

OLIGO

PRIMER ANALYSIS SOFTWARE

Version 6
for Windows & Macintosh

Wojciech Rychlik

License #: _____

Molecular Biology Insights, Inc.
18-th Edition, September 2002.

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1 Information About OLIGO

1.1 Copyright, License Agreements, Warranty, Copy Protection, & Technical Support

OLIGO is a copyrighted software program. As an OLIGO user, you are obligated to abide by the following license agreement.

1.1.1 Copyright

The OLIGO software program copyright is owned by Wojciech Rychlik.

All copyrights are protected under the US Copyright Law and International Treaty provisions. Therefore, you may not copy or reproduce the software, its documentation, or other package components with the exceptions noted under the specific licenses.

By abiding by this contract, you agree not to reverse engineer, decompile, disassemble or otherwise attempt to discover the source code of the software.

1.1.2 Usage and Licenses

The data provided by this program is for research purposes only. Program data is not intended for commercial use or redistribution for commercial use and may not be used in this manner without written permission of Dr. Rychlik.

1.1.3 Single-user License

The single-user license is intended for one principal investigator's use in his/her laboratory or other research environment. The single-user license does not allow for multiple copies of the program on various hard drives, with the exception that the principal investigator

and his/her staff (such as post doctoral and graduate students assisting with the research) may use the program on up to 4 lab, office, or home computers. The standard license (W6SF02 or M6SF02) permits loading Oligo on 2 computers. Each additional computer license needs to be purchased separately.

The single-user license requires that the principal investigator or license holder register with an authorized distributor.

1.1.4 Multi-user License

This multi-user license permits the installation of this program on the computers of several principal investigators. Each principal investigator in the multi-user agreement receives his/her own single-user license and is obligated to abide by the terms of the single-user license for 1 computer.

The multi-user license requires that all principal investigators or their assigns register with an authorized distributor.

1.1.5 Network License

This network license permits the installation of this program on your facility's network, and its use simultaneously on the number of client computers (terminals) specified in the license contract.

The license does not permit the installation of the program on computers other than those stated.

The network license requires that the network administrator, or the users register with an authorized distributor.

1.1.6 License Transfer

The authorized distributor licenses software to a particular user. You may not rent, sublicense, or lend the software to another user. You may, however, transfer the full software license and documentation package to another user under certain circumstances. If the license is transferred, contact your distributor to update the license. This assumes that the software is removed from the original machines.

The user must make all reasonable efforts to see that employees, agents, or other persons under the direction of, or in collaboration with, the user abide by the terms of this agreement.

1.1.7 Limited Warranty

Molecular Biology Insights does not and cannot warrant the performance or results you obtain as a result of using the software or documentation. Molecular Biology Insights is not liable for any special, indirect, incidental, or consequential damages in any way relating to the use, or arising from the use, of this software or program disks.

MBI gives a 30-day money back guarantee. You may return this software within 30 days from the date of purchase for a full refund (minus the shipping and handling cost).

1.1.8 Governing Laws & General Provisions

This agreement is governed by the laws of the State of Minnesota, USA.

The user agrees that the software and documentation will not be shipped, transferred, or exported to any country or used in any manner prohibited by the United States Export Administration Act or any other export laws, restrictions, or regulations.

1.1.9 Copy Protection

OLIGO 6 customers must register their software license in order to use the program.

Note

The program will not run on your computer until it is registered with an authorized distributor.

The OLIGO 6 registration process is included with the installation procedures on a separate insert, which comes with this manual. Each computer on which OLIGO 6 is installed generates a unique workstation code.

Once the workstation code is generated, contact Molecular Biology Insights, Inc. or its affiliates to submit

the workstation code and receive the access code for your OLIGO program. Each computer has a separate access code, regardless of the type of license purchased.

1.1.10 Technical Support

Technical support is available to registered users. For the assistance, please contact your OLIGO distributor for technical support. When you request technical support, have the following information available, or include it on your fax or E-mail.

- Your name
- Name of the licensed end user, if different
- Institution
- OLIGO license number
- Phone and fax numbers

The software updates may be downloaded from MBI web page www.oligo.net. This service is free of charge.

1.2 Documentation Conventions

<Key> Characters enclosed by <> are keys on the keyboard. Examples are <shift>, <page up> and <return>.

Bold Text Text in bold is text that you enter using the keyboard. Press <RETURN> when you are finished. An example is "enter **1056**." Bold text is also used in headlines throughout the OLIGO Primer Analysis Software Manual.

"QuotationMarks" Text in quotation marks is text that is displayed on the monitor at the workstation. For example, "Searching complete" is a prompt and appears in quotation marks in the manual. Titles of windows and buttons also appear in quotation marks.

Italics Menus, command options and cautions appear in italics.

1.3

Essential Criteria for Optimal PCR Primers, Sequencing Primers, and Hybridization Probes

OLIGO is a multi-functional program that searches for and selects oligonucleotides from a sequence file for polymerase chain reaction (PCR), DNA sequencing, site-directed mutagenesis, and various hybridization applications. It calculates hybridization temperature and secondary structure of oligonucleotides based on the *nearest neighbor* thermodynamic values (Ref. 1, 2, 5).

There are three essential criteria for optimal PCR, sequencing primers, and for optimal hybridization probes.

1.3.1 Specificity

Primers and probes should be highly specific for the intended target sequence and not hybridize to other sites in the nucleic acid sample. Because sequencing conditions are typically not very stringent and the formation of *primer-template duplexes* with imperfect homology may be significant, specific primers are essential. PCR or sequencing results are generally poor (because of high background) if there is *base-pairing* between the primer 3' position and sites other than the intended target in the active sequence.

1.3.2 Free of Dimer and Hairpin Structures

Another important requisite of primers, but not hybridization probes, is that they do not form *dimers* and/or *hairpins* during reactions. Oligonucleotides that form dimers or hairpins function poorly in site-directed mutagenesis and in sequencing reactions, especially if double stranded DNA is used. Dimer and/or hairpin-forming primers are particularly troublesome when 3'-termini are tied up; this can cause internal primer extension, which eliminates the primer from the reaction and may contribute to *false priming*.

1.3.3 Form Stable Duplexes

Primers and probes should form stable duplexes with the active sequence under the intended conditions. GC-rich regions are more stable than AT-rich regions.

Generally, primers should not have 3'-ends that are too stable. This increases the chance of false priming. For more information on false priming, refer to *Chapter 7 — The OLIGO Analyze Menu*.

The OLIGO program is designed specifically to search for and list the position, sequence, and other data of all oligonucleotides (or primer pairs) that optimally meet these major requirements, plus several other criteria outlined in this manual.

1.4 Hardware and Software Configurations

1.4.1 Requirements

Table 1.4.1 Hardware & Software Requirements.

	Macintosh	PC (Windows)
Computer Type	Any 68030-based Computer & up, any Power Mac	486 and up
Operating System	System 7.5 or higher	Windows 95 and higher, Windows NT v. 4 and later
Disk space, RAM	8M each (min. 4M RAM)	8M each

1.4.2 Program Information

- Number of Disks = PC: two high density disks. Macintosh: CD or two disks.
- Acceptable Nucleic Acid Sequence File Formats = *Text (ASCII)*, *EMBL*, *GenBank*, *Entrez Flat Files* and files from most sequence analysis programs capable of generating plain text files.

1.4.3 Monochrome Monitors

OLIGO is designed to work on a color monitor. Throughout this manual, you may find references to particular on-screen colors used to identify information. If you are working on a monochrome screen, you will not see these color distinctions; however, the functions work the same.

1.5 Files Present With the OLIGO Program

Following is a list of the OLIGO files, stored by default in the OLIGO Folder.

1.5.1 Oligo 6 Program

The Mac Installer contains two OLIGO 6 versions — both versions work on Power Mac computers only — one is carbonized, native to OS X, and another Classic (for systems up to 9.2). To install, just drag only one OLIGO folder to your computer. Users with old Macintosh computers (non-Power Mac) should contact the authorized distributor to receive the special version.

1.5.2 Supporting Files

By default, all files for the OLIGO 6 program are located in the OLIGO Folder. These files include:

- OLIGO 6.x Program Icon — To startup the OLIGO program
- CBP.SEQ — An example of the OLIGO file format
- pCBlu.seq — Another example. This is a general cloning vector, very efficient, only 1842 b.p., developed at NBI.
- Mouse 4E.seq — Sequence file for demonstrating the consensus oligo search function (with CBP.SEQ)
- Rabbit 4E.seq — Sequence file for demonstrating the consensus oligo search function (with CBP.SEQ)
- CBP.AMI — An example of an oligo protein sequence file. This kind of file can be loaded instead of a DNA or RNA sequence file and is automatically reverse-translated into DNA.

Uni.Primers — An example of an oligonucleotide database file
Shfolder.dll — (Windows only) a Microsoft library
Oligo.hlp and Oligo.cnt — (Windows only) help files

Frequencies Folder — Tables containing oligonucleotide frequencies assembled from GenBank
FreqSeq Folder — A collection of repetitive sequences
Tables Folder — Files containing restriction enzymes information and codon usage tables
PrimeForm Folder — The Oligonucleotide Ordering Software

After Oligo start up the following files are added:

- Oligo code — registration information
- Saved Work (Macintosh Classic version only; in all other versions this file is outside Oligo folder, see 1.5.8.)
- Preferences (always outside Oligo folder, see 1.5.8.)

1.5.3 Frequencies Folder

These files are in the Frequencies folder. They are used to create the "Sequence Frequency" window, and in "Eliminate Frequent Oligos" sub-search for PCR primers. The list of oligonucleotide frequency table files, generated from Gen Bank is given below:

GBBCTBAC.FR6 - Frequency of 6-mers from *Bacillus subtilis* sequences.
GBBCTESC.FR6 - Frequency of 6-mers from *Escherichia coli* sequences.
GBINVCAE.FR6 - Frequency of 6-mers from *Caenorhabditis sp.* sequences.
GBINVCAE.FR7 - Frequency of 7-mers from *Caenorhabditis sp.* sequences.
GBINVDRO.FR6 - Frequency of 6-mers from *Drosophila sp.* sequences.
GBMAMBOS.FR6 - Frequency of 6-mers from *Bos sp.* (cow) sequences.
GBMAMORY.FR6 - Freq. of 6-mers from *Oryctolagus sp.* (rabbit) sequences.
GBPHG.FR6 - Frequency of 6-mers from bacteriophage sequences.
GBPLNARA.FR6 - Frequency of 6-mers from *Arabidopsis sp.* sequences.
GBPLNCHL.FR6 - Frequency of 6-mers from chloroplasts sequences.

GBPLNORY.FR6 - Frequency of 6-mers from *Oryza sp.* (oat) sequences.
 GBPLNSAC.FR6 - Freq. of 6-mers from *Saccharomyces cerevisiae* sequences.
 GBPLNSAC.FR7 - Freq. of 7-mers from *Saccharomyces cerevisiae* sequences.
 GBPLNZEAFR6 - Frequency of 6-mers from *Zea sp.* (corn) sequences.
 GBPRI.FR6 - Frequency of 6-mers from Primate sequences.
 GBPRI.FR7 - Frequency of 7-mers from Primate sequences.
 GBPRIHOM.FR6 - Frequency of 6-mers from *Homo sapiens* (human) sequences.
 GBPRIHOM.FR7 - Frequency of 7-mers from *Homo sapiens* (human) sequences.
 GBROD.FR6 - Frequency of 6-mers from Rodents sequences.
 GBROD.FR7 - Frequency of 7-mers from Rodents sequences.
 GBRODMUS.FR6 - Frequency of 6-mers from *Mus sp.* (mouse) sequences.
 GBRODRAT.FR6 - Frequency of 6-mers from *Rattus sp.* sequences.

1.5.4 FreqSeq Folder

These are organism-specific repetitive sequence files included in the OLIGO program. They are used to check against potential primers.

DROSFR.LST — A list of drosophila repetitive sequences
 DROSFR.SEQ — Drosophila repetitive sequences
 HUMANFR.LST — A list of human repetitive sequences
 HUMANFR.SEQ — Human repetitive sequences
 MOUSEFR.LST — A list of mouse repetitive sequences
 MOUSEFR.SEQ — Mouse repetitive sequences
 RATFR.LST — A list of rat repetitive sequences
 RATFR.SEQ — Rat repetitive sequences
 WHEATFR.LST — A list of wheat repetitive sequences
 WHEATFR.SEQ — Wheat repetitive sequences
 YEASTFR.LST — A list of yeast repetitive sequences
 YEASTFR.SEQ — Yeast repetitive sequences

1.5.5 Tables Folder

The Tables Folder includes the following files:

Codon Usage Tables — A codon usage table, an editable text file

NICE6&UP.ENZ — A restriction enzyme site database, of six-base and greater non-degenerate cutters

REBASE.ENZ — A restriction enzyme database from NCBI

4.ENZ — A restriction enzyme site database, of sites containing least 4 -cutters

5&UP.ENZ — A restriction enzyme site database, of sites containing at least five non-degenerate cutters

7&UP.ENZ — A restriction enzyme site database, of sites containing at least 7 -cutters

1.5.6 PRIMEFORM Folder

These are files used by the PRIMEFORM Oligonucleotide Ordering Software.

The PRIMEFORM Icon to start the PRIMEFORM program

Generic.inf — The ordering form template that can be customized for ordering synthesized oligonucleotides

1.5.7 Sounds Folder

This folder (Windows version only) contains sound files used in the Edit windows.

A through Z.WAV

1.5.8 Files Present Outside the Oligo Folder

Macintosh Classic Version: Oligo Preferences file is located in the System Folder/Preferences/

Macintosh Carbon Version: Oligo Preferences file, called com.mbinsights.OLIGO.plist, is located in the Users/ (registered user folder name)/ Library/ Preferences/;

Saved Work file is located in Users/(registered user folder name)/ Documents/

Windows Version: Location of SavedWork.wrk file is variable, but usually is located in the Windows\ My Documents\ folder.

Oligo Preferences file may be found by REGEDIT (Windows system application). The

path may be: \HKey_Current_User\ Software\
Molecular Biology Insights\ Oligo

1.6 **New Features and Enhancements**

Compared to the version 5, OLIGO 6 has several changes. The major enhancements are listed below.

1.6.1 Consensus Oligo Search

This feature allows researchers to select primers or probes which target multiple sequence files across given organisms or gene families. This function will facilitate selection of consensus primers, a feature which our diverse user base has indicated is of great importance to their research.

1.6.2 Elimination of frequent sequences (a new type of subsearch for primers)

A subsearch has been developed that avoids frequent sequences (found in different subsets of GenBank) at 3' ends of the primers. This improves the chances of finding a non-false priming primer.

1.6.3 Enhancements to the search for Primers & Probes protocol

The primer and probe search protocol allows the user to "lock" every parameters such as, T_m range or 3' stability ΔG settings, so that the automatically change stringency setting, which incrementally relaxes each parameter, is more controlled during a search. In addition, the user can choose to balance PCR Primers according to the priming efficiency number rather than T_m which has been shown to provide better amplification results. The priming efficiency algorithm has been slightly modified.

1.6.4 Save Your Work

The new *Save Your Work* feature allows each user within the laboratory to save every screen in the program

and return to the identical data at a later time. A related feature allows each user to save customized parameter settings for specialized applications.

1.6.5 OLIGO Back Up

Oligo version 5 did not allow for making back up copies of the registered program. This created problems for users who had hard disk crashes or automatic back up systems. Registered Oligo 6 software is possible to back up, so you don't need to ask us for a new access code each time when something wrong happens to your computer, but only you need to re-load the backed up copy. The procedure works if you back up the entire folder and not separate components. If there is a problem with a disk space, back up individual Oligo sub-folders to one disk(s) and the remainder to another disk (a hidden file, Oligo.code, containing the registration info. must be copied).

1.6.6 Improved features

The improved graphic features for the new version allow screens to be displayed in either a bar or a dot graph. Secondly, DNA primers may contain U residues, so that you can design oligos that can be digested with uracil hydroxylase. Thirdly, more information is displayed in certain windows, such as priming efficiency number in the Key Information, PCR window, and T_m in the database. Mutagenesis window (former single line edit) has a balloon help explaining amino acid symbols.

2

Getting Started

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2 Getting Started

This section covers the basics on getting started with OLIGO.

2.1 Basic Macintosh System Elements



The OLIGO program presumes you are familiar with the basic functions of *Macintosh OS* or *Windows* interface. If you are unfamiliar with *it*, please refer to your *System* manual. If you need a brief refresher of *System* elements, review this section.

2.1.1 Mouse Pointer/Cursor

In OLIGO, the mouse pointer or cursor is either a hand with a pointing finger, a cross-hair, a vertical bar, or an arrow.

When you are on the main menu bar, the hand becomes a pointer, or arrow. Position this arrow/marker directly on the object you want to select or activate.

2.1.2 Close Box

The close box is located in the upper-left corner (Mac, ) or upper-right corner (Win, ) of most OLIGO windows, next to the title bar. When you click on this box, you close the window.

2.1.3 Title Bar

The title bar displays the name of the window. The active window title bar is marked with horizontal lines. You can choose different types of window displays from the *Window* menu. You can move most windows by clicking on and dragging the title bar.

2.1.4 Menu Bar



The menu bar is located at the top of the screen. It lists the functions available in the application. To access a function, click and drag down with the mouse to select a particular suboption.

2.1.5 Scroll Bars

The scroll bars are the long gray strips along the bottom (horizontal scroll bar) and/or side (vertical scroll bar) of a window. They contain both scroll arrows and a scroll box.

You can move through a window or document by clicking and dragging the scroll box along the bar, clicking directly on the bar, or clicking on the arrows at either end of the bar.

2.1.6 Zoom / Minimize Box

This is the button in the upper-right corner of most of the windows. Click on the button  (Mac) to change the window size or  (Win) to minimize.

2.1.7 Size Box

To resize a window, point to the box in the lower-right corner and drag. If you click and drag this box, you can resize the window horizontally and vertically.

2.2

Installing the OLIGO Program

When you install the OLIGO program, you also install PRIMEFORM Oligonucleotide Ordering Software.

2.2.1.1 Installing OLIGO on Macintosh

To load the OLIGO program to your hard drive:

1. Insert the OLIGO 6 CD into CD ROM drive.
2. Drag only ONE of the two folders to your hard disk:
 - If you're using System 9 or below and not planning to use OS X, drag "Oligo 6 Classic" folder to your HD. This version is not carbonized.
 - If you're using System 9 or System X drag "Oligo 6 Carbon" folder. Oligo 6 Carbon will also work on

Mac OS 8 (or 9) provided that CarbonLib file is installed in the System Folder: Extensions. If you don't have CarbonLib installed, we recommend to use the Classic Oligo version.
Please follow the instructions provided on a separate sheet of paper (see also Chapter 2.3).

Note

After receiving Oligo access code from your vendor, enter it to the appropriate box and click on "Confirm Code" button to access the OLIGO program (you don't need to fill out all the remaining information again).

2.2.1.2 Installing OLIGO on Windows

To load the OLIGO program to your hard drive:

1. Insert the OLIGO 6 CD Disk into computer's CD drive.
2. Installer should start automatically. If, for any reason, it won't start, double click on the SETUP.EXE icon displayed in the CD drive window. A large window of the Installshield program opens and prompts you to click on the "Next" button.
3. Click "Next". Choose the destination folder. This installer asks you whether C:\Oligo 6\ is the acceptable path. If the proposed directory does not exist, click "Yes" button when prompted "Do you want the folder to be created?".
4. Click "Next". Installer will start copying the files.
5. Once the installation is complete, double click on the OLIGO icon to register your program (using the File Manager). Alternatively you can start Oligo by choosing "Start" from the desktop, followed by "Programs". You may create a short cut icon on the desktop by clicking on the application icon with the right mouse button and dragging the cursor to the desktop - when a small window pops up, choose the 'short cut' option. Please follow the instructions provided on a separate sheet of paper (see also Chapter 2.3).

Note

If you can't find Oligo in the Programs Menu, you may add it by choosing Start-Settings-Taskbar & Start Menu-Start Menu Programs-Add-(browse and click on Oligo.exe in the Oligo folder. After receiving Oligo access code from your vendor, enter it to the appropriate box and click on "Confirm Code" button to access the OLIGO program.

2.2.2 Troubleshooting the Installation

If installation fails, check to see if one of the following conditions is apply.

