Search Results** window.
- Choose a pair in the database window by importing it.

Note: Instead of choosing a pair, let us say that you only chose a sense primer using one of the above methods. Then you are pairing the newly chosen primer with the anti-sense primer of the pair you were working on just before you made the new primer selection.

The property table updates the rating, sequence number, length of the primer, its melting temperature T_m , GC %, the degeneracy, the optical activity of sense and anti-sense primers in micrograms/OD. For the pair the length column shows the PCR product length and the T_a Opt column shows the optimum annealing temperature for PCR.


Please see **Appendix A** for the formulas used for calculating the properties.

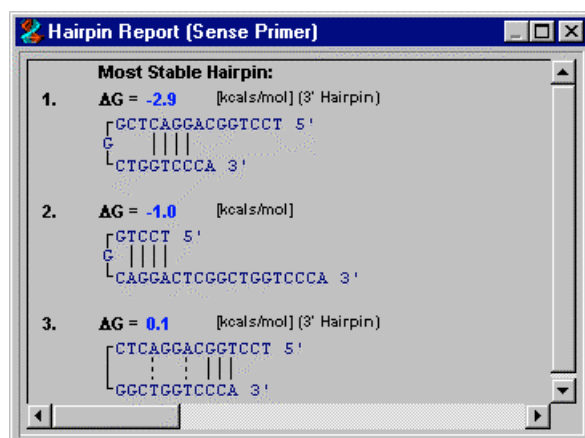
Secondary Structures

PRIMER PREMIER instantly analyzes secondary structures and the matrix of buttons shows you which structures are found. The depressed button indicates which structure is displayed in the neighboring box. Along with the secondary structure the window also displays the ΔG (in kcal/mol) of that structure.

Note: For hairpins, dimers, false priming or cross dimers to exist, it is assumed that at least three consecutive bases must bond.

The most stable structure is displayed in the box on the *PRIMER PREMIER* window. Consecutive bonds are shown with solid lines and non-contiguous with broken lines.

Pressing the  button opens the report of the structure you have selected. For example, if you were looking at the hairpins of sense primers the following window will open:



The structures appear in the order of decreasing stability, the most stable structure being the first. Scroll bars are available to study the structures in detail.

Apart from these functions there are three buttons for going directly to Search Criteria Window, Search Results Window (if a search has already been performed) and Edit Primer Window. Along with these buttons the portion amplified due to the selected primer pair is also displayed pictorially.

Edit Primer Window

Edit primer function provides you assistance in designing a primer for site directed mutagenesis and analyzing a pre-existing primer.

You can cut, copy and paste using the keystroke short cuts: CTRL-X, CTRL-C and CTRL-V for Windows and Command-X, Command-C and Command-V on Power Mac. The best way to analyze an existing primer is to delete the current primer and paste the existing primer through the clipboard. On pasting, Paste window opens, , which can used to reverse, complement or reverse complement the primer sequence. You can also type in the primer. As soon as the primer is edited, the previous results go away and the **Analyze** button becomes enabled. Clicking the **Analyze** button analyzes the current primer.

You can modify the primer by using the actual sequence or the amino

acid translation. If you use amino acid for placing bases for primer, the codon that is placed for a particular amino acid is the degenerate one.

In addition to the properties and secondary structure data (see PRIMER PREMIER window section for additional information), restriction enzyme analysis is available on this window. You can change the enzyme set by clicking the Enzyme button and modifying the set using the buttons or filter.

You can move your primer to next equal or more stable binding site. This can be achieved by pressing the Prime button. This can be used to confirm that no binding sites, which are equally or more stable than the existing site, are present.

Insider Info: If you want to change the reading frame or the codon table for the translation, close this window. Choose Function > GeneTank from the menu to make the GeneTank window active. Choose Translate > Change Parameters to open the Select Codon Table window. Change the reading frame and/or the codon table and reopen this window.

Alternate Access:
Function > Edit Primer

Search Criteria Window

The basic criteria for the search are specified here:

If you intend to use the primers for PCR, for sequencing or as hybridization probes, it is important to choose the appropriate **Search For** criteria to reflect it. *PRIMER PREMIER* adjusts the automated search parameters based on this selection.

The parameters, which optimize the ability of primer pairs to extend well, are not applicable if you are designing primers for sequencing or hybridization experiments. In addition, higher annealing temperatures are desirable. *PRIMER PREMIER* makes the adjustments to the parameters appropriate to your application when you make your selection in the **Search For** criteria. Please see the **Automatic Search Parameter** section below for the actual values used by the search algorithm.

The **Search Type** box should be used to indicate whether you want to search for single primer, both primers, primer pairs or whether you only want to search for a primer to pair with the currently selected primer.

By default, the **Search Range** is set to search the entire length of the loaded sequence. The range for PCR product length is set to a reasonable arbitrary value of 100 bp to 500 bp by default. If you are searching for single primers this value is ignored.

You can also set the **Primer Length** range for the search. Please refer to the discussion in the next section on how this parameter is used in search.

Finally, you can choose the **Search Mode** to be automatic or manual. Unless you have unique needs or desire greater control over certain parameters, you should choose the automatic search and let *PRIMER PREMIER* come up with a reasonable set of primers. You can then analyze the set in detail to pick the primers that suit your needs.

Alternate Access:

Function > Search

Search Parameter Windows

The search can be carried out using either automatic search method or manual search method.

When you want to search for primers on a sequence such that the primers should not bind (i.e., should not false prime) to any other sequence present in your reaction you can select them by pressing **Files** button.

Automatic Search

You can select which parameters are used in the search, although the *PRIMER PREMIER* determines their values automatically. Blanking the check box next to the parameter excludes the parameter as a search criterion.