Computer Configuration

Make sure your system meets the requirements listed in *Section 1.4 — Hardware and Software Configurations*. If not, you may need to upgrade your system to run the OLIGO program.

Virus Detection Software

Sometimes virus detection software can interfere with installation programs. If you have virus detection software that affects the installation, temporarily remove or disable it and try installing the OLIGO program again. Refer to the manual for your particular software package for more information on this.

Disk Compression Software

If you are using disk compression software and have a small amount of available disk space, it could interfere with installation. Try to make more space available on your hard drive or disable the compression software before attempting to reinstall the OLIGO program.

Copy Protection

It is software-based. Registration process creates a hidden file named *Oligo.code*, in the OLIGO 6 Folder. Copying this file to another disk voids the registration. In case of a hard disk crash or similar problems, please contact your OLIGO distributor to get an access code to run the OLIGO program. If you create a back up Oligo copy (see p. 16) you may be able to restore it. If you change the computer you need to apply for a new access code by contacting the vendor. There is no cost for this reinstallation.

2.3

Registering as an OLIGO User

In order to protect your copy of the OLIGO program and provide you with prompt technical support, we need you to register as an OLIGO user with your distributor prior to accessing OLIGO or seeking technical support.

The registration form takes a few minutes to complete and you should receive your access code

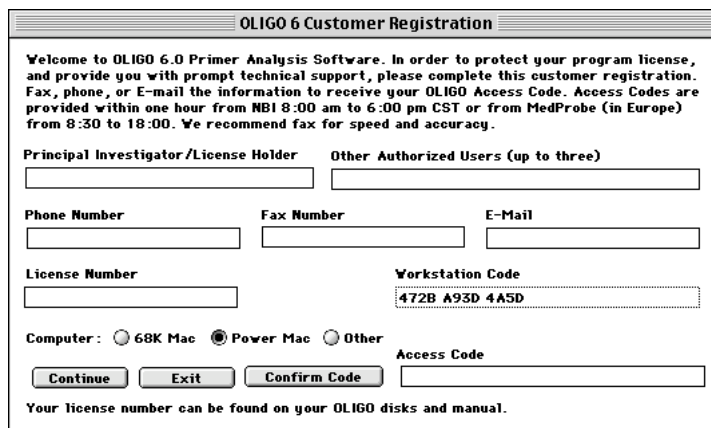
within an hour after contacting your distributor during normal business hours.

Because each computer creates a unique workstation code, you will need to register for each computer. You may want to install the program on each computer and complete the two registrations at once, if you hold a single-user license and are copying the program onto two computers. You don't need to fill out the entire registration twice. Just once is enough, and for the second computer we need its workstation code (plus the license number for identification purposes).

2.3.1 Single-user License Registration

To register your OLIGO program on each computer, follow these steps:

1. Double-click on the OLIGO icon to call up the registration procedures.
2. Once the "Customer Registration" window appears, complete each field. Use either the mouse or <Tab> to move to each field. The first window must be completed for each workstation you are registering (See *Figure 2.3.1.1 — The OLIGO Customer Registration window.*) The second window only needs to be completed the first time you register. (See *Figure 2.3.1.2 — The User Information window.*)



OLIGO 6 Customer Registration

Welcome to OLIGO 6.0 Primer Analysis Software. In order to protect your program license, and provide you with prompt technical support, please complete this customer registration. Fax, phone, or E-mail the information to receive your OLIGO Access Code. Access Codes are provided within one hour from NBI 8:00 am to 6:00 pm CST or from MedProbe (in Europe) from 8:30 to 18:00. We recommend fax for speed and accuracy.

Principal Investigator/License Holder Other Authorized Users (up to three)

Phone Number Fax Number E-Mail

License Number Workstation Code

Computer: ☐ 68K Mac ☒ Power Mac ☐ Other

Access Code

Your license number can be found on your OLIGO disks and manual.

Figure 2.3.1.1 The OLIGO Customer Registration window 1.

Figure 2.3.1.2 The OLIGO Customer Registration window 2.

Note

Your software license number is on each of the two OLIGO disks and is on the front page of this manual.

3. Print out the form and fax it to your OLIGO dealer. Faxing eliminates transcription errors, but you can register via phone or E-mail. With E-mail, you may need to wait a little longer for your access code.
4. To register second and third computers, you do not need to complete the second window each time.
5. Once your registration form is received, your OLIGO access code will be returned to you within an hour (normal business hours).
6. When you have the access code, enter it in the access code box at the bottom of the "Customer Registration" window.
7. Click "Confirm Code."

The OLIGO program is permanently unlocked and you can proceed by double-clicking on the OLIGO icon. To access the program from this screen, click on "Exit."

Note

If you ever need to re-load OLIGO, read page 16 or contact your software distributor for assistance.

2.3.2 Multi-user License Registration

Each OLIGO owner under a multi-user license must register following the instructions in 2.3.1 — *Single-user Registration*.

2.3.3 Network License Registration

Each user of network versions of OLIGO is required to register with your distributor. Specific registration information is provided with each network package. Distributor will not be able to inform you about the (free) updates without it.

2.4 The OLIGO Main Menu

If you do not load a nucleic acid sequence file, the main menu of OLIGO displays the menu bar only at the top of the screen until you load a sequence file or enter one using the *File — New* command. Once you load or enter a file, the OLIGO main screen appears. See *Section 2.5 — The OLIGO Default Screen* for more information.

To call up menu items on any of OLIGO's screens, pull down the menu using the mouse. You may also use the shortcut keys. The shortcut keys are displayed to the right of the command it represents in the menu.

As you use the OLIGO program, there are some features that are available at a given time and others that are not. OLIGO uses the standard *System* convention of "graying out" items that cannot be accessed on that particular screen or at that particular time. For information on when a menu item or option is available, refer to the individual item descriptions in this manual.

2.4.0 Main Menu — Application Name

The *Application Name* menu, located between the Apple and File menus, displayed only in the Mac running on OS X, contains the following items: About Oligo, Preferences, Hide Oligo, Hide Others, Show All, Quit Oligo. Most of these items are located also in the other menus described below.

2.4.1 Main Menu — File

The *File* menu includes options that relate to opening existing sequence files, creating new sequence files, working with oligonucleotide databases, saving data, printing, and exiting. In most cases, you will need to have a file open to perform functions listed under this and other menus.

2.4.2 Main Menu — Edit

You must have a sequence file loaded to use the *Edit* menu options. In most cases, OLIGO will prompt you to save your changes after editing.

2.4.3 Main Menu — Analyze

The *Analyze* menu contains the program options you select to analyze a single oligonucleotide, oligo groups, oligo lists, PCR, and other information pertaining to specific oligonucleotides and their uses.

2.4.4 Main Menu — Search

The *Search* menu provides multi-functional automated searches for primers and probes, hairpin loops, palindromes, and subsequences. It also provides a variety of ways to combine and manage search results.

2.4.5 Main Menu — Select

On the *Select* menu you may:

- Select Upper and/or Lower Primers (+ strand, - strand), for analysis
- Select a position on the active sequence
- Select the Memory Table to which data may be stored
- Select an oligo from a Memory Table

2.4.6 Main Menu — Change

The *Change* menu allows you to change oligo length, a DNA active sequence to an RNA active sequence (or RNA to DNA), search parameters and non-search parameters.

2.4.7 Main Menu — View

The *View* menu, Windows version only, lets you control Toolbar and Status Bar display.

2.4.8 Main Menu — Window

The *Window* menu in Windows version lets you arrange the windows and bring to front any opened window. In Macintosh, besides the features mentioned above, using the *Window* menu, you can copy graphics from any window to the "Clipboard" and then to any application that accepts graphics.

2.4.9 Main Menu — Help

This provides access to Baloon help (Macintosh) available for OLIGO. To activate/deactivate this feature choose *Show/Hide Baloons*. In the Windows version it provides help on various topics.

2.5 The OLIGO Default Screen

The OLIGO default screen consists of two windows, the "Melting Temperature" (T_m) window and the "Internal Stability" window. By default, the windows are displayed in a "bar" style, however the graphs may be displayed in a "dot" mode (use the *Options - Style* submenu to change it). In the Mac version this submenu is located just below the title bar of those windows, and in the Windows version you can pop-up this submenu by clicking the right mouse button.

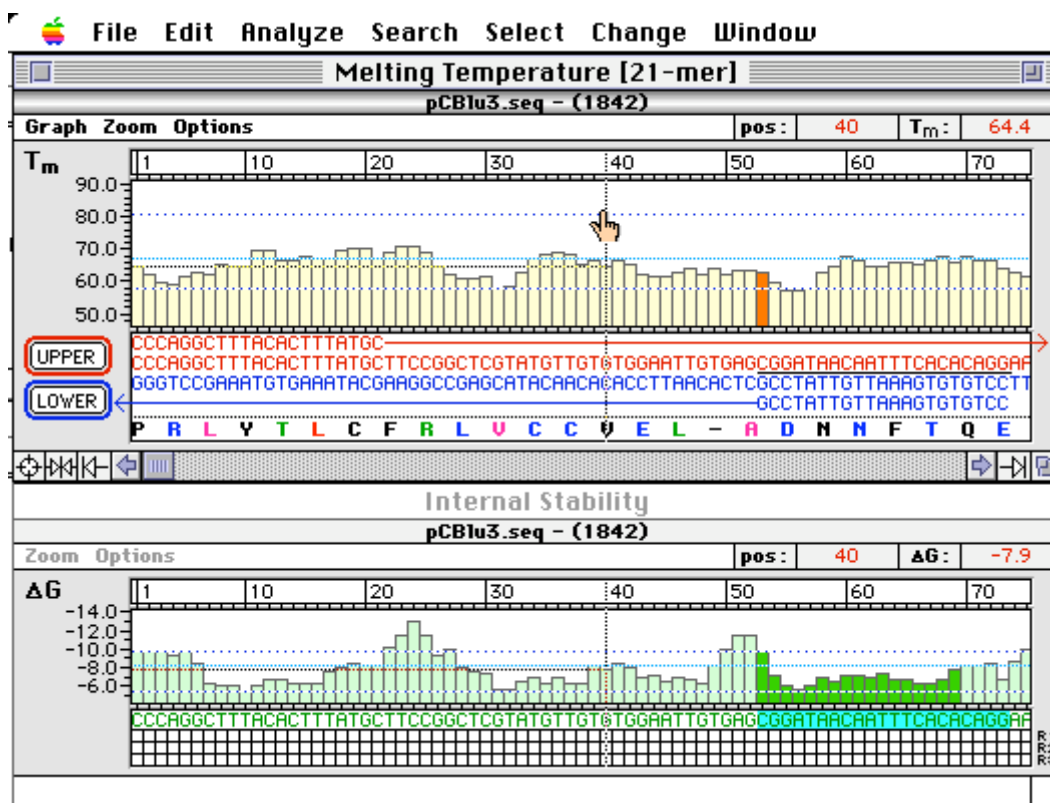


Figure 2.5 Macintosh: The OLIGO main screen. Bar graph display.

2.5.1 The Melting Temperature Window

The "Melting Temperature" (T_m) window is the core window of the OLIGO program. It is automatically displayed when a DNA or RNA sequence file is loaded, and includes the following items, functions, and capabilities in the default configuration:

- A close-up of approximately 90 nucleotides of the active sequence at the bottom of the window, both the positive strand (in red) and the negative strand (in blue). When a sequence is loaded, the (approximately; depending on the monitor resolution) 90 nucleotide 5'-terminus of the nucleic acid is displayed.
- A T_m graph of the displayed sequence segment, calculated using the nearest neighbor method. Each bar or point on the graph represents the T_m of an oligonucleotide, the 5'-terminus of which is located at that position. The T_m is calculated for an oligonucleotide length that is set in the *Current Oligo Length* command from the *Change* menu — the default is a 21-mer. The set oligo length is displayed on the window title bar.
- Cursor and cross hairs. The "Melting Temperature" window includes a mouse-driven "finger-pointer" cursor.
- The protein sequence translated from the DNA/RNA active sequence in the selected reading frame (set in *Options — Reading Frame*, T_m window sub-menu). The display of the protein sequence is optional and may be changed using the internal window sub-menu.
- The mean T_m of all the oligonucleotides of the selected length in the active sequence — the center horizontal dotted line on the graph. (Display is optional.)
- The Upper and Lower T_m selection limits as currently set for the searches — the upper and lower horizontal dotted lines on the graph. (Display is optional.)
- The Upper and Lower Primer selection buttons and other control icons for the "Melting Temperature" window.
- The *Current Oligo* — the oligonucleotide sequence underlined (Mac) or in a rectangle (Win) and in upper case letters within the active sequence. The positive and negative strand sequences are both part of the Current Oligo. The Current Oligo is always present in the

"Melting Temperature" window and advances across the *active sequence*.

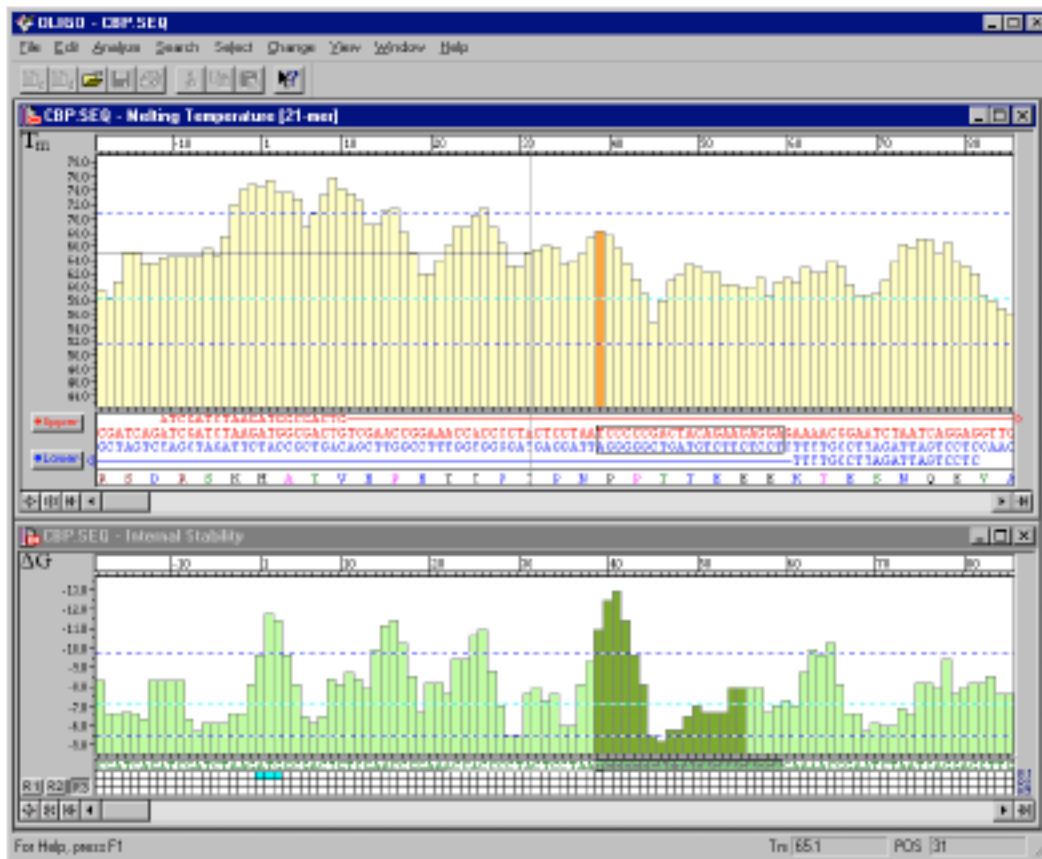


Figure 2.5.1 Windows: The OLIGO main screen. Bar graph display.

- The Upper and/or Lower Primer, when selected and located within the "Melting Temperature" window subsequence. The *Upper Primer* is displayed in red one line above the positive strand of the active sequence and includes a primer extension arrow. It is displayed in upper case letters except for nucleotides within the Upper Primer mismatched to the active sequence. These are displayed in lower case letters. The *Lower Primer* is displayed in blue one line below the negative strand of the active sequence and has the same characteristics as the Upper Primer.

2.5.2 The Internal Stability Window

The "Internal Stability" window is displayed below the "Melting Temperature" window on the main screen immediately after a sequence file is opened. Each position (either a black or red circle or different shade of green bars) on the Internal Stability graph represents the stability of a nucleotide pentamer, specifically, the ΔG values of the nucleotide directly above the position and the four nucleotides immediately downstream. The internal stability of the Current Oligo is represented by red circles or dark green bars.

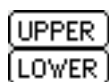
Internal stability can be used to determine an oligonucleotide's priming specificity. Oligos with a stable 5'-end and relatively unstable 3'-end typically perform best because they are stable and specific — resistant to false priming. For more information, see *Section 5.12 — Analyze — Internal Stability*.

2.6 OLIGO Icons and Definitions

There are several main screen OLIGO functions that are icon-selectable. Here is a list of all main screen icons.

2.6.1 Icons

The icons below are from the Mac version. The Windows version icons are almost identical.



The *upper and lower icons* save the Current Oligo as the Upper Primer (positive strand) and Lower Primer (negative strand), respectively. This *positioning icon* calls up a dialog box permitting the movement of the Current Oligo to a specified base position on the active sequence.

This *centering icon* centers the Current Oligo in the window.

This *5' icon* moves the Current Oligo to the 5'-end of the active sequence.



This *3'* icon moves the Current Oligo to the 3'-end of the active sequence — actually, the 5'-terminal nucleotide of the 3'-terminal oligonucleotide sequence in the file, (i.e. this position is 21 nucleotides upstream from the end of the active sequence, if the oligo length is set at 21 nt).



The *horizontal scroll bar* in the "Melting Temperature" window represents the entire length of the active sequence. Clicking and dragging the scroll bar box along the scroll bar moves the Current Oligo to an approximate position on the active sequence. Clicking to the left or right of the scroll bar box moves the Current Oligo one window length toward the 5'-end or 3'-end of the active sequence, respectively.



The *scroll bar arrows* shift the Current Oligo and the entire main window one nucleotide at a time in the designated direction.

2.6.2 Definitions Used in OLIGO

In addition to scientific terms, OLIGO has some program-specific language. Definitions of these terms are listed here. There is also a broader list of terms in *Appendix A — Glossary of Terms*.

2.6.2.1 Current Oligo

The *Current Oligo* is the oligonucleotide displayed in upper case letters and underlined (Mac) or surrounded by a rectangle (Win) in the "Melting Temperature" window on the main screen. The Current Oligo includes both the positive and negative strands and is updated with each position change on the active sequence. A Current Oligo may be saved as an Upper or Lower Primer, and/or analyzed using most options under the *Analyze* menu.

When browsing the active sequence for a new Current Oligo, you can open the "Current Oligo" window from the *Analyze-Key Info* menu. Click and drag on the "Current Oligo" window title bar to position the window in the most convenient part of the screen. The Current Oligo data is visible simultaneously with the "Melting Temperature" window and OLIGO instantly updates T_m , extinction coefficient (O.D.), and related data for each potential oligonucleotide.

2.6.2.2 ΔG

The oligonucleotide free energy, ΔG , is a measurement of nucleic acid duplex *stability*. A DNA duplex is increasingly stable as its ΔG decreases in value; it is typically a negative number. The ΔG , expressed in kcal/mol, depends on the nucleotide sequence, salt concentration, temperature, and other factors. Only the temperature can be modified within the program. Salt is fixed to 1M NaCl, except that it varies in the T_m calculations for hairpin loop. For equations, see *Appendix D*.

2.6.2.3 Dimer Length

Dimer is a fragment of double-stranded DNA. *Dimer length* refers to a string of contiguous base pairings between two strands of nucleic acid.

2.6.2.4 Duplex vs. Dimer

A *duplex* describes any hydrogen bonding between nucleotides, both intermolecular (*dimer*) and intramolecular (*hairpin*). A dimer is a duplex formed between two nucleic acid molecules.

2.6.2.5 Internal Stability

Internal stability refers to the stability of subsequences within an oligonucleotide. The "Internal Stability" window displays the ΔG of five base segments (pentamers). The stability of these pentamers is expressed in kcal/mol (ΔG). The internal stability of the 3'-end of an oligo can be used to predict its specificity in a PCR or sequencing reaction.

2.6.2.6 Loop ΔG

The hairpin *loop* ΔG value is the sum of the free energies of the stem (stabilizing) and the loop (destabilizing), see *Appendix D*. Oligonucleotides with hairpin loop ΔG more stable than the loop threshold value are rejected by the program's standard searches.

2.6.2.7 Lower Primer

The *Lower Primer* is a negative strand oligonucleotide (reverse) that has been selected for

analysis, displayed in blue on the "Melting Temperature" window. You can manually select the Lower Primer from the Current Oligo on an active sequence, enter it via the keyboard from the *Edit* menu, or select it from various position lists created by OLIGO searches, such as the PCR primer pairs table in a search for PCR primer pairs.

2.6.2.8 Memory Tables

Oligonucleotide position data are saved in one of the three *Memory Tables* — R1, R2, or R3. The position numbers in the Memory Tables are, typically, data from an OLIGO search or searches and are displayed in the "Memory Tables" window. The R1 table stores positive strand position data from an automated search. R2 stores negative strand position data. R3 stores data from other searches other than the *Primers and Probes* search when it is selected as the active table. You can also manually mark the nucleotides in the "Internal Stability" window by clicking on the grid and saving the data into a Memory Table. Marking the R3 table will not alter the last search for primers & probes result.

2.6.2.9 OD Unit

The quantity unit for nucleic acids (and other light-absorbing compounds). The *n* in nOD260 units is the amount of nucleic acid that would give absorption equal to *n* at 260nm wave length, if dissolved in a 1 ml low ionic strength buffer (pH 7.0) and measured in a cuvette with a path length of 1 cm. Approximately 30 OD260 is equal to 1 mg of single stranded DNA (depending on the length of the oligo and the sequence).

2.6.2.10 Priming Efficiency

The *priming efficiency* number is a formulation unique to the OLIGO program that quantifies the likelihood that a given oligonucleotide will prime at a given DNA site. The priming efficiency calculation is derived from an proprietary algorithm that considers nearest neighbor ΔG , duplexes, mismatches, bulge loops, and the distance of these elements from a primer's 3'-end. For additional information on P.E., see Chapters 5.5, 8.4.1.3, and 8.4.2.10.

2.6.2.11 Terminal Stability

Terminal stability is the free energy (ΔG) for an oligonucleotide's 3'-terminal pentamer and the adjacent pentamer. In OLIGO, the ΔG of both terminal 3'-pentamers of an oligonucleotide must be between the threshold values or the oligo is rejected from consideration. An oligo with a 3'-end that is too stable has a greater tendency to false prime. Too low a stability causes the primer to have a low priming efficiency. Usually a value of -8.6 (± 0.6) kcal/mol/terminal pentamer is optimal.

2.6.2.12 T_d

T_d is the temperature at which 50% of a nucleic acid probe is retained on a hybridization filter after five minutes of incubation in 1 M salt. A concentration of 100 pM oligo is used to calculate T_d by the nearest neighbor method. According to Ref. 4, $T_d = T_m - 7.6^\circ$. This value, however, has changed since the more correct initiation ΔS calculation became available (Ref. 5). OLIGO is uses an entropy initiation value of -15.1 entropy units, and T_d in this case is equal to T_m . Note that T_d may be used irrespective of salt and nucleic acid concentration (like the "2xAT+4xGC" and the %GC methods).

2.6.2.13 T_m

T_m is the temperature at which 50% of nucleic acid molecules are in duplex (and 50% denatured). For the default T_m calculations, 1 M salt (Na^+ or K^+ , neutral pH) and 100 pM nucleic acid concentrations are used in the OLIGO program to provide T_m values for oligonucleotides based on the nearest neighbor methods. Oligonucleotide T_m values calculated using the nearest neighbor method are approximately four times more accurate, than the "2xAT+4xGC" method, and approximately two times more accurate than the %GC method. T_m s of longer DNA duplexes, however, (> 50 bp) are calculated most accurately using the %GC method. Due to recently modified equations, T_m s calculated with OLIGO 6 (nearest neighbor and %GC methods) are more accurate than in the previous versions. (See *Appendix D* for equations.)

2.6.2.14 Upper Primer

The *Upper Primer* is a positive strand oligonucleotide (forward) that has been selected for analysis. You can manually select the Upper Primer from the Current Oligo on an active sequence, enter it via the keyboard from the *Edit* menu, or select it from the "Primer Pairs" window.

2.7 OLIGO Short Cut Keys

You can use OLIGO keyboard interface. Here is a listing of available accelerator keys in the OLIGO program and where these keys may be used.

2.7.1 File Menu

The following keys are available from the File menu:

Command	PC-Windows	Macintosh
New		⌘N
New Sequence	Ctrl-N	
New Database	Alt-N	
Open	Ctrl-O	⌘O
Close	Ctrl-W	
Print	Ctrl-P	⌘P
Quit (Oligo)		⌘Q
Save — Data	F2	⌘R
Save — Sequence	Ctrl-S	⌘S
Save — Your Work		⌘Y

2.7.2 Edit Menu

The following keys are available only from the Edit windows:

Command	PC-Windows	Macintosh
Upper Primer	Alt-U	
Lower Primer	Alt-L	
Entire Sequence	Alt-S	

2.7.3 Analyze Menu

The following key is available from the Analyze menu:

Command	PC-Windows	Macintosh
Key Info-Current Oligo	F4	
Key Info-Selected Primers	Shift-F4	
Duplex Formation-Curr. Oligo	Ctrl-D	
Comp. & Tm- Current Oligo	Alt-G	

2.7.4 Search Menu

The following key is available from the Analyze menu:

Command	PC-Windows	Macintosh
For Primers and Probes	F3	

2.7.5 Select Menu

The following keys are available from the Select menu:

Command	PC-Windows	Macintosh
Upper Primer	Ctrl-U	⌘U
Lower Primer	Ctrl-L	⌘D
New Current Oligo Position	F10	⌘K

2.7.6 Change Menu

The following key is available from the Change menu:

Command	PC-Windows	Macintosh
Current Oligo Length	F9	⌘L

2.7.7 Edit Submenu — Database

The following keys are available:

Command	PC-Windows	Macintosh
Cut	Ctrl-X	⌘X
Copy	Ctrl-C	⌘C
Paste	Ctrl-V	⌘V
Undo		⌘Z
Select All	Ctrl-A	⌘A

2.7.8 Edit Submenu — Edit

The following keys are available only from the Edit windows:

Command	PC-Windows	Macintosh
---------	------------	-----------

Cut		⌘X
Copy		⌘C
Paste		⌘V
Undo		⌘Z
Select All		⌘A
Accept	<enter>	<return>
Accept & Close	<shift-enter>	<enter>

2.7.9 Edit Submenu — Search

The following keys are available only from the Edit windows:

Command	PC-Windows	Macintosh
Find		⌘F
Find Next		⌘G

2.8 Help

If you use Mac *System 7.0* or higher, you may use Balloon Help. To display the help messages, select *Show Balloons* from the ? (or Help in System 8) menu on the upper right of your screen. To deactivate this feature, select *Hide Balloons* from the same menu. This help is cursor-sensitive and displays information about the item on which the cursor is pointing.

Windows has a standard help interface.

3

The OLIGO File Menu

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3 The OLIGO File Menu

The *File* menu is used to:

- Create new sequence files and databases
- Open DNA, RNA, and protein sequence files (templates) and databases
- Read Memory Tables
- Save and print
- Reset data and parameters
- Open recently closed sequence/database file
- Exit OLIGO

Note

You must open an existing file or create a new file before you can access most of the options from the Edit, Search, Select, and Analyze menus on the main menu bar.

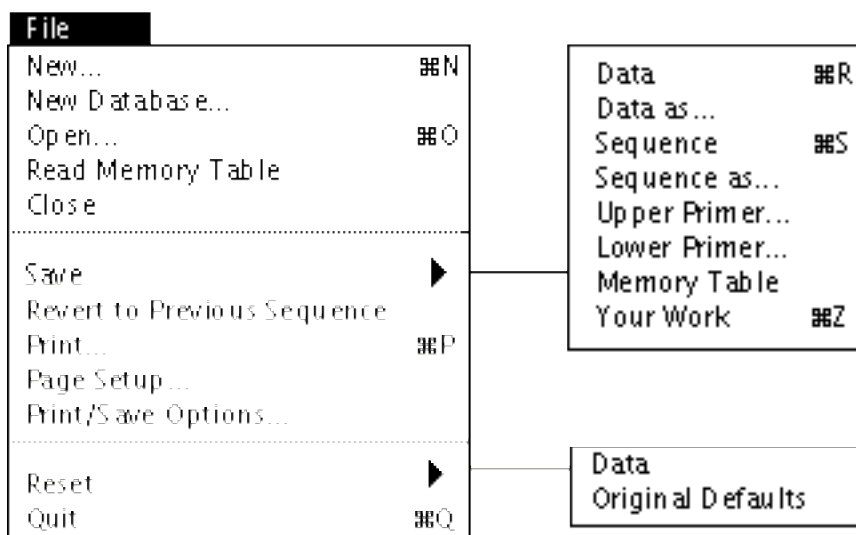


Figure 3.0 The OLIGO File menu, Mac OS 9 version.

3.1 File — New

The *File — New* (Mac) or *File — New Sequence* (Win) command opens the "New Sequence" window from the *Edit* menu and activates the text editor for

keyboard entry. Once a new active sequence is entered, you may accept,

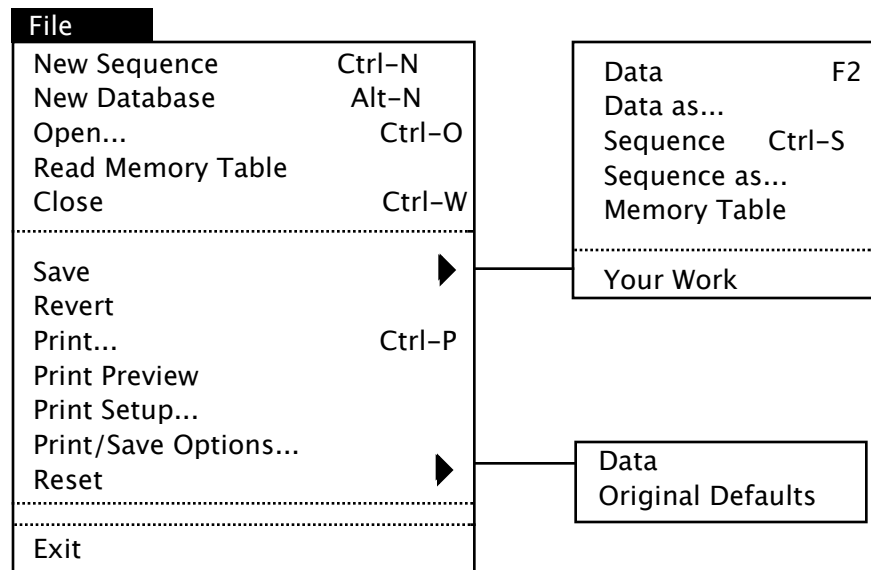


Figure 3.1 The OLIGO File menu, Win version.

discard, save, or edit it. You may accept a new sequence by choosing *Accept & Close* from the *Accept/Discard* menu, or you may save it to disk using the *Save* or *Save Sequence as* commands. In the "New Sequence" Window, most of the options in the *Edit* menu are available. These options are explained in *Chapter 4 — The OLIGO Edit Menu*.

3.2 File — New Database

The *File — New Database* command allows you to create and save new databases for storing oligonucleotides and related data.

Database									
Uni. Primers									
Link Edit Analyze Import Export									
Sorted									
Sort Sort Sort Sort Sort Sort Sort									
	#	Date	ID Number	Sequence	Ref.	3' dim. ΔG	P.E.	T _m	
1	13 Jul 1994	X02513:6309L19	GGTTTTCCAGTCACGACG	-40	-3.6	D	428/428	65.4	
2	13 Jul 1994	X02513:6290L17	GTAAAACGACGGCCAGT	-20 (univ)	-1.3	SC	402/402	57.8	
3	13 Jul 1994	X02513:6209U16	AACAGCTATGACCATG	reverse	-5.3	D	324/324	45.3	
4	13 Jul 1994	X52330:670U17	CGAGGTCGACGGTATCG	KS	-5.2	D	84/403	61.6	
5	13 Jul 1994	X52330:720L17	TCTAGAAGTATGGATC	SK	-4.7	D	31/312	40.2	
6	12 Aug 1994	X52330:774L17	ATTAACCCCTACTAAAG	T3 alt	-1.6	SC	336/336	44.9	
7	12 Aug 1994	X52330:633U21	ACTCACTATAGGGCGAATTGG	new univ...	-	SC	456/456	63.7	
8	12 Aug 1994	X52330:779L18	CGCGCAATTAACCCCTAC	new rev...	-	SC	441/441	64.4	
9	12 Aug 1994	X02514:492L18	GGAATTGTGAGCGGATAA	-36 rev	-1.9	SC	401/401	58.1	
10	12 Aug 1994	M77707:2855U17	ATTTAGGTGACACTATA	SP6	-3.5	SC	77/309	38.9	
11	12 Aug 1994	X53937:1540U18	CGCCAAGCTATTTAGGTG	SP6	-1.9	SC	45/412	58.9	
12	12 Aug 1994	X53937:1657L18	GTAAAACGACGGCCAGT	univ pEM	-1.9	SC	420/420	61.4	
13	12 Aug 1994	X53937:1529U18	GACCATGATTACGCCAAG	SP6	-	SC	400/400	58.2	
14	12 Aug 1994	X52330:669U17	TCGAGGTCGACGGTATC	KS	-1.6	D	158/384	57.8	
15	12 Aug 1994	X52330:772L20	AATTAACCCCTACTAAAGGG	T3	-7.8	D	414/414	58.2	
16	12 Aug 1994	X52330:625U22	GTAAACGACTCACTAAGGGC	T7	-3.1	D	426/426	58.4	
17	12 Aug 1994	X52330:808L19	GGAACAGCTATGACCATG	reverse	-5.3	D	387/387	57.6	
18	12 Aug 1994	X52330:720L20	CGCTCTAGAAGTATGGATC	SK	-4.7	D	31/395	56.2	

Figure 3.2 The Oligonucleotide Database window.

3.2.1 New Database — Database Fields

The fields displayed with each oligonucleotide record are described here. Each field can be sorted by clicking on the "Sort" button located above the fields. You may perform a secondary sort by pressing another "sort" button while holding the <option> key (Mac) or <Ctrl> key (Win).

N — New Entry

This field indicates whether an oligo record is new to the database since the database was last opened. A new oligo is marked by a red mark with an "N."

O — Order Form

This field, available from the *Export* menu, indicates whether an oligo record should be either printed or exported to the Order Form for synthesis or to the Memory Tables. Selected oligos are indicated by a blue mark with an "O". To select a record press a space bar.

— Number

This field indicates the record number of the oligonucleotide (record) in the database. As oligo records are deleted, the number of later entries move up accordingly, and are assigned smaller numbers.

You may have as many records as your disk space allows.

Date

This field lists the date that the oligo was downloaded to the database. The date is automatically assigned by the program using the computer's clock/calendar.

ID Number

The ID number is automatically created by OLIGO. It is a composite of the file name from which the oligo was imported, its position on the file, its strand (U = Upper, L = Lower), and its length in nt. If the file is from GenBank, the accession number is displayed rather than file name.

ID numbers are determined by the following, in order:

- The GenBank or EMBL accession number (GenBank/EMBL/ format file) or sequence name of the file from which the oligo was selected followed by a colon
- The 5' position number of the oligonucleotide on the active sequence (the 5'-end of the positive strand)
- The letter U or L to identify whether it is an Upper or Lower Primer
- The length of the oligonucleotide

Accession Number	5' Position Number	Upper/Lower Designation	Length of Oligo
L02459:	10200	U	25

Figure 3.2.1.1 An explanation of an oligonucleotide ID number L02459:10200U25 using the GenBank file format.

Sequence Name	5' Position Number	Upper/Lower Designation	Length of Oligo
LAMBDA:	10200	U	25

Figure 3.2.1.2 An example of an oligonucleotide ID number using the sequence name (non-standard file format).

Sequence

This field lists the oligonucleotide sequence, 5' to 3'.

Ref. — Reference

This is a field in which you can enter your initials, a literature reference, and/or synthesis specifications or other short notes you want tied to the record. You are able to view only a few characters in the "Reference" field in the "browse mode," but can enter/view all of the field (up to 256 characters) in the "edit" mode by double-clicking on the record or selecting *Edit — Modify*.

Comments

This field is available for extended notes; you have approximately six lines to enter extended notes. These are not displayed in the "browse" mode, but can be entered and/or viewed in the "edit" mode by double-clicking on the record.

3' ΔG

This field displays the 3' ΔG value of the oligonucleotide record and its dimer formation potential. Also, if the multiplex function in the database is selected, the field reports oligos cross-compatible to user-selected oligos plus the ΔG value of cross-compatibility.

The ΔG value of the 3' dimer formation is displayed on the left side of the field, while the oligo's dimer-forming propensity, either at the 3' end or overall, is displayed on the right side. "SC" indicates that oligo is unlikely to dimerize to itself (self-compatible), while a red "D" indicates that dimer formation (3' or overall) is likely (exceeds the current search parameter threshold).

Cross-compatibility for multiplex PCR assays can be identified in an oligo database by highlighting an oligo of interest and then selecting "Multiplex" from the "Analyze" database window menu.

The multiplexing analysis checks the selected oligo against all others in the database and displays the results in the "3' ΔG " field, of each oligo's dimer forming potential with the selected (multiplexed) oligo. In order

to select a multiplex oligo, it must first be free of self-dimerizing potential ("SC" is displayed), if not, relax "Acceptable 3' Dimer ΔG " or/and "Maximum Length of Acceptable Dimers" and "3'-terminal Nucleotides Checked for Dimers" search parameters.

After the multiplex analysis is run, oligos that are cross-compatible with the selected multiplex oligo display a blue "C" in the "3' ΔG " field, while a green "M" is displayed adjacent to the multiplex oligo itself. Oligos that are not cross-compatible continue to display either "SC" or "D".

The 3' ΔG values displayed following a multiplex analysis now report the ΔG of cross-dimerization between the selected multiplex oligo and all other oligos rather than self-dimerization ΔG . In order to identify 3, 4, or more oligos from a database suitable for a multiplex assay, however, you must continue the multiplex operation by clicking on oligos displaying "C" and converting them to "M." Once they display "M," they are multiply cross-compatible to all other oligos displaying "M."

Oligos not cross-compatible with each subsequent multiplex selection no longer display a blue "C" and revert to "SC".

After two or more multiplex oligos have been selected, the 3' ΔG value displayed for each oligo now reports the 3' cross-dimerization ΔG between that oligo and the most stable of all the selected multiplex ("M") oligos.

3' dimer ΔG values less stable (greater than) -1.0 (meaning no likelihood of dimerization) are displayed as a "dash" (-). Oligos can be removed from a given group of multiplex oligos by highlighting them and choosing *Deselect multiplex* under *Analyze* from the database window menu.

P.E. — Priming Efficiency

This field reports the priming efficiency check of either the selected oligonucleotide record if "Selected Oligonucleotides" is chosen from the Analyze , or all

oligos in the database if "All Oligonucleotides" is selected.

The priming efficiency check tests database oligos against every potential priming site on the active sequence — within set search ranges — and reports the highest priming number on the most stable site for each oligo.

After the highest priming number is listed, OLIGO provides the highest theoretical priming number, given a perfectly homologous target. Oligos with high priming efficiencies may be exported from the database as Upper and Lower Primers where they will be displayed on their most stable targets.

Priming efficiency checks of large oligo databases or groups of databases can be used to find previously synthesized primers that can be reused on other active sequences, particularly for sequencing.

T_m — Melting Temperature

This field reports the T_m of each oligonucleotide using the nearest neighbor method. The nucleic acid and concentrations used to calculate T_m can be found in the Non-Search Parameters window (Change Menu) of the linked sequence file.

3.2.2 (New) Database — Items

The menu items for the *Database* menu are described here. In Macintosh they appear as submenus attached to the database window, in Windows version the entire menu changes when the Database window is active.

Link

With this , you can associate a given database with any open sequence file. After linking, export, import, and priming efficiency functions are available.

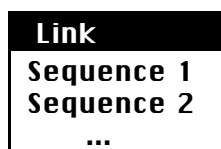


Figure 3.2.2.1 The Link menu.

Edit

The *Edit* allows you to modify, delete, and view oligonucleotide records in a full window format, rather than in the "browse" format.