Primer 5.0 Manual Reference

Automatic search starts with the stringency control set **Very High**. If the search does not yield any primers, the stringency is dropped a notch until primers are found. The following table shows the initial (Very High Stringency) values used in automatic search when you are searching for **PCR Primers**. The T_m range is based on the average of T_m 's of all possible primers for the sequence within the sequence number range selected for the search:

<input checked="" type="checkbox"/> T_m	Min: <input type="text" value="51.0"/> °C	Max: <input type="text" value="63.0"/> °C
<input checked="" type="checkbox"/> GC	Min: <input type="text" value="40.0"/> %	Max: <input type="text" value="60.0"/> %
<input checked="" type="checkbox"/> Degeneracy	Reject if last 2 3'end bases degenerate Reject if \geq <input type="text" value="1"/> degenerate bases within 6 of 3'end Reject if \geq <input type="text" value="20.0"/> % degenerate bases in primer	
<input checked="" type="checkbox"/> 3' end Stability	Min: <input type="text" value="-9.0"/> kcal/mol	Max: <input type="text" value="-6.5"/> kcal/mol
<input checked="" type="checkbox"/> GC Clamp	Reject if no pentamer with $\Delta G \leq$ <input type="text" value="-10.0"/> kcal/mol	
<input checked="" type="checkbox"/> Repeats/Runs	Reject 3 dinucleotide repeat (i.e. ATATAT) Reject if \geq <input type="text" value="3"/> base run (i.e. GAAAAT)	
<input checked="" type="checkbox"/> Dimer/Hairpin	Reject if \geq <input type="text" value="3"/> bonds within 6 bases of 3'end Reject if \geq <input type="text" value="4"/> consecutive bonds in primer	
<input checked="" type="checkbox"/> False Priming	Reject if \geq <input type="text" value="6"/> consecutive bonds at 3'end <input type="button" value="FILES"/> Reject if \geq <input type="text" value="8"/> consecutive bonds in primer	

Note: T_{ave} is the average of T_m 's of all possible primers for the sequence within the sequence number range selected for the search.

If you are searching for **Sequencing Primers** the values which differ from the above table are:

Primer

<input checked="" type="checkbox"/> Tm	Min: <input type="text" value="61.0"/> °C	Max: <input type="text" value="100.0"/> °C
<input checked="" type="checkbox"/> GC	Min: <input type="text" value="50.0"/> %	Max: <input type="text" value="100.0"/> %
<input checked="" type="checkbox"/> Degeneracy	Reject if last 2 3'end bases degenerate	
	Reject if >= <input type="text" value="1"/>	degenerate bases within 6 of 3'end
	Reject if >= <input type="text" value="20.0"/>	% degenerate bases in primer
<input checked="" type="checkbox"/> 3' end Stability	Min: <input type="text" value="-9.0"/> kcals/mol	Max: <input type="text" value="-6.5"/> kcals/mol
<input checked="" type="checkbox"/> GC Clamp	Reject if no pentamer with $\Delta G \leq$ <input type="text" value="-10.0"/> kcals/mol	
<input checked="" type="checkbox"/> Repeats/Runs	Reject 3 dinucleotide repeat (i.e. ATATAT)	
	Reject if >= <input type="text" value="3"/>	base run (i.e. GAAAAAT)
<input checked="" type="checkbox"/> Dimer/Hairpin	Reject if >= <input type="text" value="3"/>	bonds within 6 bases of 3'end
	Reject if >= <input type="text" value="4"/>	consecutive bonds in primer
<input checked="" type="checkbox"/> False Priming	Reject if >= <input type="text" value="6"/>	consecutive bonds at 3'end
<input type="button" value="FILES"/>	Reject if >= <input type="text" value="8"/>	consecutive bonds in primer

If you are searching for **Hybridization Probes** the values which differ from the table for PCR primers are:

<input checked="" type="checkbox"/> Tm	Min: <input type="text" value="67.0"/> °C	Max: <input type="text" value="100.0"/> °C
<input checked="" type="checkbox"/> GC	Min: <input type="text" value="50.0"/> %	Max: <input type="text" value="100.0"/> %
<input checked="" type="checkbox"/> Degeneracy	Reject if last 2 3'end bases degenerate	
	Reject if >= <input type="text" value="1"/>	degenerate bases within 6 of 3'end
	Reject if >= <input type="text" value="20.0"/>	% degenerate bases in primer
<input checked="" type="checkbox"/> 3' end Stability	Min: <input type="text" value="-12.0"/> kcals/mol	Max: <input type="text" value="0.0"/> kcals/mol
<input checked="" type="checkbox"/> GC Clamp	Reject if no pentamer with $\Delta G \leq$ <input type="text" value="-11.0"/> kcals/mol	
<input checked="" type="checkbox"/> Repeats/Runs	Reject 3 dinucleotide repeat (i.e. ATATAT)	
	Reject if >= <input type="text" value="3"/>	base run (i.e. GAAAAAT)
<input checked="" type="checkbox"/> Dimer/Hairpin	Reject if >= <input type="text" value="3"/>	bonds within 6 bases of 3'end
	Reject if >= <input type="text" value="4"/>	consecutive bonds in primer
<input checked="" type="checkbox"/> False Priming	Reject if >= <input type="text" value="6"/>	consecutive bonds at 3'end
<input type="button" value="FILES"/>	Reject if >= <input type="text" value="8"/>	consecutive bonds in primer

Insider Info: If you select **Manual Search** and click the **Stringency** buttons, you can get an on-line display of the tables above.

Manual Search (Science of Primer Design)

PCR methods have been evolving over the past ten years to allow the most efficient amplifications possible. Improvements in our understanding of primer stability, primer dimer formation, and optimal primer length have led to progressively higher PCR efficiencies while maintaining adequate specificity. The *PRIMER PREMIER* search algorithm maintains a balance between the goals of specificity and efficiency.

Primer Length

Along with the annealing temperature of the PCR reaction, the length of the primer primarily controls the specificity. For most experiments, primers between 18 and 24 bases are optimal to use. Short primers of 15 bases or less are sometimes used for mapping simple genomes or other specialized library protocol. 28-35 bases long primers are necessary for amplifying sequences encoding closely related molecules or cloning genes from different species.

Longer PCR primers result in increased specificity during amplification. This allows the use of lower annealing temperatures, improving the sensitivity of the reaction. However, longer primers are more likely to form secondary structures including hairpins, dimers, and self-complementation.

Ideally, the shortest primer with melting temperature in the desired range provides the best balance between specificity and efficiency.

PRIMER PREMIER ships with reasonable values for the primer length range. If you change them, it remembers your new values and continues using them until you change them again. It is, therefore, important for you to enter a range based on the specificity required by your application.