Edit	Edit
Undo ⌘Z	Undo
Cut ⌘X	Cut Ctrl-:
Copy ⌘C	Copy Ctrl-c
Paste ⌘V	Paste Ctrl-v
Clear	Delete Del
Select All ⌘A	Select All Ctrl-A
Add	Add
Modify	Modify
	Renumber

Figure 3.2.2.2 The Edit submenus for Mac (left) and Windows.

Edit — Undo/Redo Modify

The *Undo* option is a toggle that enables you to undo/redo the last edit action. After changing a record, you may undo those changes by selecting *Undo Modify*. Similarly, you can *Undo* any other edit function such as *Cut* or *Clear*. After "undoing," you may "redo" a specific task.

Edit — Cut

The *Cut* option copies the selected record to the Clipboard and deletes it from your database.

Edit — Copy

The *Copy* option copies the selected record to the Clipboard. You may paste this record to another database at a later time.

Edit — Paste

The *Paste* option copies a database record from the Clipboard to the database. You need to use the *Cut* or *Copy* functions to activate this item.

Edit — Clear

The *Clear* or *Delete* (in Windows) command deletes the selected record from your database. You may use *Undo — Clear* to reverse this action.

Edit — Select All

This function selects/deselects all records for other edit functions. To select a single record, click on it. To select a group of continuous or non-continuous records, use a shift-click combination or a ⌘-click (Mac), Ctrl-click (Win) combination.

Edit — Add

This function allows you to add a new record to the database. This opens the same format window as in *Edit — Modify*.

Edit — Modify

This function allows you to change an existing record in the database.

The screenshot shows a window titled "Modify Record #12". It contains the following fields and controls:

- Date:** A date picker with a dropdown for the day (showing "12"), a dropdown for the month (showing "August"), and a text box for the year (showing "1994").
- ID Number:** A text box containing "X53937:1657L18". To its right, it says "Length: 18".
- Sequence:** A text box containing "GTAAACGACGCCAGTG". It is flanked by "5*" on the left and "3*" on the right.
- Reference:** A text box containing "Bartolomeetal.(1991)Gene102,75-78".
- Comments:** A text box containing "AlsoinPromegapGEM32andInvitrogenpCRII;T7-alt alternativeprimer('universal')".
- Buttons:** "Cancel" and "OK" buttons at the bottom.

Figure 3.2.2.3 The "Modify Record" window.

You can change the date, ID number, and sequence in the record. You can also add and edit references and comments that are tied to the record.

The edit database record windows also can be called up by double clicking on the database record.

In addition, standard editing features — *Cut*, *Copy*, and *Paste* are available in the *Edit — Modify Record* window.

Edit — Renumber

This function allows you to renumber existing records in the database. Useful after sorting. Available only in the Windows version.

Analyze — Priming Efficiency

The *Analyze — Priming Efficiency* option calculates the priming efficiency for the selected primer(s) or for all the

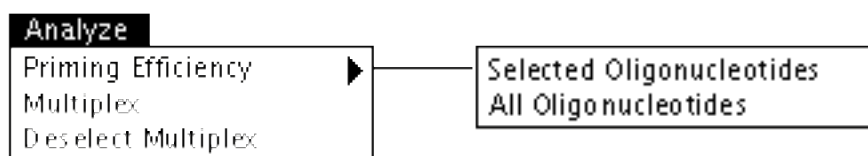


Figure 3.2.2. The "Analyze" Database submenu.

oligonucleotides in the database. Once the actual P.E. for the primer against a linked sequence, and the highest theoretical P.E. values are calculated, these values are displayed in the far right field in the oligonucleotide record. For example, if the P.E. of a given primer is equal to 104/403, this means that its priming efficiency on a sequence linked to this database, within selected search ranges for this sequence, is 104 and the maximal PE number for this oligo (perfect match) is 403.

Analyze — Multiplex

The *Analyze — Multiplex* option checks the selected record against other database records for multiplex PCR experiments. "M" appears in the "3 prime (3')" field to indicate this record is compatible with all the "C"-marked oligos in the database.

Following the multiplex check, primers with a "C" in the 3' column are cross-compatible with all "M" - marked primers. Primers with "SC" in this column following a multiplex check are not cross-compatible with every oligo from the multiplex group. The lowest ΔG value of the most stable 3'-end dimer for all multiplexed primers is given, as well. Note that the letter "C" means that the 3'-terminal duplex is less stable than the value set in the "Search Parameters" window and that a dimer of the length (or longer) specified in the "Search Parameters" window does not exist for a given pair (set) of primers.

Analyze — Deselect Multiplex

Multiplexed primers may be "un-multiplexed" by choosing the *Analyze — Deselect Multiplex* option. This will deselect the primer that has been multiplexed last, regardless of which oligonucleotide record is highlighted.

Import

Using the *Import* menu, you can add selected upper and lower primers, LCR oligos, and multiplexed primers as records to your database. To add a record, click on the primers or primer sets you want to add. OLIGO automatically adds the currently selected primer to the database.

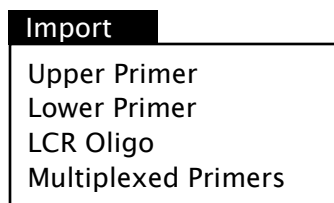


Figure 3.2.2.5 The *Import* menu.

Import — Upper Primer

If you have selected an upper primer in the OLIGO program, choose *Upper Primer* from the *Import* menu to add that upper primer as a record in the database. It is added at the end of all previously entered oligo records or at the lowest available empty record.

Import — Lower Primer

If you have selected a lower primer, choose *Lower Primer* from the *Import* menu to add that lower primer as a record in the database. It is added at the end of all previously entered oligo records or at the lowest available empty record.

Import — LCR Oligos

If you have designed and selected LCR oligos in the OLIGO program and have not closed that window, choose the *LCR Oligos* option from the *Import* menu to add the selected LCR oligos to the database.

Import — Multiplexed Primers

If you have selected multiplexed primers in the OLIGO program, or you would like to import all oligos found in the last search, select the *Multiplexed Primers* option from the *Import* menu to add these primers to the database. This button is active when either the R1 or R2 Memory Tables contain marked positions. Selecting the *Multiplexed Primers* option without opening the "Multiplexing" window transfers all the oligonucleotide sequences indicated by the marked positions in the R1 and R2 Memory Tables to the database.

Caution

The length of primers loaded into the database is equal to the length of the Current Oligo when this option is used.

Export

The Export menu options allow you to transfer database primers to the OLIGO program or to the PRIMEFORM Oligonucleotide Ordering Software to create an order form to send oligos to a synthesis facility.

Export
Upper Primer Lower Primer
Select Order Form Memory Table

Figure 3.2.2.6 The Export menu.

Export — Upper Primer

To transfer a primer from the database to other OLIGO windows as an upper primer, select the primer by clicking on that record in the database and then selecting *Upper Primer* from the *Export* menu. When you go back to the main menu of the OLIGO program, you can work with this primer in any of OLIGO's functions. When several sequence files are opened, make sure that the intended sequence file is linked to the Database.

Export — Lower Primer

To transfer a primer from the database to other OLIGO windows as a lower primer, select the primer by clicking on that record in the database and then selecting *Lower Primer* from the *Export* menu. When you go back to the main menu of the OLIGO program, you can work with this primer in any of OLIGO's functions. When several sequence files are opened, make sure that the intended sequence file is linked to the Database.

Export — Select/Deselect

To select a primer for export to the order form or memory tables, highlight the primer and choose *Select* from the *Export* menu. Selected primers are marked by a blue dot in the "O" field. To deselect primers to export to the order form, choose *Deselect* or highlight the record and press the space bar.

Export — Order Form

To transfer your primers to an order form, select the oligos to transfer using the *Select* function and then choose *Order Form*. For more information on using the order form, see *Chapter 10— PRIMEFORM Oligonucleotide Ordering Software*.

Export — Memory Table

To transfer your primers to a memory table, select the oligos to transfer using the *Select* option and

choose "Memory Table." The export function to the Memory Tables depends on the primer name that OLIGO assigns to each primer in the database. For example, a primer that has been assigned the name: "SEQNAME:27U21" is exported to the R1 Memory Table at position 27, while "SEQNAME:440L18" is exported to the R2 Memory Table and, because it is a lower primer, its 3'-end is at pos. 440.

3.3 File — Open

The *File — Open* command selects, by default, a nucleic acid sequence file from disk. By default, only OLIGO-generated files are displayed in the "File Open" dialog box, however, you may view all the files by unchecking the "Show OLIGO files only" box (Fig. 3.3.1). You may open also a protein file, memory table, oligonucleotide database, or previously saved work file by using *File type* menu from the dialog box.

3.3.1 File — Open - Nucleic Acid

This option selects DNA or RNA sequence files from disk. Sequence files that have acceptable file formats may originate from several sources including GenBank, EMBL, and other database files. Text files generated and saved without formatting in a word processing program, files generated from most DNA sequence analysis programs, and, of course, sequence files created in OLIGO, are also acceptable files.

Note

Sequence files from other software programs that do not open using this function may not be text files or may contain incompatible characters. Sequences interrupted with periods, semicolons, greater than and less than symbols, or some hidden word processing characters cannot be loaded.

To correct a file that will not open and you suspect has incompatible characters:

1. Remove the characters using any standard word processing package.
2. In the word processing package, save the file as plain text.

Choose any of the nucleic acid sequence files in the "File Open" dialog box by double clicking on the sequence file name, or by highlighting it with a single click and then clicking the "OK" button. The name of the selected sequence file is displayed on the title bar.

Fig. 3.3.1 shows the File-Open dialog in Macintosh. A different appearance has the Windows dialog (Fig. 3.3.4), but functionality is similar.

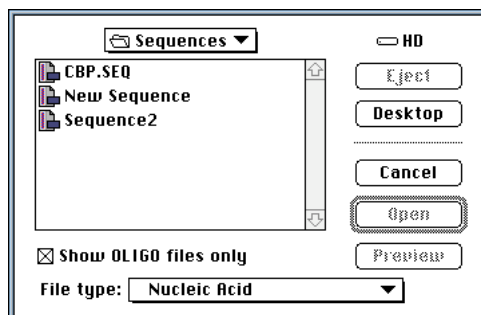


Figure 3.3.1 The File — Open dialog box. Open Nucleic Acid file was selected, Macintosh.

3.3.2 File — Open - Protein

Protein sequences of various text formats can be opened using this option. The sequence is automatically reverse-translated into DNA (it is important that the rev. translation method you wish to use is chosen using the Change-Rev. Translate Method before the protein sequence is loaded). Once the protein sequence is translated into DNA, OLIGO functions are the same as with any native nucleic acid sequence file.

3.3.3 File — Open - Memory Table

This command opens a memory table file, previously saved with *File-Save-Memory Table...* option, along with the appropriate nucleic acid sequence file, as opposed to *File-Read Memory Table* option, which just reads the memory table into an opened sequence.

3.3.4 File — Open - Oligonucleotide Database

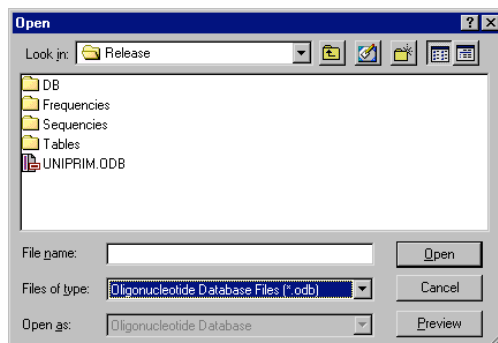


Figure 3.3.4 The File — Open dialog box. Open Oligonucleotide Database was selected, Windows.

You may store, import, export, edit, analyze, and manage oligonucleotides generated by OLIGO in one or more user-created oligonucleotide databases. The File — Open - Database command opens a previously saved database of choice. The "Oligonucleotide Database" window permits you to download and save Upper and Lower Primers, multiplexed primers, and LCR primers to the database. You can also transfer them from the database to the OLIGO program, or transfer them to PRIMEFORM Oligonucleotide Ordering Software.

The database stores oligonucleotide sequences, sequence data (such as length and name, user initials and comments), and the date the sequence was downloaded to the database. This data is automatically generated by OLIGO, with the exception of user initials and comments, which must be entered manually.

All functions of the database are described in detail in File- New Database chapter.

3.3.5 File — Open - Previously Saved Work File

This feature reconstructs all the sequences and windows that were displayed at the time of the last *File-Save-Your Work* command. If a sequence file has been modified after using the *File-Save Your Work* function,

then the previous sequence (before modifications) will be loaded.

3.4 File — Read Memory Table

The *File — Read Memory Table* command opens a file previously saved via the *Save Memory Table* command and loads its position data into the active Memory Table. The OLIGO Memory Tables (R1, R2, and R3) contain position data from OLIGO searches or positions that you have entered using the keyboard. (See Memory Table descriptions p. 208)

Note

Any position data in the active Memory Table is lost when you use this function unless you first copy it to another table (e.g. R1 to R3), or to a file.

3.5 File — Close

The *File — Close* command closes all windows associated with one, currently analyzed sequence or closes a database.

3.6 File — Save

The *File — Save* commands save a variety of data to disk.

3.6.1 File — Save — Data

The *Data* command can save data from any or all analysis windows. The *File — Print/Save Options* command calls up a dialog box where you can specify what window information is saved. Each subsequent window saved is appended to an open data file. After saving, you can open this file and edit or print its contents with any word processor, but not with OLIGO.

3.6.2 File — Save — Data As

The *Data As* command saves any open window or set of windows to a file with a new name that you are prompted to enter.

3.6.3 File — Save — Sequence

The *Sequence* command saves the active nucleic acid sequence to disk. You may use this function whenever you enter a new sequence via the *New* command or when you alter a sequence using the *Edit* function.

3.6.4 File — Save — Sequence As

The *Sequence As* command saves the active sequence under a new name. Using this command, you may modify a sequence and save it without altering the original file.

3.6.5 File — Save — Database

The *Database* command saves all changes to an open oligonucleotide database file. This option is displayed instead of Save-Sequence when a database window is activated.

3.6.6 File — Save — Database As

The *Database As* command saves all changes to an open oligonucleotide database under a new file name you enter. This option is displayed instead of *Save-Sequence As* when a database window is activated.

3.6.7 File — Save — Upper Primer

The *Upper Primer* command saves the Upper Primer sequence to disk. The primer sequence is saved in a plain text file.

If you intend to transfer primers to a PRIMEFORM Oligonucleotide Ordering Software order form, or use them for other purposes which do not involve direct transfer to a DNA synthesizer, it is more convenient to save the primers to an OLIGO oligonucleotide database.

3.6.8 File — Save — Lower Primer

The *Lower Primer* command saves the Lower Primer sequence to disk. The primer sequence is saved in a text file, which is compatible with most DNA synthesis instrumentation.

If you intend to transfer primers to a PRIMEFORM Oligonucleotide Ordering Software order form, or use them for other purposes which do not involve direct transfer to a DNA synthesizer, it is more convenient to save the primers to an OLIGO oligonucleotide database.

3.6.9 File — Save — Memory Table

The *Memory Table* command saves to disk all oligonucleotide positions stored in the current Memory Table. The current Table is indicated by a button in the Internal Stability window.

3.6.10 File — Save — Your Work

This option saves all the open sequences, windows and parameters, so that you may return to your saved work after either somebody else used OLIGO after you or the computer was shut off.

Note

By default, quitting OLIGO saves "Your Work" file automatically. This may be changed using the Change-Preferences menu.

3.7 File — Revert (to Previous Sequence/Database)

The *File —Revert* (Win) or *File —Revert to Previous Sequence/Database* (Mac) command changes the active sequence or database (if its window is active) back to its unaltered state.

3.8 File — Print...

The *File — Print* command generates a printout of an open window or group of windows as specified in the *Print/Save Options* dialog box.

To print Macintosh window graphics, use the *Take a Snapshot* command from the *Window* menu. See *Chapter 9.3, Window — Take a Snapshot*. To print graphics from PC-Windows version, copy the window contents to the Clipboard by pressing <Alt-Print Screen> key. Then print the Clipboard picture directly or paste the Clipboard contents into a document opened with another application and print from it.

3.9 File — Print Setup

The *File — Print Setup* command calls up a dialog box where you can change printer specifications and the printer port. Using this dialog box, you can also select the paper orientation and the resolution of printer graphics.

3.10 File — Print/Save Options

Print and/or save any OLIGO window(s) with the *File — Print/Save Options* command. These settings are effective every time you print, save or open the checked windows (*Analyze - All Checked*), until you change them.

In the dialog box, the column between the "Upper" and "Lower" columns applies to both the Upper and Lower Primers.

3.10.1 Print/Save Options — Selected Windows

When this selection is active, only windows that are checked in the "Print/Save Options" dialog box on the left of the screen are printed or saved.

3.10.2 Print/Save Options — All Open Windows

When this selection is active, all open windows — whether they are visible or not — are saved and/or printed.

3.10.3 Print/Save Options — Current Window

When the "Current" (window) option is selected, only the active window can be printed or saved. This option allows you to print any open window, including those that are not specified in this dialog box. The ⌘W key in Macintosh (*Analyze - All Checked*) is still functional when this option is activated. (*Analyze - All Checked* option opens all the analysis windows checked in this dialog.)

Print/Save Options

	Current	Upper	U/L	Lower
Oligo Info:	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Duplex Formation:	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
Hairpin Formation:	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Composition & T _m :	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
False Priming Sites:	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Internal Stability:	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Nucleic Acid:	<input type="checkbox"/>	LCR:	<input type="checkbox"/>
Protein:	<input type="checkbox"/>	Current Graph:	<input type="checkbox"/>
Selected Oligos:	<input type="checkbox"/>	Comp. PCR:	<input type="checkbox"/>
Primer Pairs:	<input type="checkbox"/>	Comp. Sequence:	<input type="checkbox"/>
Multiplex Primers:	<input type="checkbox"/>	Hybridization Time:	<input type="checkbox"/>
PCR:	<input checked="" type="checkbox"/>	Concentrations:	<input type="checkbox"/>
Frequencies:	<input type="checkbox"/>		

Memory Tables: ☐ 1 ☐ 2 ☐ 3

Primers & Probes Search Data: ☐

Restriction Sites: ☐

Restriction Sites in Protein: ☐

Primer Pairs: ☒ List Only ☐ Full Analysis

Database: ☒ List Only ☐ Full Analysis ☒ Selected Primers Only

OK Cancel

☒ Selected ☐ All Open ☐ Current

Figure 3.10.3 — The Print/Save Options window in Macintosh.



The saved file as well as the printout of the database records and the Primer Pairs window could be enhanced by checking the "Full Analysis" button.

3.11 File — Reset

There are two levels of reset in the OLIGO program.

3.11.1 Reset — Data

The *Data* command resets all user-generated results, calculations, and accumulated data generated since opening the active sequence. This erases all data in the Memory Tables, closes relevant windows, erases any selected primers, and resets the temperature for ΔG calculations to 25°. It does

not, however, change search parameters and other program parameters, except search ranges.

3.11.2 Reset — Original Defaults

The *Original Defaults* command changes all search parameters and other parameters back to the original program defaults. It does not reset data or close windows.

3.12

File — Quit OLIGO File — Exit

The *File — Quit OLIGO* (Mac) or *File — Exit* (Win) command quits the OLIGO program. By default, all open windows, sequences and parameters are automatically saved in "Your Work" file, so that you may go back to the same window arrangement as they were at the time you quit the program.

In the Mac OS X interface, the Quit Oligo item has been moved to the "Application" menu.



Figure 3.10.5 — The Application Menu in Mac OS X.

In Mac OS X a new default menu is added by the operating system, that is called the Application menu. It contains items that existed in other menus of OS 9 and below. About Oligo has been moved from the Apple menu, Preferences from the Change menu (see p. 164), Services (new system-added item, not functional in Oligo), interface options: Hide Oligo, Hide Others, and Show All, and the last item - Quit Oligo.

4

The OLIGO Edit Menu

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4

The OLIGO Edit Menu

The *Edit* menu on the main menu bar allows you to enter, edit, merge, translate, and back translate nucleic acid sequences.

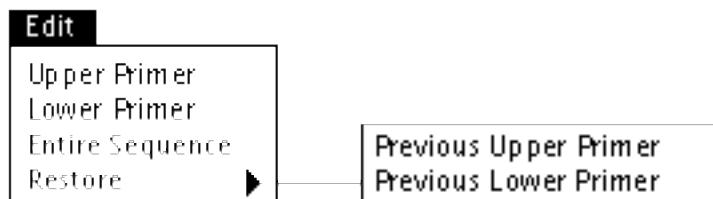


Figure 4.0 The Edit menu.

There are four commands under the *Edit* menu: *Upper Primer*, *Lower Primer*, *Entire Sequence*, and *Restore*. Within the first three options, most the editing features of OLIGO are available to you.

Full Screen and Mutagenesis Edit Modes

There are two Edit modes (screens). For editing the Upper and Lower Primer, the default is the mutagenesis mode; for the entire sequence it is the full screen edit mode. You may edit active sequence in either a mutagenesis or full screen edit window. You select the mode in which you wish to edit from the *Edit - Edit* submenu.

When you select *Edit-Upper Primer* or *Edit-Lower Primer* or *Edit-Entire Sequence* Oligo will change its main menu bar in the Windows version or, in the Macintosh version, the Edit window will have its own menu. The new items in the Main Windows menu and the Macintosh Edit submenu are described below. Although it is technically incorrect, for simplicity, the new items in the Windows version menu are called "Edit Submenu".

4.1

Edit Submenu— Accept/Discard

The *Accept/Discard* submenu is where you decide the disposition of your changes to the edited sequence.

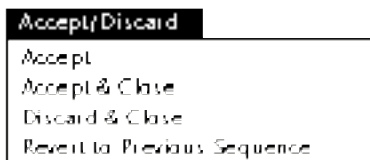


Figure 4.1 The Edit — Accept/Discard submenu.

4.1.1 Accept/Discard — Accept

The *Accept* command temporarily saves changes in oligonucleotide sequences made in the edit window, without closing the window. The new primer or sequence is saved only in RAM at this point. Sequences may be saved to disk only by using one of the save commands from the *File* menu. The short cut key is <enter> (PC) or <return> (Mac).

4.1.2 Accept/Discard — Accept & Close

The *Accept & Close* command accepts new changes to the sequence and exits from the "Edit" window to the main screen. The new primer or sequence is saved only in RAM at this point. Sequences may be saved to disk by using only one of the save sequence commands from the *File* menu. The short cut key is <shift-enter> (PC) or <enter> (Mac).

4.1.3 Accept/Discard — Discard & Close

The *Discard & Close* command discards any changes to the edited primer or sequence and closes the edit window.

4.1.4 Accept/Discard — Revert to Previous Sequence

This command reverts the edited Upper or Lower Primer to the sequence that was in the window prior to any editing.

4.2 Edit Submenu — Edit

All operations under the *Edit* command are performed on the base immediately to the left of the cursor. More than one base can be selected for editing by highlighting them as a group using the click and drag technique with the mouse. When at least three characters are displayed in the "Edit" window, all codon probabilities for a given amino acid (and a given organism) may be observed by positioning the cursor on any portion of the sequence or protein.

The edit functions in the "Edit" window are accessible from the *Edit* menu or from the icons listed here. Most of these functions are not accessible until at least one base has been entered and highlighted. These edit functions are standard Windows/Macintosh functions and perform in the same manner in the OLIGO program as they do in other Windows/Macintosh applications.

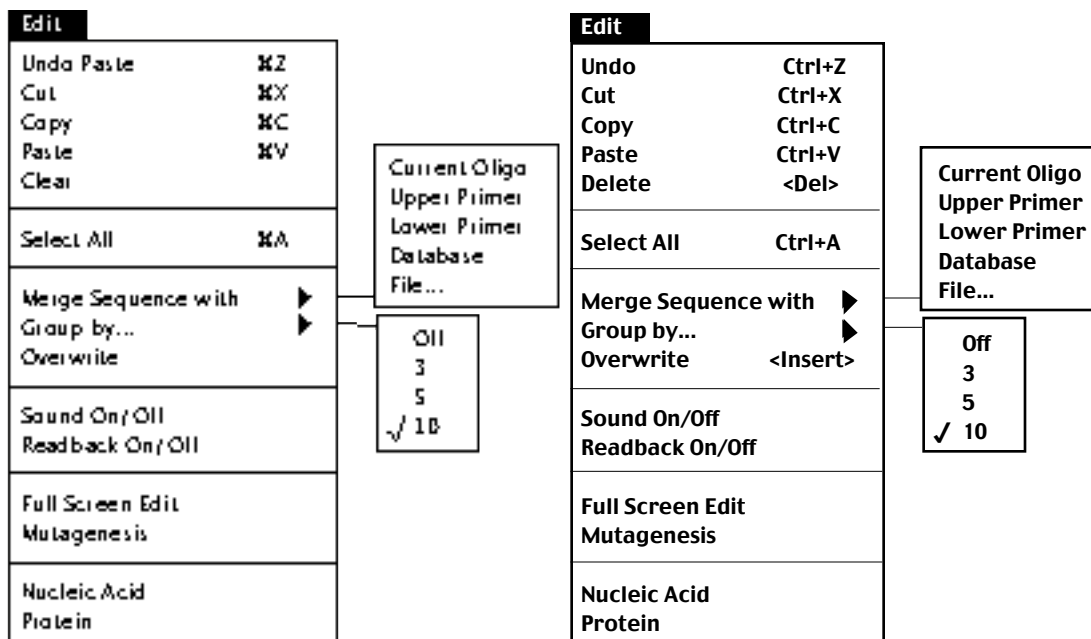


Figure 4.2 The Edit — Edit submenus. Macintosh (left) and Windows (right).

4.2.1 Edit — Undo/Redo



This icon (left Mac, right, Win) reverses the last single command, including *Clear*, if it is selected immediately after that command.

4.2.2 Edit — Cut



This icon removes any highlighted portion of text from the screen and places it in the Clipboard.

4.2.3 Edit — Copy



This icon places a copy of any highlighted text to the Clipboard where it can be pasted to the active cursor position.

4.2.4 Edit — Paste



This icon inserts all Clipboard contents at the current cursor position. It is also possible to paste a sequence that has been copied from another application.

4.2.5 Edit — Clear or Edit — Delete



This icon (Mac only) deletes any highlighted text. This text can be retrieved with the "Undo" function if "Undo" is selected immediately after the *Clear* command, or with *Revert*.

4.2.6 Edit — Select All

The *Select All* command highlights the entire sequence for cutting, copying, or clearing.

4.2.7 Edit — Merge Sequence With

The *Merge Sequence with* command merges the sequence from the Current Oligo, Upper Primer, Lower Primer, database, or a sequence file to the "Edit" window.

To load a protein sequence, open an empty edit window using *File — New*. Then position the cursor on the "protein edit line" (lower) while editing in the mutagenesis edit mode, or choose *Protein* from the *Edit* submenu. Then load the protein file using *Merge Sequence with* and selecting *File*.

Merge Sequence With — Current Oligo

The *Merge Sequence with — Current Oligo* merges the Current Oligo with an edited sequence at the current cursor position.

Merge Sequence With — Upper Primer

The *Merge Sequence with — Upper Primer* merges the Upper Primer with an edited sequence at the current cursor position.

Merge Sequence With — Lower Primer

The *Merge Sequence with — Lower Primer* merges the Lower Primer with an edited sequence at the current cursor position.

Merge Sequence With — Database

The *Merge Sequence with — Database* merges the highlighted database record into the active sequence at the cursor position.

Merge Sequence With — File

The *Merge Sequence with — File* merges a sequence file with the edited sequence at the cursor position. When the Upper or Lower Primer is merged with a file, the total length of the combined sequences cannot be longer than 200 nt.

This option calls up the "Merge From File" window where the desired DNA, RNA, or protein file is selected.

4.2.8 Edit — Group By

The *Group By* command changes the base grouping number in the "Full-Screen Edit" window to either three, five, or ten contiguous bases, or "off" — displaying a string that is not interrupted by spaces.

4.2.9 Edit — Overwrite/Insert

The *Overwrite/Insert* command toggles between the two editing modes. In the "Overwrite" mode, typed characters replace the old. In the "Insert" mode, newly typed characters are inserted at the cursor position.

4.2.10 Edit — Sound On/Off



The *Sound On/Sound Off* command activates/deactivates sequence read-out.

4.2.11 Edit — Readback On/Off



The *Readback* command "reads back" the key presses you make while entering a new sequence (Mac only; this function is available under Windows but only from the edit menu).

4.2.12 Edit — Full Screen Edit

The *Full Screen Edit* command displays a primer or the current sequence in a full screen format for viewing or editing. Either nucleic acid or protein sequences can be edited here.

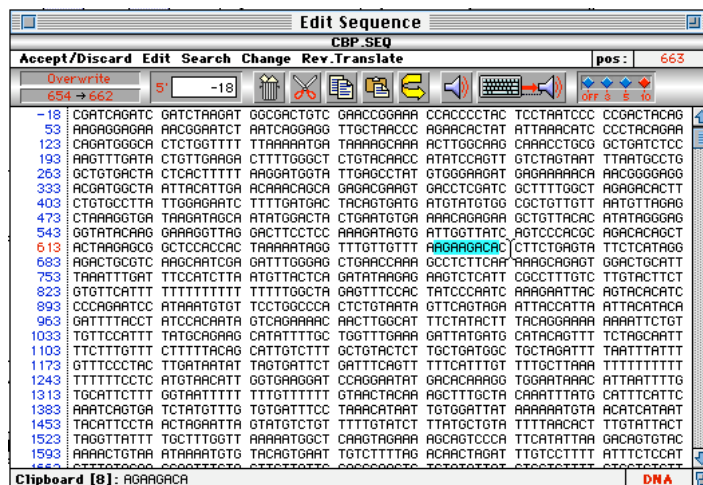


Figure 4.2.12.1 Full Screen Edit window.

The Toolbar functions are listed in Fig. 4.2.12.2. Beside the Toolbar information, the full screen edit window displays:

- The edited sequence with the first nucleotide in each line numbered
- Contents of the Clipboard
- The type of the edited sequence (DNA, RNA, Protein)

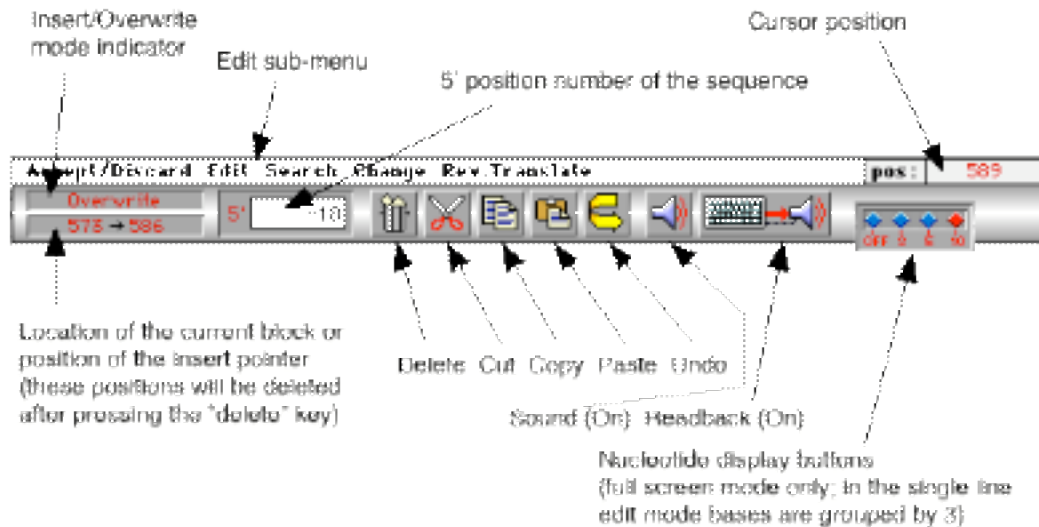


Figure 4.2.12.2 Functions of the Edit windows Toolbar.

4.2.13 Edit — Mutagenesis

The *Mutagenesis* command displays a single line of 50-100+ bases at a time for viewing or editing, depending on the width of the "Edit" window. You can switch from a nucleic acid sequence to a protein sequence in this edit mode by re-positioning the cursor. This window also displays the most stable hairpin, codon probabilities, and several other characteristics of the displayed sequence.

Beside the Toolbar information, single line edit information includes:

- Sequence Length
- Active Reading Frame
- Degeneracy

- T_m
- ΔG
- Loop T_m
- Loop ΔG
- Reverse Translation Method
- Codon Frequency for a Given Organism (the sum of all 64 codons is equal to 1,000)
- Nucleotide Sequence with Position Numbers
- Translated Protein Sequence
- The Strongest Hairpin Loop of the Displayed Sequence
- Contents and size of the Clipboard — the sequence that can be inserted with the "Paste" command

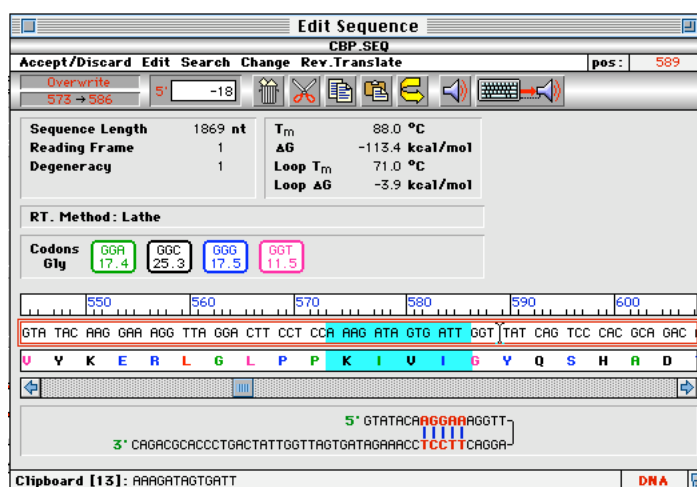


Figure 4.2.13.1 Mutagenesis Edit window.

The various colors of residues in the amino acid sequence represent the codon frequencies of each residue according to the codon table selected in the *Change* submenu. The following six colors designate the relative codon frequencies in OLIGO:

- Black — Most frequent codon for the selected organism
- Blue — Second most frequent codon for the selected organism
- Green — Third most frequent codon for the selected organism
- Pink — Fourth most frequent codon for the selected organism
- Brown — Fifth most frequent codon for the selected organism
- Red — The least frequent Leu, Arg, or Ser codon for the selected organism

4.2.14 Edit — Nucleic Acid

In the "Full Screen Edit" window, the *Nucleic Acid* command activates the DNA/RNA sequence editor.

4.2.15 Edit — Protein

In the "Full Screen Edit" window, the *Protein* command activates the protein sequence editor.

As a protein sequence is edited its adjoining nucleic acid sequence is modified according to the current reverse translation method; however, this is not displayed in the protein sequence editor in the "Full Screen Edit" window, but only in the Mutagenesis window.

4.3

Edit Submenu — Search

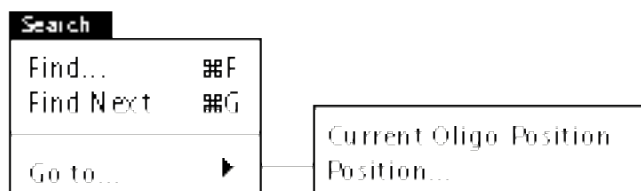


Figure 4.3 The Edit - Search submenu.

4.3.1 Search — Find

The *Find* command searches for a sequence string, entered from the keyboard, on the sequence displayed in the window.

The first occurrence of the sequence is highlighted. Use the *Find Next* command to search for the next occurrence.

4.3.2 Search — Find Next

This command repeats the last search for a sequence string you conducted.

4.3.3 Search — Go to

The *Go to* command moves you to the Current Oligo position or prompts you for a new position number in the active sequence and moves to the position you enter by base number.

Go to — Current Oligo Position

Use this option to automatically move to the "Current Oligo Position."

Go to — Position . . .

Use this option to enter a base number to which you want to move.

4.4 Edit Submenu — Change

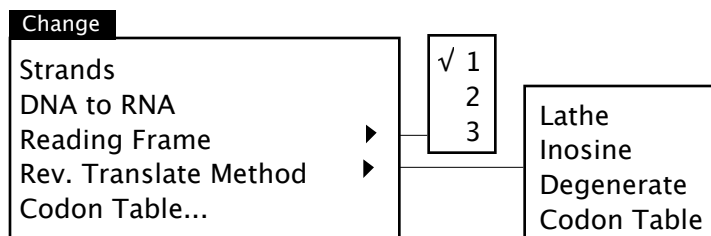


Figure 4.4 The Edit - Change submenu.

4.4.1 Change — Strands

The *Strands* command creates and displays the complementary strand of the edited sequence.

4.4.2 Change — DNA to RNA

The *DNA to RNA* command translates a DNA sequence to RNA and vice versa. The menu display toggles back and forth between DNA and RNA.

4.4.3 Change — Reading Frame

The *Reading Frame* command changes to the first, second, or third Translation Reading Frame.

Reading Frame — 1

Reading Frame 1 begins with base one and the reading frame includes bases one, two, and three.

Reading Frame — 2

Reading Frame 2 begins with base two and the reading frame includes bases two, three, and four.

Reading Frame — 3

Reading Frame 3 begins with base three and the reading frame includes bases three, four, and five.

4.4.4 Change — Rev. Translate Method

Lathe

The Lathe method is based on the most probable codons in mammalian mRNA. (Ref. 14.)

Inosine

When the Inosine method is selected, an inosine (universal substitute nucleotide) is inserted at the third base position in certain degenerate codons. (Ref. 11.)

Degenerate

This method gives all possible oligonucleotide combinations using the standard biochemical symbols for degenerate codons — N, Y, R, etc.; see Appendix D, Table 7. (Ref. 12.)

Codon Table

This back translation method creates a non-degenerate guess-mer sequence from the most frequent codons for a given organism. This is the default reverse translation method used in OLIGO.

OLIGO includes these codon tables:

- | | |
|----------------------------|--------------------------|
| • Arabidopsis | • Pseudomonas |
| • Bacillus | • Rabbit |
| • Barley | • Rat |
| • Caenorhabditis elegans | • Rhizobium |
| • Chicken | • Rice chloroplast |
| • Clostridium | • Salmonella typhimurium |
| • Cow | • Soybean |
| • Distyostelium discoideum | • Staphylococcus |
| • Drosophila | • Streptomyces |
| • E. coli | • Tobacco |
| • HIV | • Tomato |
| • Human | • Trout |
| • Influenza virus A | • Vaccinia virus |
| • Mouse | • Wheat |
| • Neurospora crossa | • Wheat chloroplast |
| • Pea | • Xenopus laevis |
| • Pig | • Yeast (S. cerevisiae) |
| • Plasmodium | |

4.4.5 Change — Codon Table

The *Codon Table* command selects the codon frequency table for a given organism. To add a new codon frequency table to the list, modify the codon table file "Codon Usage Tables."

There are four lines in each codon table file:

1. The name of the organism
2. The codon frequencies for that organism as described in *Nucleic Acid Research* (Ref. 18.)
3. The codons listed in order of frequency in the organism
4. The amino acids

A time-saving way to add a new user-specified codon table is to copy the last codon table in a file, and then change the organism name and codon frequencies.

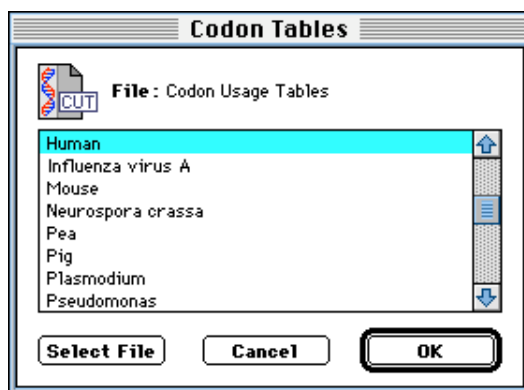


Figure 4.4.5 The Codon Tables window.

To add or modify a codon table, open "Codon Usage Tables" file with a word processor, copy and paste (duplicate) the last record, and change one of them. The recent codon tables can be found on a web page:

<http://www.dna.affrc.go.jp/~nakamura/CUTG.html>

Note

When you change the codon table, the reverse translation method automatically sets to "Codon Table".

4.5

Edit Submenu — Reverse Translate



Figure 4.5 The Edit - Rev. Translate submenu.

Caution

Reverse translation is automatic when you load or enter any protein (by the last method set). If you reverse translate an amino acid sequence when a degenerate nucleic acid sequence is displayed, you may create a "nonsense" nucleic acid sequence.