PRIMER PREMIER starts the search with the primer lengths at the minimum acceptable value. If the T_m is lower than the desired T_m range or GC % is not within the desired range, it adds a nucleotide making it longer. It recalculates the T_m and GC % and tests them to see if they fall within the acceptable ranges. If not, it continues adding to the length until the maximum length is reached. If the T_m and GC % are still not within the acceptable range, it rejects the primer. This search algorithm ensures finding the shortest possible primers consistent with desired T_m and GC %.

Primer

In designing an efficient primer, several parameters should be considered. The order in which *PRIMER PREMIER* searches these parameters follows:

T_m (Melting Temperature)

PRIMER PREMIER calculates melting temperature using nearest neighbor theory. Please see Appendix A of this manual for the formula. The range of T_m selected should provide sufficient thermal window for annealing. The algorithm used by *PRIMER PREMIER* for choosing a range for automatic search is:

First, T_m's of all possible primers for the sequence along the region selected for the search are calculated. An average T_m is computed. A range for each stringency is calculated around this average by using reasonably tight numbers.

A reasonable range for values of T_m for PCR is 56-63 °C according to the published experimental data^[8]. The above algorithm is devised to guarantee finding reasonable number of primers within the desired range.

GC %

Around 50% GC content is reasonable for PCR, while for sequencing primers and hybridization probes the GC content should be at least 50% or higher. The tables in the **Automatic Search** section above show the exact numbers used for each type of search.

Degeneracy

When designing degenerate primers, primers with least degeneracy are more desirable because they provide greater specificity. It is also critical to avoid degeneracy at the 3' end, because even a single base mismatch can prevent extension.

*For further details, please see the **Degeneracy** chapter of this manual.*

3' End Stability

The stability of the primer determines its false priming efficiency. An ideal primer has a stable 5' end and an unstable 3' end.

If the primer has a stable 3' end, it will bond to a site that is complementary to it other than the target with its 5' end hanging off the edge. It may then lead to secondary bands.

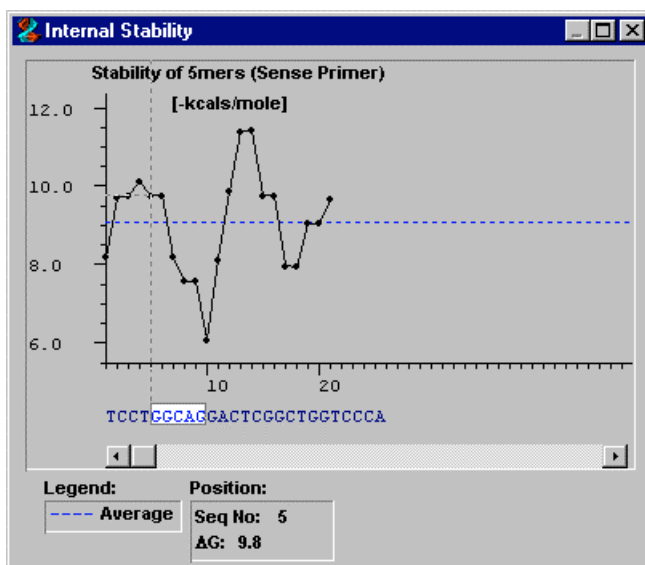
Primer 5.0 Manual Reference

Primers with low stability at the 3' ends function well because the 3' end bonding to false priming sites are too unstable to extend.

GC Clamp

Stability of the 5' termini allows for efficient bonding of the primer to the target site. This stable 5' region is called the GC Clamp. It ensures adequate binding of the primer to the template. Use of primers with optimal stability allows for the use of lower annealing temperatures without the production of secondary bands.

The internal stability graph of a good primer using **Report > Internal Stability** menu item is shown:



Notice that the 3' end has low stability and the 5' end has a strong GC clamp.

Repeats and Runs

PRIMER PREMIER automatically rejects any primers with 3 or more dinucleotide repeats. For example, if the primer contains ATATAT, dinucleotide AT is repeated three times, the primer is rejected. You can set the number of single base runs that are acceptable.


Secondary Structures

Primer

An important factor to consider in the design of a primer is the presence of secondary structures. Secondary structure can greatly reduce the number of primers available for bonding in the reaction. The presence of hairpin loops in a primer reduces its efficiency by limiting its ability to bond to the target site. Primers that have formed loops are unavailable for bonding to the PCR products.

Hairpin:

A hairpin loop is formed when primer folds back upon itself and is held in place by intramolecular bonding. Because hairpin loop formation is an intramolecular reaction, it can occur with as few as 3 consecutive homologous bases. To measure the stability of the hairpin loop formed, measure the free energy. The free energy of the loop is based upon the energy of the intramolecular bond and the energy needed to twist the DNA to form the loop. If this free energy is greater than 0, the loop is too unstable to interfere with the reaction. If the free energy is less than 0, the loop could reduce the efficiency of amplification. The

Hairpin Report accessed using the  button on the *PRIMER PREMIER* window allows you to avoid primers containing secondary structure.

Dimer:

Dimers occur when a region of homology is present within a primer or between two primers. This results in bonding of the two primers, increasing production of the primer dimer artifact and reducing product yields.

Dimers can occur within a primer when two copies of the primer bind to each other or when a primer binds to the other primer in the pair. This is particularly problematic when the homology occurs at the 3' end of either primer. The 3' end will extend readily leading to primer dimer artifact. The Dimer Report can be used to test for formation of dimer duplexes.

False Priming:

If the primer binds anywhere other than the target site, the amplification specificity is reduced significantly. The product is weak or a smear is observed. *PRIMER PREMIER* examines the entire sequence for each primer to identify potential false priming sites. The propensity for false priming is higher if the 3' end can form consecutive bonds than if the same number of bonds can

form upstream of the 3' end. You can choose separate screening criteria for number of bonds at the 3' end and for the entire primer.

Pair Rating

Poorly matched primers are less specific because the primer with lower T_m determines the specificity of the amplification. The primer with higher T_m has a greater chance of mispriming resulting in secondary banding. *PRIMER PREMIER* calculates a rating for each pair, 100 being a perfect match, and presents the pairs in the order of decreasing rating. Appendix A contains the formulas used for the rating calculation of primer pairs as well as individual primers.

Search Results Window

When you accept the results of the search by clicking OK on the Search Progress window the Search Results window opens. It shows all the primers and or pairs found with the search.

As you select a primer or a pair, it is instantly analyzed and the analysis appears on the *PRIMER PREMIER* window. The location and the sequence of the primer with the selected strand appear in the Direct Select box.

Insider Info: Change the size and the location of the Search Results window relative to the *PRIMER PREMIER* window which shows the analysis so that the properties you are interested in are not blocked by the window.

Alternate Access:

Function > Search Results

Multiplex/Nested Primer Window

Nested PCR is often successful in reducing unwanted products while dramatically increasing sensitivity. It is often used when the actual quantity of target DNA is very dilute or not very pure.

You first run a search using automatic mode or tailoring it to your experimental needs. You are then ready to select a compatible set of nested primers from this pool. *PRIMER PREMIER* assists you in selecting multiple or nested primers from the pool by ensuring that they are free of cross dimers.

Primer

After you accept the results from a search, choose Function > Multiplex/Nested Primers from the menu to open this window: The entire sequence and all the primers found are shown in graphical form at the top of the window. The box indicates the portion of the sequence magnified in the center of the window. You can change the region magnified by using the horizontal scrollbar at the bottom of the magnified region.

The available primers appear as vertical lines in the top box and as blue and red hollow triangles in the magnified region. As always, blue represents sense primers and red anti-sense.

Click on the triangle representing a primer you want to select for your multiplex experiment. The triangle changes from hollow to filled indicating your selection. That primer appears in the bottom box as a selected primer both in graphical and tabulated form.

Notice that as soon as you make your selections the cross dimers are displayed which are formed between the selected primers. *PRIMER PREMIER* instantly analyzes all the selected primers and displays the cross dimers that are formed in between the primers you have selected. Continue your selection until you have a set with no or less stable cross dimer. To deselect a previously selected primer, simply click on the solid triangle representing it and it will be deselected.

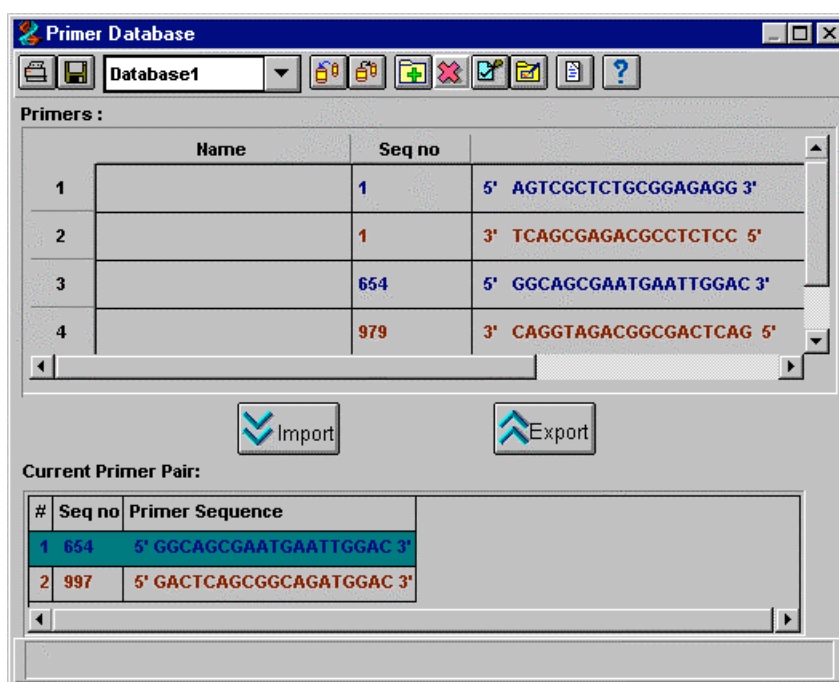
You can also add primers manually. This is useful if you already have primers in your lab or working with published primer sequences. You can add all your primers manually and check any combination of primers in the pool that form cross dimers.


You can define that the input oligo is a sense primer or an anti-sense primer, its sequence number and the sequence. If the sequence number is not specified, it is defaulted to 5' sequence number of first base. If you wish to delete a primer, select it and press the Delete button.

You can print the target sequence showing all primers on the sequence. Select primers are displayed as filled triangles, unselected primers as hollow triangles. Primers appearing in table and all possible cross dimers can also be printed.

Database Window

The Primer Database allows you to store the sequences of primers. You can name individual primers and save them to a new database set or append them to an existing database set. Primers can be ordered directly from database window using the Order Primers button.



There are two primary ways to get your selection into the database. You can mark the primers on the Search Results window and then import the primers into the database using the  (Get Marked Primers) button or you can select a primer directly and export it through the Current Primer Pair box.

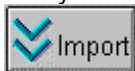
The database remembers the sequence number, sequence, strand and the name of the primer. Editing of any of the primers name,

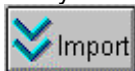
Primer

sequence, sequence number and strand can be done by selecting the primer to be edited and then selecting edit primer option from menu




If you open a database set by selecting it from the drop down box, with previously saved primers, you can analyze them with the currently loaded sequence by selecting the primer and making it the



current selection, using  button. Once the primer is selected, you can use *PRIMER PREMIER* window to analyze it or edit it. You can automatically find the most stable priming site on the new sequence by using the prime button on the Edit Primer Window.

You can add new database sets with different names. This makes it easy to keep primers stored by target gene or projects. Similarly you can also delete any primer database set which was created earlier.

New primers can be added directly by pressing the  (Add Primer) button. This will popup a window where you enter the primer name, strand, sequence number and the oligo sequence. If the sequence number is not given it is defaulted to 5' sequence number of first base.

Reports

Several reports are available through the Reports menu. In addition to the secondary structure reports which are also available by clicking



on the *PRIMER PREMIER* window, an activity report and an internal stability report is available for the currently selected primers.

When you select Optical Activity from Report menu a window displaying the Molecular Weight and Activity (in nmol/OD and μ G/OD) for the current primer pair selected opens.


When you move the cursor on the internal stability graph, a broken line snaps to the nearest valid sequence position and the readout box is updated. The pentamer is highlighted as well.

The graph window can be resized to adjust the amount of detail viewed.

A window opens when you select Hybridization Time from Report menu. You can enter the primer length and concentration of interest and click the Analyze button to get hybridization time.

Synthesis Order Form

After searching and analyzing primers, if you decide to order primers for synthesis, you can use the synthesis order form. A sample form is provided for your convenience.