4.5.1 Reverse Translate — Entire Sequence

The *Entire Sequence* command reverse translates the entire current protein sequence into DNA/RNA according to the active reverse translation method.

4.5.2 Reverse Translate — Selected Block

The *Selected Block* command reverse translates only the selected block of amino acids into DNA/RNA, according to the active reverse translation method.

4.6 Edit — Upper Primer

The *Edit — Upper Primer* command opens the editing window for the keyboard entry or editing of the Upper Primer. Positive strand oligonucleotides, including those not on the active file, are entered or modified here, translated, and selected for analysis.

To choose an Upper Primer on the positive strand of the active sequence, use one of the following methods:

- Choose it manually from the *Select* menu.
- Choose it by clicking on the **UPPER** button in the "Melting Temperature" window.
- Enter the Upper Primer in the Edit screen.
- Select an Upper & Lower Primer pair from the "Primer Pairs" window.
- Select a Current Oligo (usually a sequencing primer or hybridization probe) from the "Selected Oligos" window, and press ⌘U (Mac) or <Ctrl-U> (Win) to choose the Upper Primer.

4.7 Edit — Lower Primer

The *Edit — Lower Primer* command opens the editing window for the keyboard entry or editing of the Lower Primer. Negative strand oligonucleotides, including those not from the active file, are entered or modified here, translated, and selected for analysis.

To choose a Lower Primer on the negative strand of the active sequence, use one of the following methods:

- Choose it manually from the *Select* menu.
- Choose it by clicking on the **LOWER** button in the "Melting Temperature" window.
- Enter the Lower Primer in the Edit screen.
- Select an Upper & Lower Primer pair from the "Primer Pairs" window.
- Select a Current Oligo (usually a sequencing primer or hybridization probe) from the "Selected Oligos" window, and press ⌘L (Mac) or <Ctrl-L> (Win) to choose the Lower Primer.

4.8 Edit — Entire Sequence

The *Edit — Entire Sequence* command opens the editing window for editing the entire active sequence. After you choose the *Entire Sequence* command, the active sequence is displayed in the "Full Screen Edit" window where individual nucleotides or strings may be modified. You may, alternatively, choose the "Mutagenesis Edit" window to obtain better control over translation, or to view the secondary structure.

While in the "edit" mode, you can save modifications to the entire sequence using the *Accept* or *Accept & close* commands.

You can also use the *Save Sequence As* command from the *File* menu. Use the *Save Sequence As* command here unless you want to overwrite the sequence as it was prior to your changes. Use the *New* function from the *File* menu to enter a completely new sequence.

4.9 Edit — Restore

This command restores any Upper or Lower Primer that may have been overwritten using one of the following program functions:

- Selecting either *Upper Primer* or *Lower Primer* from the *Select* menu
- Editing in any of the windows
- Conducting an automatic search

4.9.1 Restore — Previous Upper Primer

This command restores the previous Upper Primer — the one that was selected just prior to the currently displayed sequence.

4.9.2 Restore — Previous Lower Primer

This command restores the previous Lower Primer — the one that was selected just prior to the currently displayed sequence.

5

The OLIGO Analyze Menu

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5 The OLIGO Analyze Menu

The *Analyze* menu allows you to perform analyses of oligonucleotide primers and probes, the active sequence(s), PCR products, and primer specific experimental conditions.

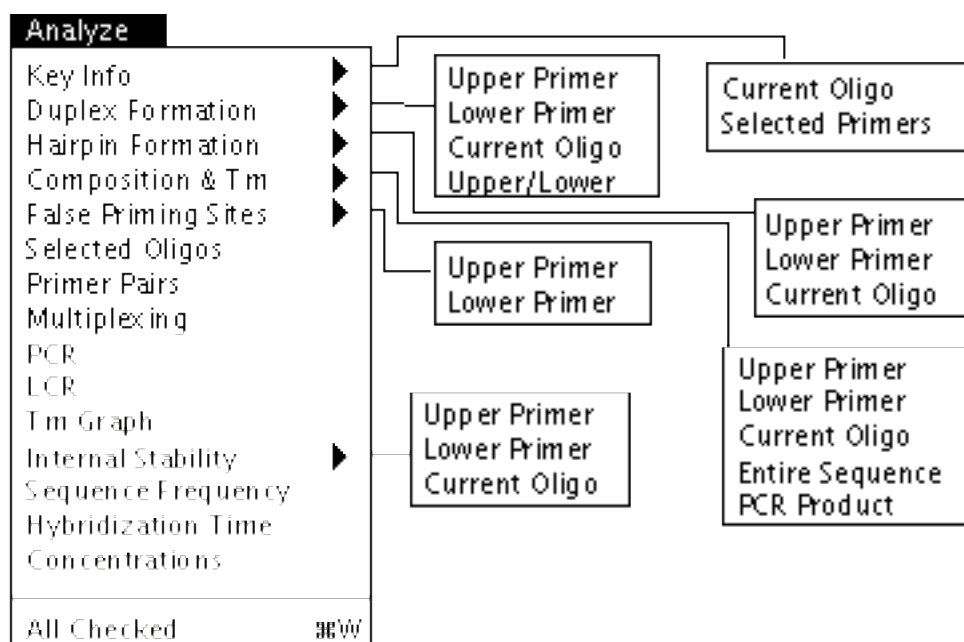


Figure 5.0 The Analyze menu.

5.1 Analyze — Key Info

5.1.1 Key Info — Current Oligo

This option calls up the "Current Oligo" window that displays the following Current Oligo calculations and specifications:

- The length of the active sequence
- The positive strand sequence of the Current Oligo, displayed from the 5' to the 3' end
- The Current Oligo length

- The 5' position of the Current Oligo positive strand and the 3' position of its negative strand complement
- The Current Oligo's T_m calculated by the nearest neighbor method using the salt and nucleic acid concentrations displayed in the "Non-Search Parameters" dialog box (*Change* menu)
- The ΔG (free energy) calculated using temperature displayed in the "Non-Search Parameters" dialog box (*Change* menu)
- The degeneracy number for the Current Oligo
- The Priming Efficiency number of the positive strand oligo (P.E. #; for the explanation see section 5.5)
- The extinction coefficient properties of the positive strand Current Oligo, expressed in nmol/O.D. at 260 nm and in $\mu\text{g/O.D.}$ at 260 nm
- The negative strand sequence of the Current Oligo
- The priming efficiency and extinction properties of the negative strand Current Oligo

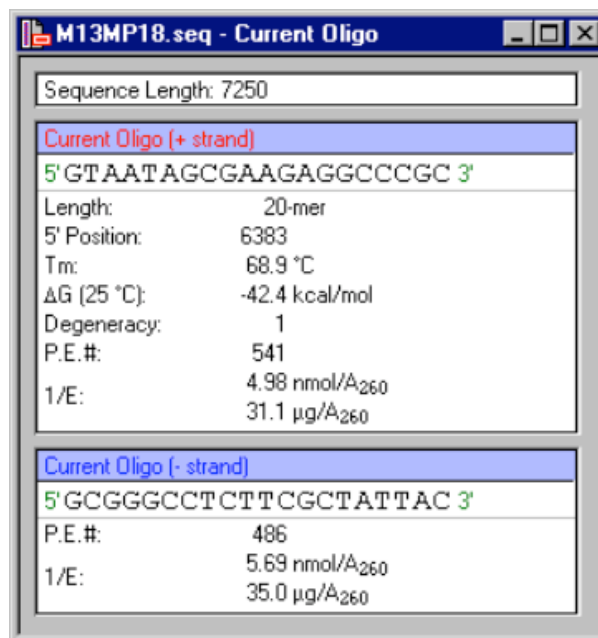


Figure 5.1.1 The Current Oligo window (Win).

When analyzing Current Oligos on the active sequence, it may be helpful to get instant data updates from the "Current Oligo" window as you change position. Click and drag the "Current Oligo" window title

bar to position the window below the "Melting Temperature" window on the screen so that the Current Oligo can be changed without obscuring the contents of the "Current Oligo" window.

"Current Oligo" window data saved in a file also include the salt and nucleic acid concentrations used to calculate Current Oligo T_m .

5.1.2 Key Info — Selected Primers

The *Selected Primers* command calls up the "Selected Primers" window that displays the following calculations and specifications of the selected Upper and Lower Primers:

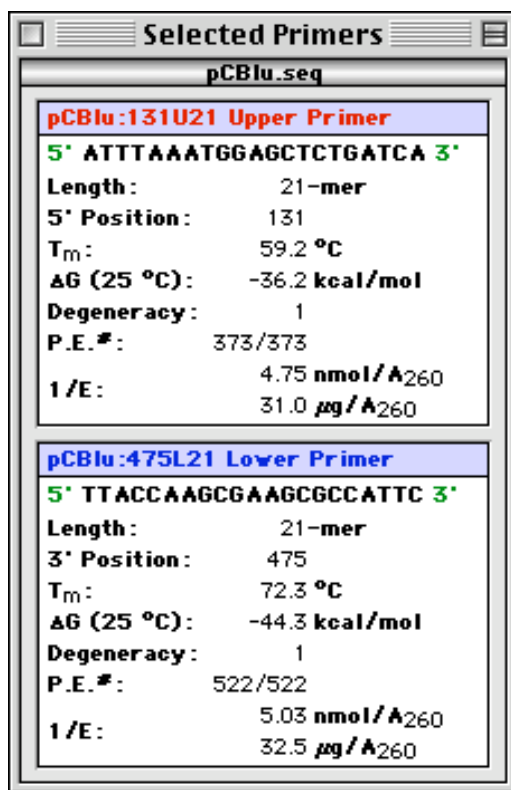


Figure 5.1.2 The Selected Primers window (Mac).

- The names of the Upper and Lower Primers assigned by OLIGO
- The sequences of the Upper and Lower Primers, displayed from the 5' to the 3'-end
- The length of the Upper and Lower Primers

- The 5' position of the Upper Primer and the 3' position of the Lower Primer
- The Current Oligo's T_m calculated by the nearest neighbor method using the salt and nucleic acid concentrations displayed in the "Non-Search Parameters" dialog box (*Change* menu)
- The ΔG (free energy) of the Upper and Lower Primer calculated using the temperature displayed in the "Non-Search Parameters" dialog box (*Change* menu)
- The number of degeneracies in the Upper and Lower Primer
- The Priming Efficiency numbers (P.E. #; for the explanation see Chapter 5.5) of the Upper and Lower Primer. The first value is the actual number and the second is max. theoretical value. If the primer is not 100% complementary to the main sequence (template) than those two values are different.
- The extinction coefficient properties of the Upper and Lower Primer, expressed in nmol/O.D. at 260 nm and in $\mu g/O.D.$ at 260 nm

5.2 Analyze — Duplex Formation

The *Analyze — Duplex Formation* command calls up the "Duplex Formation" window that displays potential duplexes in the Upper Primer, Lower Primer, and Current Oligo. Potential dimers between the selected Upper and Lower Primer may also be displayed.

ΔG and hairpin loop T_m values are calculated and displayed in association with each duplex (dimer or hairpin). If the T_m is not displayed for a given hairpin, then it is not likely that it can form ($T_m > 0$).

Note

The minimum number of contiguous bases required for the display of a potential dimer structure in this formation is two. If fewer than two contiguous bases match, a "no dimer formation" message appears.

5.2.1 Duplex Formation — Upper and Lower Primer

The information shown in this window includes:

- The most stable 3' terminal dimer alignment of the primer
- The most stable primer dimer alignment overall

- The most stable hairpin structure in the primer, if any
- The stability values of the most stable uninterrupted duplex (shown in bold) in each alignment, and of the hairpin structure, expressed in kcal/mol; T_m of the hairpin is displayed when it is greater than 0°

5.2.2 Duplex Formation — Current Oligo

The "Current Oligo" window displays:

- The most stable 3' terminal dimer alignment of the current positive strand oligo
- The most stable 3' terminal dimer alignment of the current negative strand oligo
- The most stable dimer alignment overall in the current positive strand oligo
- The most stable hairpin structure in the positive strand of the Current Oligo
- The stability values of the most stable uninterrupted duplex (shown in bold) in each alignment, and of the hairpin structure, expressed in kcal/mol; T_m of the hairpin is displayed when it is greater than 0°

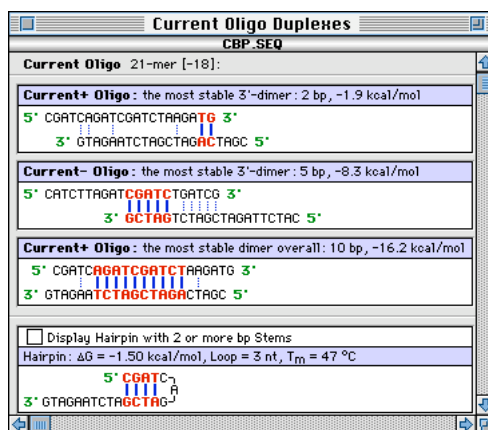


Figure 5.2.2 The Current Oligo Duplexes window (Mac). When the "Display Hairpin with 2 or more b.p." box is not checked, only the hairpins with stems 3 b.p. and longer are displayed.

The "Display Hairpin with 2 or more bp Stems" check box controls the length of the shortest hairpin stem that is displayed in the "Analyze — Duplex Formation" and "Analyze — Hairpin Formation" windows. If the box is

checked, hairpins with two or more base pair stems are displayed. If it is not checked, only three and longer base pair stems are displayed.

5.2.3 Duplex Formation — Upper/Lower

The "Upper/Lower" window displays the following dimer alignments between the Upper and Lower Primers:

- The most stable dimer alignment pairing the 3'-terminus of the Upper Primer to any segment of the Lower Primer
- The most stable dimer alignment pairing the 3'-terminus of the Lower Primer to any segment of the Upper Primer
- The most stable dimer structure overall between the Upper and Lower Primer
- The stability values of the most stable uninterrupted duplex in each alignment, and of the hairpin structure, expressed in kcal/mol; T_m of the hairpin is displayed when it is greater than 0°

5.3 Analyze — Hairpin Formation

The *Analyze — Hairpin Formation* command displays potential hairpin loop structures in the Upper Primer, Lower Primer, or Current Oligo. The hairpin stems are displayed in descending order of stability, expressed in hairpin loop ΔG values. The default setting for the minimum length of a hairpin stem displayed is three base pairs. You may, however, adjust the minimum hairpin stem displayed to two base pairs within this window.

5.3.1 Hairpin Formation — Upper Primer

The *Hairpin Formation — Upper Primer* command displays hairpin structures in the Upper Primer. The structures are displayed in the "Upper Primer Hairpin Stems" window. At the default setting, all hairpins with stems that are three base pairs (minimum) are displayed in order of stability (ΔG). Each individual hairpin display includes:

- The hairpin stem with nucleotide positions
- The oligonucleotide, if the Upper Primer is less than 60 nt

- The stem length (base pairs)
- The duplex strength (ΔG)
- The size of the hairpin loop (nt)

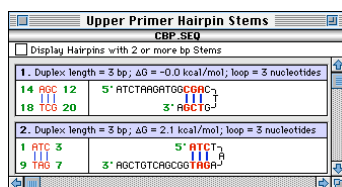


Figure 5.3.1 The Hairpin Formation - Upper Primer window. When the "Display Hairpin with 2 or more b.p." box is not checked, only the hairpins with stems 3 b.p. and longer are displayed.

If you want to display two base pair and above structures, check the box located below the title bar.

5.3.2 Hairpin Formation — Lower Primer

The *Hairpin Formation — Lower Primer* command displays hairpin structures in the Lower Primer. The structures are displayed in the "Lower Primer Hairpin Stems" window. At the default setting, all hairpins with stems that are three base pairs (minimum) are displayed in order of stability (ΔG). Each individual hairpin display includes:

- The hairpin stem with nucleotide positions
- The oligonucleotide, if the Lower Primer is less than 60 nt
- The stem length (base pairs)
- The duplex strength (ΔG)
- The size of the hairpin loop (nt)

If you want to display two base pair and above structures, check the box located below the title bar of this window.

5.3.3 Hairpin Formation — Current Oligo

The *Hairpin Formation — Current Oligo* command displays hairpin structures in the Current Oligo. The structures are displayed in the "Current Oligo Hairpin Stems" window. At the default setting, all hairpins with stems that are three base pairs (minimum) are

displayed in order of stability (ΔG). Each individual hairpin display includes:

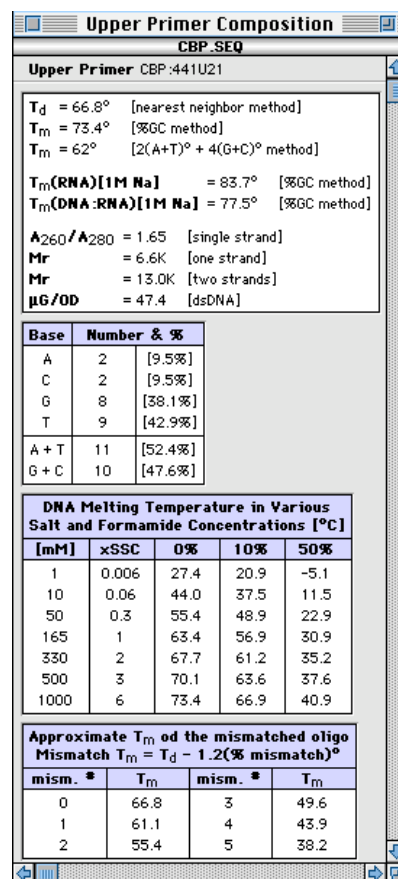
- The hairpin stem with nucleotide positions
- The oligonucleotide, if the Current Oligo is less than 60 nt
- The stem length (base pairs)
- The duplex strength (ΔG)
- The size of the hairpin loop (nt)

If you want to display two base pair and above structures, check the box located just below the title bar.

5.4 Analyze — Composition and T_m

The *Analyze — Composition and T_m* command displays the base composition and melting temperature of the selected Upper or Lower Primer, Current Oligo, active sequence, or PCR product at various salt, nucleic acid, and formamide concentrations. Molecular weights, absorption data, and the T_m s of mismatched oligos are also displayed.

Figure 5.4.1.
The *Analyze — Composition and T_m* window.



5.4.1 Composition and T_m — Upper Primer

The *Composition and T_m — Upper Primer* command calls up the "Upper Primer Composition" window that displays:

- The melting temperature calculated by three methods — the nearest neighbor method, the % GC method, and the "2 plus 4" ($2(A+T)+4(G+C)$) method. The nearest neighbor T_m value in this window is always given for 100pM oligo and 1M salt regardless of the global settings in the OLIGO program. (T_m in these conditions is equal to T_d , as described in Ref. 4.)

- The melting temperature of the Upper Primer as an RNA sequence (%GC method) in 1 M Na^+
- The melting temperature of the Upper Primer as a DNA-RNA hybrid (%GC method) in 1 M Na^+
- The A260/A280 absorption ratio of the Upper Primer

Note *The A260/A280 absorption data have been generated using a limited number of oligos.*

- The molecular weight of the Upper Primer, both single stranded and double stranded
- The number and percentage of each base in the Upper Primer plus the number and percentages of A+T and of G+C in the sequence
- The melting temperature (%GC method) of the Upper Primer at various salt concentrations (expressed both as mM and as multiples of the *SSC buffer*) at 0%, 10%, and 50% formamide
- A calculation of melting temperature (nearest neighbor method) for the Upper Primer with up to five mismatches

5.4.2 Composition and T_m — Lower Primer

The *Composition and T_m — Lower Primer* command calls up the "Lower Primer Composition" window that displays:

- The melting temperature calculated by three methods — the nearest neighbor method, the % GC method, and the "2 plus 4" ($2(A+T)+4(G+C)$) method. The nearest neighbor T_m value in this window is always given for 100pM oligo and 1M salt regardless of the global settings in the OLIGO program. (T_m in

- these conditions is equal to T_d , as described in Ref. 14.)
- The melting temperature of the Lower Primer as an RNA sequence (%GC method) in 1 M Na^+
- The melting temperature of the Lower Primer as a DNA-RNA hybrid (%GC method) in 1 M Na^+
- The A260/A280 absorption ratio of the Lower Primer

Note

The A260/A280 absorption data have been generated using a limited number of oligos.