You can open a new form and enter your shipping and billing information and save it under a new name. You can use this form as a template for all of your orders. The Database window is used to select the primers you wish to order. Simply select the primers to be ordered, open the form, by pressing  button, with the correct shipping and billing information, *PRIMER PREMIER* will automatically add the sequence of the primers you have selected on the Database window in the appropriate place on the synthesis form. Add remarks, print it and send it on its way.

Save the form so that you can re-open it.

Optical Activity

The absorbance at 260 nm, A_{260} , is proportional to the primer concentration, with a value of 0.02 units per microgram DNA per milliliter. The molecular weight of a primer is also available. This makes it easy to calculate dilution factors for newly synthesized oligonucleotides.

- Molecular Weight - This is the molecular weight of both the primers in grams/mol. It is calculated from the table of molecular weights.
- nmol/A_{260} - This is the concentration of primer in nanomoles per unit absorbance (OD) at 260 nm. It is calculated from table of extinction coefficients and molecular weights.
- $\mu\text{g}/A_{260}$ - This is the concentration of primer in micrograms per unit absorbance (OD) at 260 nm. It is calculated from table of extinction coefficients.

Degeneracy

Some PCR applications require that primers bind to an unknown sequence. Here are several possible situations:

- Expressed protein known but DNA has not been sequenced.
- Template DNA is poorly sequenced or the sequencing is incomplete.
- The coding region that needs to be amplified has been identified in another organism.
- Consensus sequence from multiple species for the template DNA is available.

When designing degenerate primers, along with all the parameters mentioned in the Search Parameters, the degree of degeneracy within the primer must be considered. The primers with the least degeneracy are more desirable because they provide greater specificity. It is also critical to avoid degeneracy at the 3' end, because even a single base mismatch can inhibit extension. *PRIMER PREMIER* automatically scans the target DNA sequences and designs primers in regions of low degeneracy that are checked for the overall percentage of ambiguous bases as well as number of ambiguous bases occurring at the critical 3' end⁽¹³⁾.

It has been reported that the PCR thermal profile can dramatically alter the success rate of degenerate PCR. The preferred degenerate PCR amplification profile starts with a non-stringent annealing temperature (35-45 °C) for 2-5 cycles, followed by 25-40 cycles at a more stringent annealing temperature. The relaxed annealing conditions allow the short complementary primer portion to hybridize to the target. After the second cycle of amplification, the 5' extension becomes incorporated into the amplified product and will serve as the template for subsequent rounds of amplification. By shifting to a more stringent annealing temperature, increased specificity can be achieved⁽⁹⁾.

Graphs

The graphs of T_m , GC % and internal stability for the entire sequence are available through the **Graph** menu. T_m is the melting temperature of all potential primers with the default length of primer as set in Preferences window. GC % is the GC content of all potential primers.

Primer 5.0 Manual Reference

Internal stability is the free energy of pentamers located at each position of the sequence.

When you move the cursor on the graph, a broken line snaps to the nearest valid sequence position and the readout box is updated. The primer or the pentamer is highlighted as well.

Restriction Enzyme Analysis

A set of approximately 900 common enzymes is supplied with *PRIMER PREMIER* for your convenience. Easy to use facilities to add to this set are provided as well. By accepting/rejecting enzymes from the available enzyme set, you can create a current set. Alternatively, you can use filter to specify properties of enzymes to create the current set. The current set is used for restriction enzyme analysis. The analysis is displayed in several formats: table, map or sequence.

The function has the following sections:

Restriction Enzyme Analysis Window
Filter Window
Edit Window


Restriction Enzyme Analysis Window

Approximately 900 commonly used enzymes have been supplied with *PRIMER PREMIER* for your convenience. You can add or delete enzymes to the All Enzymes set using the **Edit** function.


The **Selected Enzymes** box displays the currently selected set of enzymes, which is subset of **All Enzymes**. The **Selected Enzyme** set is used for analysis. There are two ways of choosing the current set:

1. Select enzymes you want to add to the current set by selecting them in the **All Enzymes** box. You can use CTRL key on Windows and Power Mac while clicking to choose multiple enzymes. To choose a range of enzymes, select the first enzyme by clicking and then select the last enzyme in the range by pressing SHIFT key while clicking.



Press  button to add the selected enzymes to the current set. Selecting enzymes in **Selected Enzyme** box and pressing



 button deletes the selected enzymes from the current set. You can also double click on an enzyme in the **All Enzyme** box to add it to **Selected Enzyme** box or double click on an enzyme in **Selected Enzyme** box to delete it.

2. Use filter window to choose the current set by specifying the desired properties. See the section on the **Filter** window for details.

Restriction Sites Window

Pressing **OK** button on the **Restriction Enzyme** window opens up the **Restriction Sites** window.

Available displays are:

- **Table**: Lists the enzyme, its recognition sequence, number of cut sites and the location of cut sites.
- **Sequence**: Shows the exact location of the cut site for each enzyme on the sequence.
- **Map**: Represents the sequence graphically with a rectangle and shows the location of the cut sites for each enzyme with vertical lines.
- **Non-cutters**: Lists the enzymes of the current set which do not cut the sequence at all or which do not qualify the set criterion.
- You can filter the display of enzymes by selecting to display enzymes with specific number of cut sites. For example, if you choose the 4 box, the enzymes that cut the sequence 5 or more times will be eliminated from the display.

Note: If an enzyme cuts the sequence for more than 50 times, only the first 50 cuts are shown and the rest are ignored.

You can print Table, Sequence or Non-cutters by selecting **File > Print** from the menu.

Edit Enzyme Window

To modify an enzyme in the **All Enzymes** set, select the enzyme and press **Edit** button. This will bring up Edit Enzyme window where enzymes name, recognition site or heat activation can be changed. To add an enzyme, click on **Add** button and a window opens with a default name and recognition site for the enzyme. The **Delete** button will delete the selected enzyme.

Press the **OK** button to accept all changes and return to the **Restriction Enzyme Analysis** window.

Note: There is no need to press **OK** after each change. All changes are remembered as you make them. Press **OK** to accept them all or **Cancel** to undo all the changes you made in this session.

Filter Window

You can create the current set for use in the analysis by specifying the desired properties of the enzymes.

Filter Enzymes

Selected Enzymes:

- Afa16RI
- Afa22MI
- BspCI
- EagBI
- ErhB9I
- NblI
- Ple19I
- Psu161I
- PvuI
- RshI
- XorII

Recognition Sites:

☒ Recognition Site Length

☐ 4 bp ☒ 5 bp ☒ 6 bp ☐ 7 bp ☐ 8 bp

☐ 9 bp ☐ 10 bp ☐ 11 bp ☐ 12 bp ☐ 13 bp

Overhang:

☒ Overhang ☒ Overhang Sequence

☒ 3' ☐ 5' ☐ Blunt

5' AT 3'

Heat Inactivation:

☐ Inactivated by 20° at 60 °C

OK Cancel Help

Specify the recognition site length between 4 bp to 13 bp by choosing the appropriate boxes and the **Selected Enzymes** box is instantly updated to include only the enzymes that satisfy your criterion.

You can specify the type of overhang and the overhang sequence if you wish. The table showing the conventions used to specify the overhang follows:

Enzyme	Recognition Site	Type of Overhang	Overhang Sequence
AatII	5'..G ACGT^C.3' 3'..C^TGCA G.5'	3'	ACGT
AccIII	5'..T^CCG GA.3' 3'..A GGC^CT.5'	5'	GGC
Aor51HI	5'..AGC^GCT.3'	Blunt	-

You can also choose whether the **Selected Enzyme** set is heat sensitive or not by choosing **Heat Inactivation** box.

When you press **OK**, the **Selected Enzyme** set becomes the current set of Selected Enzymes on the **Restriction Enzyme Analysis** window and you are ready to analyze the cut sites using this set.

Motif Analysis

A set of approximately 165 common motifs is supplied with *PRIMER PREMIER* for your convenience. Easy to use facilities to add to this set are provided as well. By accepting/rejecting motifs from the available motif set, you can create a current set. The current set is used for motif analysis. The analysis is displayed in several formats: table, map or sequence.

The function has the following sections:


Motif Analysis Window
Edit Window


Motif Analysis Window

Approximately 150 commonly used motifs have been supplied with *PRIMER PREMIER* for your convenience. You can add or delete motifs to the All Motifs set using the **Edit** function.

The **Selected Motifs** box displays the currently selected set of motifs, which is subset of **All Motifs**. The **Selected Motifs** set is used for analysis. You can choose the current set in the following manner:

Select motifs you want to add to the current set by selecting them in the **All Motifs** box. You can use CTRL key on Windows and Power Mac while clicking to choose multiple motifs. To choose a range of motifs, select the first motif by clicking and then select the last motif in

the range by pressing SHIFT key while clicking. Press  button to add the selected motifs to the current set. Selecting motifs in

Selected Motif box and pressing  button deletes the selected motifs from the current set. You can also double click on an motif in the **All Motif** box to add it to **Selected Motif** box or double click on an motif in **Selected Motif** box to delete it.

Motif Sites Window

Pressing **OK** button on the **Motif Analysis** window opens up the **Motif Sites** window.

Available displays are:

- **Table:** Lists the motif, its recognition sequence, number of sites and the location of sites.
- **Sequence:** Shows the exact location of the site for each motif on the sequence.
- **Map:** Represents the sequence graphically with a rectangle and shows the location of the sites for each motif with vertical lines.
- **Motifs Absent:** Lists the motifs of the current set which do not appear in the sequence at all or which do not qualify the set criterion.

You can filter the display of motifs by selecting to display motifs with specific number of sites. For example, if you choose the 4 box, the motifs that appear 5 or more times in the sequence will be eliminated from the display.

Note: If a motif appears in the sequence for more than 50 times, only the first 50 sites are shown and the rest are ignored.

You can print Table, Sequence or Motifs Absent by selecting **File > Print** from the menu.

Edit Motif Window

To modify a motif in the **All Motifs** set, select the motif and press **Edit** button. This will bring up Edit Motif window where motif name, recognition sequence and description can be changed. To add a motif, click on **Add** button and a window opens with a default name and recognition sequence for the motif. The **Delete** button will delete the selected motif.

Press the **OK** button to accept all changes and return to the **Motif Analysis** window.

Note: There is no need to press **OK** after each change. All changes are remembered as you make them. Press **OK** to accept them all or **Cancel** to undo all the changes you made in this session.

Menu Commands

Each of the main functions has its own menu:

GeneTank Menu
Primer Menu
Enzyme Results Menu
Motif Results Menu

GeneTank Menu

File

New Sequence: Opens GeneTank window with a blank sequence.

Open Sequence: Prompts you for a file to use and opens the sequence.

Save: Saves the loaded sequence to a file. You can open a saved file without going through the Import Window.

Save As: Saves a copy of the loaded sequence to the chosen file.

Degenerate Bases: Lists the table of all allowed degenerate bases with their meanings.

Print: You can print the sequence in various formats with various combinations.

Quit: Exits *PRIMER PREMIER*.

List of last four opened files.

Edit

Standard cut, copy, and paste menu items.

Preferences: Lets you choose parameters that apply to the entire product.

View

Show Header: Displays the header of the loaded sequence in the GeneTank window.

Sense Strand: Displays the sense strand of the loaded sequence.

Anti-sense Strand: Displays the anti-sense strand of the loaded sequence.

ds DNA: Displays both the sense strand and anti-sense strand of the loaded sequence.

Grouping: Choose the grouping of the bases of the sequence to be 3 or 10.

5' Seq no: Renumbers bases of the sequence starting from 5' end.

Search

Find: Finds occurrences of the pattern within the sequence starting from the current position.

Find Next: Finds the next occurrence of the pattern within the sequence.

Go To Position: Places the cursor at the specified position in the sequence.

Function

Primer Design: Designs primers according to parameters set after selecting this function.

Restriction Enzymes: Performs restriction enzyme analysis according to the set parameters.

Motifs: Performs motif analysis according to set parameters.

Bias majority by: When no single base has a majority the sequence from this base is used.

Speaker: Voice readback of sequence.

The menu lists the functions available to you, except the function you are using.

Translate

To DNA: Translate loaded protein sequence to DNA.

To Protein: Translate loaded DNA sequence to protein.

Change Parameters: Change reading frames or codon table for translation.

Edit Codon Table: You can add, delete or modify codon tables.

Window

The menu lists all of the windows that are open, except the one that is active. When you select a window in the list, it becomes the active window.