- The molecular weight of the Lower Primer, both single stranded and double stranded
- The number and percentage of each base in the Lower Primer plus the number and percentages of A+T and of G+C in the sequence
- The melting temperature (%GC method) of the Lower Primer at various salt concentrations (expressed both as mM and as multiples of the SSC buffer) at 0%, 10%, and 50% formamide
- A calculation of melting temperature (nearest neighbor method) for the Lower Primer with up to five mismatches

5.4.3 Composition and T_m — Current Oligo

The *Composition and T_m — Current Oligo* command calls up the "Current Oligo Composition" window that displays the information listed here. If the Current Oligo is longer than 59 nt, the nearest neighbor values are not displayed.

- The melting temperature calculated by three methods — the nearest neighbor method, the % GC method, and the "2 plus 4" ($2(\text{A}+\text{T}) + 4(\text{G}+\text{C})$) method. The nearest neighbor T_m value in this window is always given for 100pM oligo and 1M salt regardless of the global settings in the OLIGO program. (T_m in these conditions is equal to T_d , as described in Ref. 14.)
- The melting temperature of the Current Oligo as an RNA sequence (%GC method) in 1 M Na^+
- The melting temperature of the Current Oligo as a DNA-RNA hybrid (%GC method) in 1 M Na^+
- The A260/A280 absorption ratio of the Current Oligo

Note

The A260/A280 absorption data have been generated using a limited number of oligos.

- The molecular weight of the Current Oligo, both single stranded and double stranded
- The number and percentage of each base in the Current Oligo plus the number and percentages of A+T and of G+C in the sequence
- The melting temperature (%GC method) of the Current Oligo at various salt concentrations (expressed both as mM and as multiples of the SSC buffer) at 0%, 10%, and 50% formamide
- A calculation of melting temperature (nearest neighbor method) for the Current Oligo with up to five mismatches

5.4.4 Composition and T_m — Entire Sequence

The *Composition and T_m — Entire Sequence* command calls up the "Entire Sequence Composition" Window that displays:

- For oligos longer than 60 bp, the melting temperature is calculated by two methods — the % GC method, and the "2 plus 4" ($2(A+T) + 4(G+C)$) method. When the sequence is shorter than 60 bp the nearest neighbor method calculation is displayed, as well. The nearest neighbor T_m value in this window is always given for 100pM oligo and 1M salt regardless of the global settings in the OLIGO program. (T_m in these conditions is equal to T_d , as described in Ref. 14.)
- The melting temperature of the entire sequence as an RNA sequence (%GC method) in 1 M Na^+
- The melting temperature of the entire sequence as a DNA-RNA hybrid (%GC method) in 1 M Na^+
- The A260/A280 absorption ratio of the entire sequence

Note

The A260/A280 absorption data have been generated using a limited number of oligos.

- The molecular weight of the entire sequence, both single stranded and double stranded
- The number and percentage of each base in the entire sequence plus the number and percentages of A+T and of G+C in the sequence

- The melting temperature (%GC method) of the entire sequence at various salt concentrations (expressed both as mM and as multiples of the SSC buffer) at 0%, 10%, and 50% formamide
- A calculation of melting temperature (nearest neighbor method) for the entire sequence with one, two, three, four, and five mismatches

5.4.5 Composition and T_m — PCR Product

The *Composition and T_m — PCR Product* command calls up the "PCR Product Composition" Window that displays:

- For PCR products longer than 60 bp, the melting temperature is calculated by two methods — the % GC method, and the "2 plus 4" ($2(A+T) + 4(G+C)$) method. When the PCR product sequence is shorter than 60 bp the nearest neighbor method calculation is displayed, as well. The nearest neighbor T_m value in this window is always given for 100pM oligo and 1M salt regardless of the global settings in the OLIGO program. (T_m in these conditions is equal to T_d , as described in Ref. 14.)
- The melting temperature of the PCR product as an RNA sequence (%GC method) in 1 M Na^+
- The melting temperature of the PCR product as a DNA-RNA hybrid (%GC method) in 1 M Na^+
- The A260/A280 absorption ratio of the PCR product

Note

The A260/A280 data have been generated using a limited number of oligos.

- The molecular weight of the PCR product, both single stranded and double stranded
- The number and percentage of each base in the PCR product plus the number and percentage of A+T and of G+C in the sequence
- The melting temperature (%GC method) of the PCR product at various salt concentrations (expressed both as mM and as multiples of the SSC buffer) at 0%, 10%, and 50% formamide

5.5 Analyze — False Priming Sites

The *Analyze — False Priming Sites* command searches for and displays potential false priming sites

for the Upper or Lower Primer in both the sense and antisense strands of the active sequence.

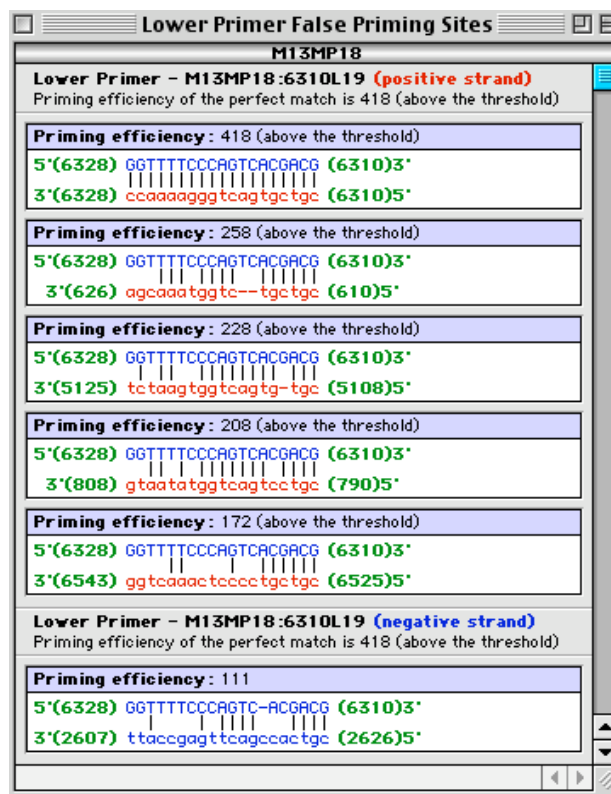


Figure 5.5 An example of a False Priming Sites window (Win). The site with P.E. of 258 points is the real false priming site(20).

The *False Priming Sites* search performs the following:

- A search for potential sites using a homology algorithm similar to FAST-N
- A calculation of the priming efficiency number of potential sites found in the search

Priming Efficiency

The priming efficiency number (P.E. #) is a formulation unique to the OLIGO program (a proprietary algorithm) that quantifies the likelihood that a given oligonucleotide will prime at a given site on the template. It has been determined that matching PCR primers by their P.E. # gives better results than matching the primers by their T_m s. This is especially

important in designing multiplex PCR experiments. The priming efficiency considers:

- The stability (ΔG) of primer-template duplexes and their distance from the 3'-end
- Bulge loops and mismatches and their distance from the 3'-end
- The overall primer length

A priming efficiency number (P.E. #) of 210 or higher for a given priming site may result in priming during PCR or cycle sequencing conditions. A P.E. # of 170 is the approximate lower limit for priming (or false priming) when using SEQUENASE®.

5.5.1 False Priming Sites — Upper Primer

False Priming Sites — Upper Primer calls up the "Upper Primer — False Priming Sites" window that displays:

- The priming efficiency of the Upper Primer on a perfectly matched sequence (complement)
- The priming efficiency and alignment of the most stable false priming sites followed by the alignments and priming efficiencies of other false priming sites, in order of stability
If the false priming site has a value higher than the priming efficiency setting in the "Search Parameters" window, the message "Above the threshold" appears.

5.5.2 False Priming Sites — Lower Primer

False Priming Sites — Lower Primer calls up the "Lower Primer — False Priming Sites" window that displays:

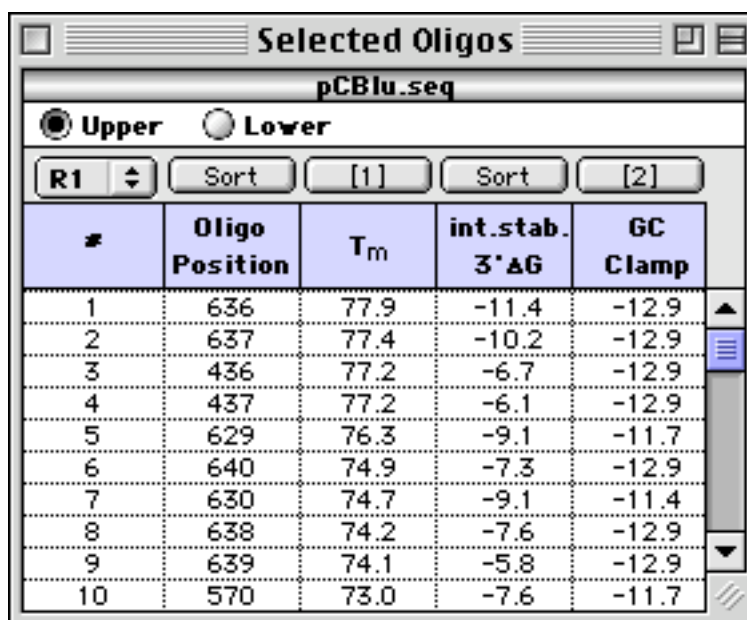
- The priming efficiency of the Lower Primer on a perfectly matched sequence (complement)
- The priming efficiency and alignment of the most stable false priming sites followed by the alignments and priming efficiencies of other false priming sites, in order of stability
If the false priming site has a value higher than the priming efficiency setting in the "Search Parameters" window, the message "Above the threshold" appears.

5.6

Analyze — Selected Oligos

The *Analyze — Selected Oligos* command calls up the "Selected Oligos" window in a table listing all oligonucleotide selections from the most recent search. After the search for primers and probes, R1 contains all positive strand oligonucleotide positions. R2 displays all negative strand oligonucleotide position numbers and R2 contains negative strand position numbers.

Check the "R1" button at the upper left corner of the window to display all positive strand oligonucleotide positions. Checking "R2" displays all negative strand positions.



The screenshot shows a window titled "Selected Oligos" with a sub-header "pCBlu.seq". It has radio buttons for "Upper" (selected) and "Lower". Below are buttons for "R1" (selected), "Sort", "[1]", "Sort", and "[2]". The table below lists 10 oligonucleotides with columns for index, position, T_m, 3' ΔG, and GC clamp.

#	Oligo Position	T _m	int.stab. 3'ΔG	GC Clamp
1	636	77.9	-11.4	-12.9
2	637	77.4	-10.2	-12.9
3	436	77.2	-6.7	-12.9
4	437	77.2	-6.1	-12.9
5	629	76.3	-9.1	-11.7
6	640	74.9	-7.3	-12.9
7	630	74.7	-9.1	-11.4
8	638	74.2	-7.6	-12.9
9	639	74.1	-5.8	-12.9
10	570	73.0	-7.6	-11.7

Figure 5.6. The Selected Oligos window (Mac).

Although PCR primer search data can be displayed using this option, the window is primarily for displaying sequencing primer and hybridization probe search data. Accordingly, the data table lists T_m, 3' ΔG (specificity), and GC clamp stability associated with each oligonucleotide position.

At the default setting, the window sorts oligonucleotides by ascending position number. You may also sort the table by T_m, 3' ΔG, or GC clamp by

clicking on the sort button above that column. You may implement secondary, tertiary sorts and so on.. Simply hold down the <option> key (Mac) or <Ctrl> key (Win) and click on the button labeled "Sort". The primary sort field button will be labeled "1", and the secondary "2", tertiary "3" etc.

The T_m and 3' ΔG columns sort in descending order; the GC clamp column sorts in ascending order. The 3' ΔG values in the table are always negative.

You can display either selected oligos from the upper or lower strand by clicking on the "upper" or "lower" button at the top of the "Selected Oligos" window.

As you click on different positions in the table, the Current Oligo position is updated accordingly.

5.7 Analyze — Primer Pairs

The *Analyze — Primer Pairs* command calls up the "Primer Pairs" window containing a table of primer pairs data from the most recent PCR primer search. The following information is included in the table:

- The primer pair number
- The primer pair's positions on the + and - strand active sequences
- The length of the PCR product the pair would generate
- The calculated optimal annealing temperature recommended for PCR using this primer pair
- The percent GC content of the PCR product generated by this pair

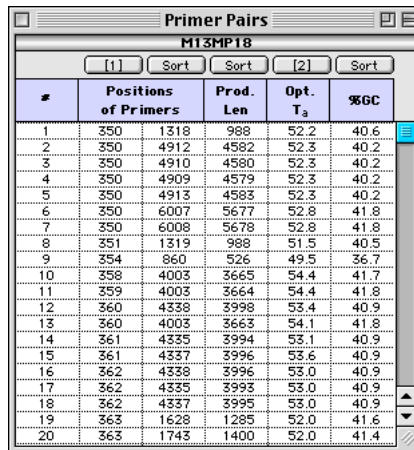
By default, primer pairs are displayed by position number in ascending order. Sorting the primer pairs by optimal annealing temperature (T_a), product length, or %GC content (all in ascending order) is performed by clicking on the button just above the respective column.

By clicking on any of the listed primer pairs in the table, you select that pair as the Upper and Lower Primer. Clicking on a pair also calls up the PCR window that displays a graphic of the PCR product's

location on the active sequence, along with other data for running PCR with the selected primer pair.

Note

The "Primer Pairs" window is not accessible unless a PCR primer search is performed first.



#	Positions of Primers	Prod. Len.	Opt. T _a	%GC
1	350 1318	988	52.2	40.6
2	350 4912	4582	52.3	40.2
3	350 4910	4580	52.3	40.2
4	350 4909	4579	52.3	40.2
5	350 4913	4583	52.3	40.2
6	350 6007	5677	52.8	41.8
7	350 6008	5678	52.8	41.8
8	351 1319	988	51.5	40.5
9	354 860	526	49.5	36.7
10	358 4003	3665	54.4	41.7
11	359 4003	3664	54.4	41.8
12	360 4338	3998	53.4	40.9
13	360 4003	3663	54.1	41.8
14	361 4335	3994	53.1	40.9
15	361 4337	3996	53.6	40.9
16	362 4338	3996	53.0	40.9
17	362 4335	3995	53.0	40.9
18	362 4337	3995	53.0	40.9
19	363 1628	1285	52.0	41.6
20	363 1743	1400	52.0	41.4

Figure 5.7 The Primer Pairs window (Mac). By pressing the <option> key (Mac) or the <Ctrl> key (Win) and clicking "Sort" simultaneously you can perform secondary sorts.

5.8 Analyze — Multiplexing

With the *Analyze — Multiplexing* command, you can select a series of cross-compatible primers suitable for applications requiring more than two primers in the same tube.

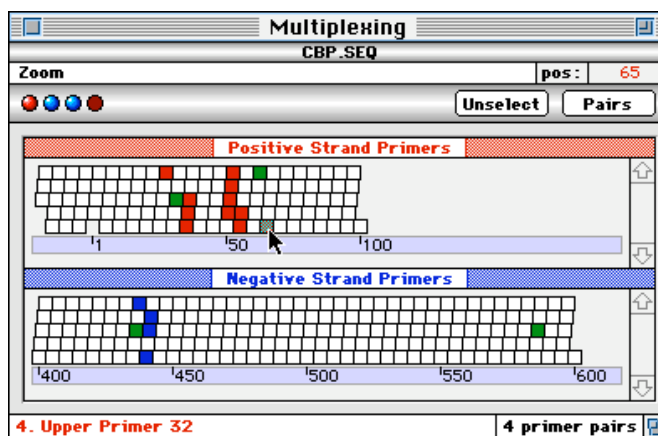


Figure 5.8 The Multiplexing window.

The *multiplexing* command finds primers that are cross-compatible — will not form dimers in all combinations. For example, four cross-compatible primers are dimer-free between primers 1 to 2, 1 to 3, 1 to 4, 2 to 3, 2 to 4, and 3 to 4.

While the multiplexing command only selects on the basis of cross-compatibility (no 3'-end dimer formation between the same primer is checked), the list of positive strand and negative strand primers that comprise the data set from which the option selects are generated in the *Search for PCR Primers Pairs*; therefore, this data set will be "pre-optimized" for other PCR primer traits (as listed in the "Search for Primers and Probes" dialog box). The stringency of the cross-compatible selection process can be modified in the "Search Parameters" dialog box from the *Change* menu. The specific parameters that affect this stringency are:

- Acceptable 3' dimer ΔG
- Minimal length of acceptable dimer
- Check (X) 3' terminal nucleotides for dimers

The last two parameters are used to check for all dimers, not only those that begin at the 3'-end of an oligo.

To select multiplexed primers:

Choose *Analyze — Multiplexing* after a search for PCR primer pairs. Potential positive strand primer positions are automatically stored in the R1 Memory

Table and potential negative strand primer positions are stored in the R2 Memory Table.

The "Multiplexing" window consists of two grids:

- A positive strand primer grid where the red squares represent the positions of the optimized positive strand primers (5'-end) in the PCR search
- A negative strand primer grid with blue squares that represent the positions for the optimized negative strand primers (3'-end)

The empty, or white, squares within both grids represent the positions of primers rejected in the PCR search, or during multiplexing.

To select multiplexed primers:

1. Click on one of the red or blue squares at a desired position.
Once selected, this square turns green, becoming the first of the primer set. Colored squares (both red and blue) not compatible with this selected primer are erased from the grid. All squares remaining on the screen are compatible with the selected (green-marked) primer.

Note

Click on the "Deselect" button to undo your last selection.

2. Click on another positive (red) or negative (blue) strand primer at a desired position.
This second selection also turns green. Now, only primers compatible with both of these selections remain (in red and blue) on the grids.
3. Continue to select primers. As they are selected, a green circle is displayed for each selected primer at the top left of the window and the selected box in the grid is displayed in green.
4. When your selections are complete, click on the "Primer Pairs" button to open the "Multiplex Primers" list.

Every primer on the list is now cross-compatible, (will not form dimers with other primers) based on the current search parameters, and may be used for PCR in the same reaction mix. If too many primers are removed during the multiplexing, to create the desired group of cross-compatible primers, reduce the search

stringency setting in the "Search Parameters" window and run the search again.

A multiplexed primer set can be downloaded to an OLIGO database as a group via the "Multiplexed Primers" command of the *Import* menu.

5.9 Analyze — PCR

The *Analyze — PCR* command displays various data for PCR based on a user-selected PCR primer pair. The primer pair must be selected before this window can be called up.

Optimal annealing temperature for PCR T_a^{OPT} and the maximum annealing temperature (T_a^{max}) are given. For an initial experiment, use T_a^{OPT} , since it usually gives the highest yield. The T_a^{max} should be used mainly for diagnostic purposes because PCR is more specific in these conditions.

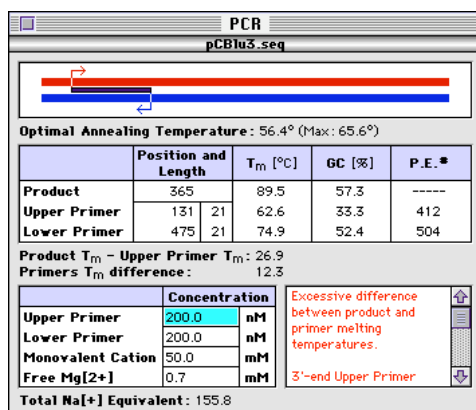


Figure 5.9 The *Analyze — PCR* window.

The following is provided relative to the prospective PCR product. This data is also listed on the "Primer Pairs" window:

- Length in base pairs
- GC content

- T_m (GC% method)
- For the "Upper and Lower Primer" the following information is given:
- Length in nucleotides
 - 5'-end position number for the Upper Primer and 3'-end position number for the Lower Primer
 - T_m at concentrations specified in the lower portion of the window — these are interactive and can be defined by you

Note

The primer and salt concentrations in this window are independent from the global salt and nucleic acid conditions specified in Change — Non-Search Parameters. For the T_m^{primer} calculations in the PCR window, the expression $C/4$ is replaced by C in the equation I, Appendix D, since the primer and the active sequence concentrations are different.

- Priming efficiency number (P.E. #)

PCR primers should be balanced by using their P.E. # rather than by their T_m s. Excellent results have been obtained for multiplex PCR (10 pairs) when all the primers were within a 400-450 P.E. # range. Balancing the primers using T_m (55-60°) produced less optimal results. More on P.E. in *Analyze — False Priming Sites.*, Chapter 5.5.