Help

Menu Commands

Use this menu to view on-line help. The information contained in the help is substantially the same as the information available in this manual. It has been formatted for on-screen viewing and contains hypertext links to enable you to jump to relevant topics. It also contains search facility to locate topics.

Primer Menu

File

Preferences: Lets you choose parameters that apply to the entire product.

Degenerate Bases: Lists the table of all allowed degenerate bases with their meanings.

Print: You can print the sequence in various formats with various combinations.

Quit: Exits *PRIMER PREMIER*.

Edit

Find: Finds occurrences of the pattern within the sequence.

Find Next: Finds next occurrence of the pattern with in the sequence.

Function

Search: Opens the Search Criteria window to set parameters and start primer search.

Search Results: Makes the Search Results window active if you have completed a search.

Edit Primer: You can add, delete, modify the primer sequence and analyze it instantly. You can also move the primer to the next best false priming site.

Multiplex/Nested: Choose compatible nested sets of primers from search results and can also add or delete new primers and check with the existing set of selected primers.

Database: You can add, delete or modify the existing database of primers. You can edit the strand, name, sequence number or sequence of a primer. You can also order primers of from any database.

After the line that separates the above menus, three functions (GeneTank, Restriction Enzymes and Motifs) are listed.

Report

Hairpin: Shows all hairpin structures for currently selected sense or anti-sense primer.

Dimer: Shows all dimer structures for currently selected sense or

anti-sense primer.

False Priming: Shows all false priming sites for currently selected sense or anti-sense primer.

Internal Stability: Draws a graph of internal stability of pentamers along the currently selected sense or anti-sense primers.

Optical Activity: Shows the molecular weight and optical activity of the primer.

Hybridization Time: This will calculate the hybridization time for a specific primer length and concentration.

Graph

Melting Temp (T_m): Draws a graph of the T_m for the entire sequence.

GC %: Draws a graph of the GC% for the entire sequence.

Internal Stability: Draws graphs of stability of 5 mers in (-Kcal/mol).

Window

Please refer to the explanation in GeneTank.

Help

Please refer to the explanation in GeneTank.

Enzyme Results Menu

File

Preferences: Lets you choose parameters that apply to the entire product.

Degenerate Bases: Lists the table of all allowed degenerate bases with their meanings.

Print: You can print the sequence in various formats with various combinations.

Quit: Exits *PRIMER PREMIER*.

Function

Restriction Enzymes: Performs restriction enzyme analysis according to set parameters.

GeneTank: Displays GeneTank window.

Primer Design: Design primers according to parameters set after selecting this function.

Motifs: Performs motif analysis according to set parameters.

Window

Please refer to the explanation of the same menu in GeneTank.

Help

Please refer to the explanation of the same menu in GeneTank.

Motif Menu

File

Preferences: Lets you choose parameters that apply to the entire product.

Degenerate Bases: Lists the table of all allowed degenerate bases with their meanings.

Print: You can print the sequence in various formats with various combinations.

Quit: Exits *PRIMER PREMIER*.

Function

Restriction Enzymes: Get restriction enzyme analysis done according to set parameters.

GeneTank: Displays GeneTank window.

Primer Design: Design primers according to parameters set after selecting this function.

Motifs: Get motif analysis done according to set parameters.

Window

Please refer to the explanation of the same menu in GeneTank.

Help

Please refer to the explanation of the same menu in GeneTank.

Appendices

- Appendix A: Formulas and Algorithms
- Appendix B: Degenerate Code
- Appendix C: Amino Acid Symbols
- Appendix D: Software Limits
- Appendix E: References
- Appendix F: System Specifications

Appendix A: Formulas and Algorithms

Formula for Determining Melting Temperature of Primer (T_m of Primer)

Melting temperatures are based on nearest neighbor thermodynamic theory. The formulas are from the paper by Freier et al (7). These are highly accurate nearest neighbor based T_m calculations. Several corrections have been incorporated into the formula to yield the most accurate results possible.

$$T_m = \Delta H / (\Delta S + R * \ln (C/4)) + 16.6 \log ([K^+]/(1 + 0.7 [K^+])) - 273.15$$

ΔH is the enthalpy for helix formation.

ΔS is the entropy for helix formation.

C is the DNA concentration.

$[K^+]$ is the salt concentration.

ΔH and ΔS for the respective oligo are calculated using the values of nearest-neighbor thermodynamics from Breslauer, K.J. (2). Since the thermodynamic values calculated by Breslauer, K.J. are at 1 M Na^+ , it was necessary to add a term to correct for salt concentration, $[K^+]$. $[K^+]$ is calculated using both the monovalent ion concentration and the free Mg^{2+} concentration. The value of $[K^+]$ is determined using the formula:

$$[K^+] = \text{Monovalent ion concentration} + 4 \times \sqrt{\text{Free } Mg^{2+} \text{ ion concentration} \times 1000}$$

The salt concentration i.e., $[K^+]$ is represented as Total Na^+ Equivalent in the preferences window and it gets updated according to the changes made in either Monovalent ion concentration or Free Mg^{2+} ion concentration. The default values of Monovalent ion concentration and Free Mg^{2+} ion concentration are 50.0 mM and 1.5 mM res., which gives a total salt concentration equal to 204.9 mM.

In the formulation of Freier *et al.* (7), c is the total molar concentration of the annealing oligos, when oligos are not self-complementary. It is neither the primer concentration nor the initial template concentration. It is based on the PCR product concentration. Since the concentration of template changes dramatically during the course of the PCR, it is difficult to define the value of " c ". It has been empirically determined that setting the value of $c = 250$ pM, as suggested by Rychlik, W. *et*

al. (11), gives good results in a wide variety of PCR and sequencing reactions. This is set as the default value though you can change this value from the Preferences window, which can be activated from File menu.

Formula for Determining Melting Temperature of Product (T_m of Product)

The melting temperature of the PCR product cannot be calculated using the nearest neighbor model as it is only accurate for short sequences such as oligonucleotides. For calculating T_m of long duplex DNA, the formula of Wetmur, J.G. is employed: (12):

$$T_m \text{ product} = 81.5 + 16.6 \log ([K+]/(1+0.7[K+])) + 0.41 (\%G + \%C) - 500/\text{length}$$

The salt concentration is taken from the Preferences Window (Total Na [·] Equivalent), GC % are calculated and length of product is also determined.

Formula for Determining Optimum Annealing Temperature T_a Opt

PRIMER PREMIER automatically calculates the predicted optimal annealing temperature for any given primer pair on a particular target using the formula of Rychlik, W. (11). Use of this temperature in the annealing step of the PCR cycle usually results in optimal PCR product yield with minimum false product production.

$$T_a \text{ Opt} = 0.3 \times (T_m \text{ of primer}) + 0.7 \times (T_m \text{ of product}) - 25$$

where T_m of primer is the melting temperature of the less stable primer-template pair and T_m of product is the melting temperature of the PCR product, both calculated as shown above.

Formula for Determining Free Energy ΔG

Entropy and enthalpy calculations are based upon the thermodynamic library of all 10 Watson-Crick DNA nearest-neighbor interactions determined by Breslauer *et al.* (2). Using this method, the free energy of an oligo is calculated from the enthalpies and entropies of each dinucleotide in the oligo and the temperature defined in the Preferences Window. The temperature setting on the Preferences Window should be set to ambient room temperature. The experiments are based on a 1M NaCl concentration in the PCR

reaction.

$$\Delta G = \Delta H - T \times \Delta S$$

ΔH is the enthalpy.

T is the temperature.

ΔS is the entropy.

Secondary structures form either due to bonds at 3' end and due to internal bonding. The 3' end blocking secondary structures reduce efficiency more than the internal ones. Thus to take that into account, ΔG of 3' end secondary structures is decreased by 1.

Formula for Determining Single Primer Rating

$$\text{Rating} = 100 - (\Delta G (\text{Dimer}) \times 1.7 + \Delta G (\text{Hairpin}) \times 1.4 + \Delta G (\text{False Priming}) \times 1.5)$$

After a search for primers is completed, it is difficult to determine which primer from the selected set will yield optimal amplification. *PRIMER PREMIER* automatically rates and sorts primers based upon the stability of the secondary structures. The rating is based on the stability of secondary structures formed by the primers. If the secondary structures formed by a primer are stable then it is rated low and if they are less stable then it is rated high. *PRIMER PREMIER* rates the primers based on the thermodynamic stability values of secondary structures formed by primers. Each value is multiplied with a specified weight, determined experimentally, for that term. These weights can be modified on the Preferences Window.

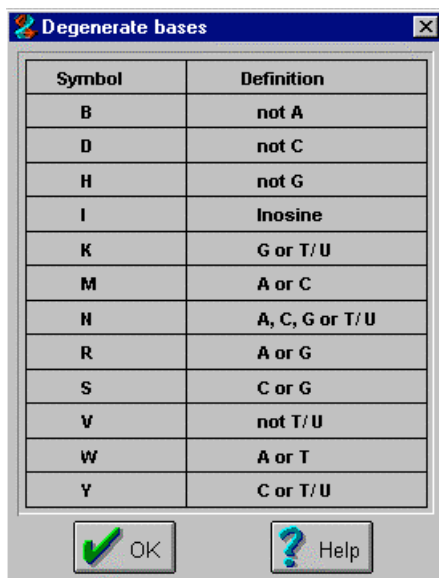
Formula for Determining Primer Pair Rating

$$\text{Rating} = \text{Average of sense and anti-sense primer rating} - ((T_m \text{ difference between primers}) \times 1.7) - (\text{DG Cross Dimer}) \times 1.3)$$

The Pair Rating is calculated from the average single primer rating of the sense and anti-sense primer, the T_m matching between the two primers, and the presence of any cross dimers between the sense and anti-sense primers.

Appendix B: Degenerate Code

This window displays the allowed degenerate bases for DNA.



Symbol	Definition
B	not A
D	not C
H	not G
I	Inosine
K	G or T/U
M	A or C
N	A, C, G or T/U
R	A or G
S	C or G
V	not T/U
W	A or T
Y	C or T/U

Appendix C: Amino Acid Symbols

Following are the single letter and three letter symbols for the respective amino acids:

Single Letter Symbol	Three Letter Symbol	Amino Acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic Acid
E	Glu	Glutamic Acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Appendix D: Software Limits

Primer length	70 bp
Sequence length	50 kb
Recognition site length of restriction enzyme	19
Maximum number of cut sites shown	50
Maximum number of primer pairs searched	1000

Appendix E: References

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Appendix F: System Specifications

For Windows Operating System (Windows 98/NT 4/2000):

	Required	Recommended
CPU	Pentium/ 100 MHz	Pentium 166 MHz
RAM	32 MB	64 MB

For Power Macintosh Operating System (7.0 or later):

	Required	Recommended
CPU	Power PC	PowerPC G3
RAM	56 MB	64 MB

Service and Support

PREMIER Biosoft International is committed to providing the best service possible. As a part of our commitment we offer free, lifetime technical support to all **PRIMER PREMIER** users. Our highly rated technical support staff offers assistance by telephone, fax, or electronic mail.

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Index

A

Alignment Sequences, 9
Appendices, 46
Appendix A, 47
Appendix A, Formulas and Algorithms, 47
Appendix B, 50
Appendix B, Degenerate Code, 50
Appendix C, 51
Appendix C, Amino Acid Symbols, 51
Appendix D, 52
Appendix D, Software Limits, 52
Appendix E, 53
Appendix E, References, 53

C

Copyright, i

D

Database Window, 28
Degeneracy, 31

E

Edit Codon Table Window, 13
Enzyme Menu, 44

F

Filter Window, 35

G

GeneTank, 3
GeneTank Menu, 39
GeneTank Window, 3
Getting Started, 1
Graphs, 31

M

Menu Commands, 39
Motif Edit Window, 38
Motif Menu, 45
Multiplex/Nested Primer Window, 26

N

Navigational Basics, 1

O

Optical Activity, 30

P

Paste, 7
Paste Window, 7
Preferences Window, 7
Primer, 14
Primer Menu, 42
Primer Premier Window, 15

R

Rating Parameters, 8
Reports, 29
Restriction Enzyme Analysis, 33, 37
Restriction Enzyme Analysis Window, 33, 37
Restriction Enzyme Edit Window, 34

S

Search Criteria Window, 18
Search Parameter Windows, 19
Search Results Window, 26
Synthesis Order Form, 30
System Specifications, 54

T

Translation, 12