- T_m difference between the product and the less stable primer

Avoid a primer-product difference of more than 22° since this will produce too much competition between template-template and primer-template annealing.

- T_m difference between the primers

Avoid designing primers with a high T_m difference whenever possible. When the selected primers have disparate T_m s (greater than 10°), the more stable primer does not work optimally because the annealing temperature has to be reduced to compensate for the less stable primer. Removing nucleotides from the 5' end of the more stable primer in order to match T_m s does not affect PCR. On the other hand, increasing the length of the less stable primer to match the T_m of the more stable primer will typically improve PCR.

Note

The OLIGO program has an automatic primer T_m matching feature (on the "Search Parameters" dialog box from the Change menu) where nucleotides are removed from the 5'-end of the more stable primer until T_m s or PEs match.

The warning scroll-box on the bottom-right (when displayed in red letters) indicates possible problems that may arise from using selected oligos as PCR primers.

5.10 Analyze — LCR

LCR (Ligase Chain Reaction) is a diagnostic technique designed to confirm the sequence in a target DNA sample. The LCR function designs two pairs of complementary LCR primers for a wild-type DNA sample, and optionally, an additional pair to detect a point mutation.

To use the LCR feature in OLIGO:

1. Load the sequence file containing the target oligo sequence.
2. Locate the site of the potential point mutation, and position the 5'-end of the Current Oligo at that position.
3. Choose *LCR* from the *Analyze* menu.
4. Click on "Select" to automatically select the four probes.

The two sets of probe pairs overlap by one base, creating 5' recessed ends. The mutation-specific probes have an approximate T_m of 65°C, while the common probes have an approximate T_m of 70°C. OLIGO automatically adds a non-complementary tail to mutagenic probes so you can distinguish them from the wild-type probes.

5. Click on one or more of the remaining three buttons to select the appropriate mutation. The melting temperature of the probes detecting a mutated DNA listed in this window is calculated only for the template-matching portion of the probe. The length of the non-complementary tail has no effect on T_m calculations.

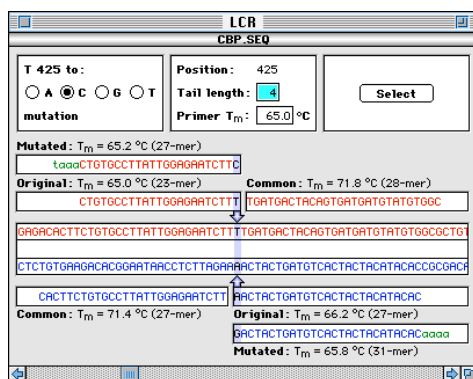


Figure 5.10 The Analyze — LCR window.

To modify the probes (the "fill-in" LCR variation):

1. Load the probes to an OLIGO database using the LCR Oligos command from the database Import menu.
2. Edit the probes using Modify command from the database Edit menu.

Note

Changing salt and nucleic acid concentrations in the "Non-search Parameters" dialog box affects the size of the selected probes.

5.11 Analyze — T_m Graph

The T_m Graph command reopens the "Melting Temperature" window (the top window on the default screen) if you previously closed it by clicking the "Go Away" box at the top left corner of the window. There are two display styles available for this window: "bar" and "dot" that may be switched using the Options-Style (the bar graph display is shown in Fig. 2.5.1).

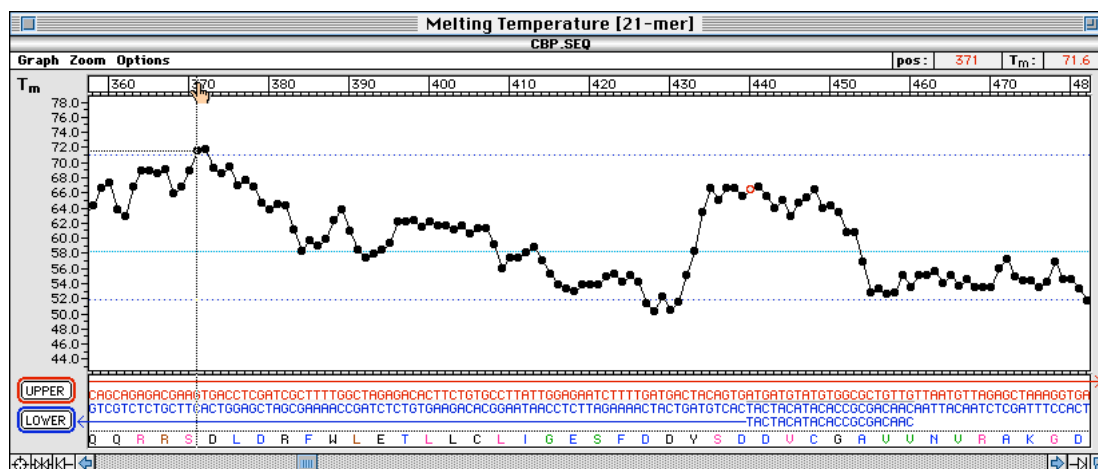


Figure 5.11.1 The Melting Temperature window, dot style, using Zoom 1 option (Mac).

The "Melting Temperature" window is the core window of the OLIGO program. It is automatically displayed when a DNA or RNA sequence file is loaded, and includes the following displays, functions, and capabilities in the default configuration:

- A close-up of approximately 90 nucleotides of the active sequence at the bottom of the window, both the positive strand (in red) and the negative strand (in blue). This display can be zoomed-out using the Zoom option.

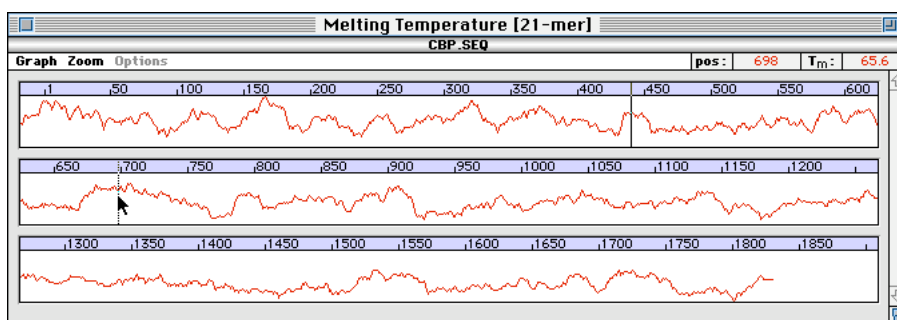


Figure 5.11.2 The Melting Temperature window using Zoom 3 option (Mac).

- A T_m graph of the displayed sequence segment, calculated using the nearest neighbor method. Each point or bar on the graph represents the T_m of an oligonucleotide, the 5'-terminus of which is located at that position. The T_m is calculated for

- an oligonucleotide length that is set in the "Current Oligo Length" option from the *Change* menu — the default is a 21-mer. The set oligo length is displayed on the window title bar.
- Cursor and cross hairs. The T_m window includes a mouse-driven "finger-pointer" cursor.

By default, the "Melting Temperature" window displays the T_m plot of every 21-mer of the active sequences. However, there are two other display modes — ΔG values, %GC composition, or, for degenerate sequences, it may display degeneracy. You can change the display from the *Graph* window .

The "Melting Temperature" window submenus, displayed at the top portion of the window (Mac) or when pressing the right mouse button (Win) are shown in Fig. 5.11.3. The word "Stability" in the Mac *Graph* menu is replaced by ΔG .

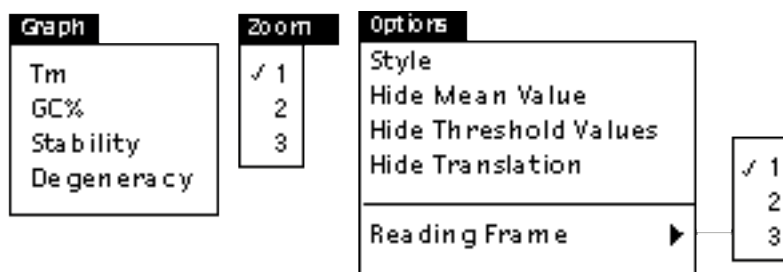


Figure 5.11.3 The T_m Graph window submenus (Win).

The Graph, Zoom and the Options submenu items are displayed in Figure 5.11.3. The T_m window *Options* allow you to change the features displayed in the window.

- Style*. This option permits you change the appearance of the T_m window from bar to dot and vice versa. In order to change the style, click on the appropriate graph, see Fig. 5.11.4.

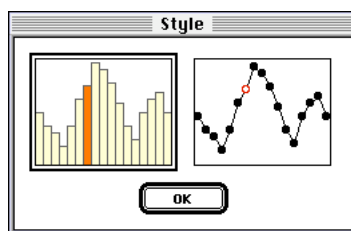


Figure 5.11.4 The Options-Style window.

- *Hide/Show Mean Value.* When the "show" option is selected, a dotted line is displayed in the "Melting Temperature" window. The line represents the mean T_m , ΔG , %GC or degeneracy of all the oligonucleotides of the selected length in the active sequence. This mean value is shown as the center line on the T_m plot.
- *Hide/Show Threshold Values.* The other two lines represent the upper and lower T_m threshold settings currently controlling the "Oligonucleotides Within Selected Stability Limits" subsearch which is used by the PCR primer, sequencing primer, and hybridization probe searches.
- *Hide/Show Translation.* By default, OLIGO translates the active DNA (RNA) active sequence in the designated reading frame and displays the amino acid sequence below the sequence in the "Melting Temperature" window.
- *Reading Frame.* "1," "2," and "3" options indicate the reading frame in which the nucleic acid sequence in the "Melting Temperature" window is translated.

5.12 Analyze — Internal Stability

The *Analyze — Internal Stability* command plots the ΔG of pentamers (internal stability) of the active sequence, Current Oligo, and Upper and Lower Primers. The "Internal Stability" (Current Oligo) window is displayed along with the "Melting Temperature" window on the main screen immediately after an active sequence is opened. There are two display styles available for this window: "bar" and "dot" that may be switched using the Options-Style (the bar graph display is shown on Fig. 2.5.1).

Each base of the Current Oligo pentamer is represented by an open red circle or a deep-green bar, while the rest of the sequence pentamers are represented by solid black circles or light green bars. Each pentamer is marked at the 5'-end of the positive strand.

The "Internal Stability" window can be used to check that an oligo is specific and will prime efficiently on its intended target. An oligo with a relatively unstable 3'-end, but with higher stability along the remainder of its length, should possess both these characteristics, assuming that other design characteristics are optimized.

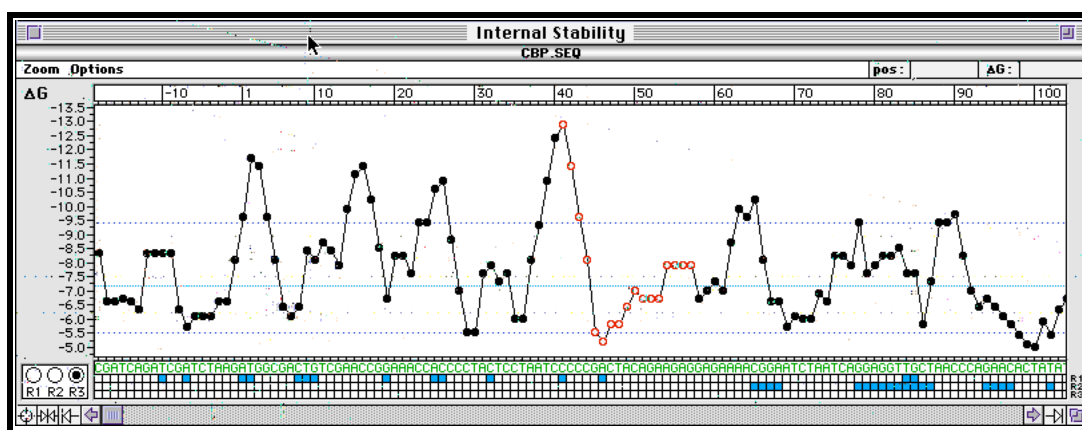


Figure 5.12 The Internal Stability window (Mac).

At the bottom of the "Internal Stability" window are the "Memory Tables," which mark the specific positions listed in the memory table as blue squares. Typically, these are positions of oligonucleotides selected in OLIGO searches. To select a memory table as current (for manual search purposes) click R1, R2, or R3 button.

The "Internal Stability" window has a submenu similar to the Melting Temperature window submenu, shown on Fig.5.11.3.

5.13



Figure 5.13 The Frequencies window, bar style display (Mac).

This graph shows the relative frequency of nucleic acid subsequences (6- or 7-mers) throughout the entire loaded sequence. Oligonucleotides having 3'-ends which are common in a specific database (subset of GenBank) have a greater likelihood of false priming. The tables of frequencies (located in the Frequencies folder) are user-selected and contain normalized frequencies of GenBank sub-sequences (6- or 7-mer). The header of each frequency file describes in detail how each table was created. For example, the first 20 lines of GBPRIHUM.FR6 are shown below.

GBPRI.SEQ Genetic Sequence Data Bank

15 April 1997

NCBI-GenBank Flat File Release 100.0

Primate Sequences

68547 loci, 90213435 bases, from 68547 reported sequences

Organism: Homo

Frequency of 6-mers from 57478 records greater than 99 nt. (tot. 86452438 nt.) expressed as $\text{HITS} \times 1000 \times 4096 (= \text{Table size}) / K$. The average frequency for a given 6-mer is 1000. K, the number of analyzed 6-mers is 85736235.

1	AAAAAA	10020
2	AAAAAC	2138
3	AAAAAG	2857
4	AAAAAT	4055
5	AAAACA	2699
6	AAAACC	1536
7	AAAACG	395
8	AAAACT	1812
9	AAAAGA	2945
10	AAAAGC	1529

In this particular example, the frequency number for the sequence AAAACG is 395, meaning that this particular hexamer is found 8268 times ($395 \times 85736235 / 4096,000$) in human sequences from GenBank, release 100. By choosing oligos having 3'-ends which are infrequent in a given database, you are decreasing the likelihood of selecting a primer that primes on many sites in a complex substrate, such as genomic DNA. In other words using oligos with low frequency numbers decreases chance of unspecific PCR. No significant difference in PCR performance was found when primers were selected from a 7-mer frequency table or a 6-mer frequency table. A frequency of 1000 is the average hexamer (or heptamer) frequency for a given database. These data tables are also used in the "Eliminate Frequent Oligos" sub-search for primers.

5.14

Analyze — Hybridization Time


Hybridization Time

pCBlu.seq

DNA Length: 21 nt.

Concentration: 200.0 nM

1.298 µg/ml

 T_{1/2} = 45.4 sec

T = 4 min 46.9 sec

Figure 5.14 The Hybridization Time window (Mac).

The *Analyze — Hybridization Time* command calculates oligonucleotide hybridization time for various user-selected lengths and concentrations in the "Hybridization Time" dialog box. OLIGO uses the following formula:

$$t_{1/2} = \frac{N \ln 2}{3.5 \times 10^5 \times \sqrt{L} \times C_N}$$

Where N is the total number of base pairs in a non-repeating sequence (molecular complexity), L is the oligonucleotide length (in nt), and C_N is the oligonucleotide concentration (mol nucleotides/liter).

For simplicity, $N = L$; therefore,

$$t_{1/2} = \frac{\sqrt{L} \times \ln 2}{3.5 \times 10^5 \times C}$$

Where C is oligonucleotide concentration (moles/liter). (Ref. 15.)

When a new length or concentration value is entered, a new hybridization time is displayed.

Note

Because of high secondary structure potential and the presence of sequence repeats, hybridization times for long DNA fragments may be underestimated in this formula. The hybridization time for standard length primers should be reasonably accurate.

5.15

Analyze — Concentrations

Concentrations
CBP SEQ

☒ Constant Concentration
☐ Constant Volume

☒ Current +Oligo: 4.69 nmol/OD, 30.8 µg/OD
☐ Current -Oligo: 5.11 nmol/OD, 32.9 µg/OD
☐ Entire Sequence: 0.042 nmol/OD, 49.0 µg/OD
☐ Upper Primer: 4.62 nmol/OD, 30.2 µg/OD
☐ Lower Primer: 5.11 nmol/OD, 32.9 µg/OD
☐ PCR Product: 0.146 nmol/OD, 47.8 µg/OD

30.8 µg
or 1.0 OD(260)
or 4.686 nmol
in 468.6 µL
yields 10.0 µM

Figure 5.15 The Concentrations window.

The *Analyze — Concentrations* command calls up the "Concentrations" window and calculates concentration, volume, absorption, and molecular

weight conversions for the following sequences from the OLIGO program:

- Current Oligo, positive strand
- Current Oligo, negative strand
- Entire sequence (active sequence)
- Upper Primer
- Lower Primer
- PCR product

There are two calculation modes in the "Concentrations" window:

- Constant concentration, where the concentration of a nucleic acid sample in solution is held constant while allowing the other values to vary
- Constant volume, where the total solution volume of a nucleic acid sample is held constant while allowing other values to be recalculated

The right side of the "Concentrations" window provides five data entry boxes where μg , OD, mole, μL and μM values may be entered. Once a new value is entered in a given data box, you can click the mouse in any other box and initiate a recalculation of all other boxes, except the volume or concentration box, whichever was set constant.

Note

The window should read: "X micrograms of DNA/RNA or Y optical density (O.D.) units or Z nanomoles in K microliters yields an M micromolar solution."

5.16 Analyze — All Checked

The *Analyze — All Checked* command (⌘W in Mac) opens all the windows checked in the *File-Print/Save* option. If those windows are already opened, this option closes them. This toggle works even when the printing of the "Current" window is selected in the "Print/Save" dialog.

6

The OLIGO Search Menu

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6 The OLIGO Search Menu

The *Search* menu includes OLIGO's automatic searches: the search for primers and probes, the search for hairpin loop stems, the search for sequence strings, the search for palindromes, and the search for restriction sites in DNA and protein sequence files.

The *Search* menu also provides access to OLIGO's three Memory Tables — R1, R2, and R3 where search position data and user-selected position data are stored. The R1 table stores positive strand search results; R2 stores negative strand search data; and R3 stores manually selected positions or positions selected by hairpin loop or palindrome searches when R3 is the active table. Positions marked in the Memory Tables can be compared and modified. The Memory Table management commands are accessible from the *Search* menu.

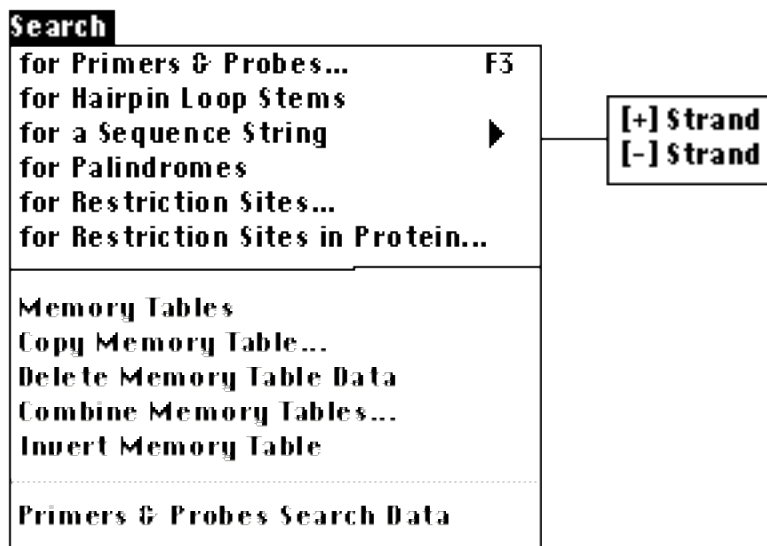


Figure 6.0 The *Search* menu (Win).

6.1

Search — Primers and Probes

The *Search for Primers and Probes* command (<F3> is the short cut key in the Windows version) calls up the "Search for Primers and Probes" dialog box from which you may search for optimized PCR primer pairs, sequencing primers, and hybridization probes in the active nucleic acid sequence. Each of these searches is a composite consisting of several subsearches, each of which proceeds according to preset search parameters that you may adjust. You can change the search parameter values individually or globally using the "Search Stringency" settings in the "Search Parameters" dialog box.

Next to each search option in the "Search for Primers and Probes" dialog box is a button to activate or deactivate that search option. When a button is activated, the individual subsearches that are components of the search are indicated by checked boxes.

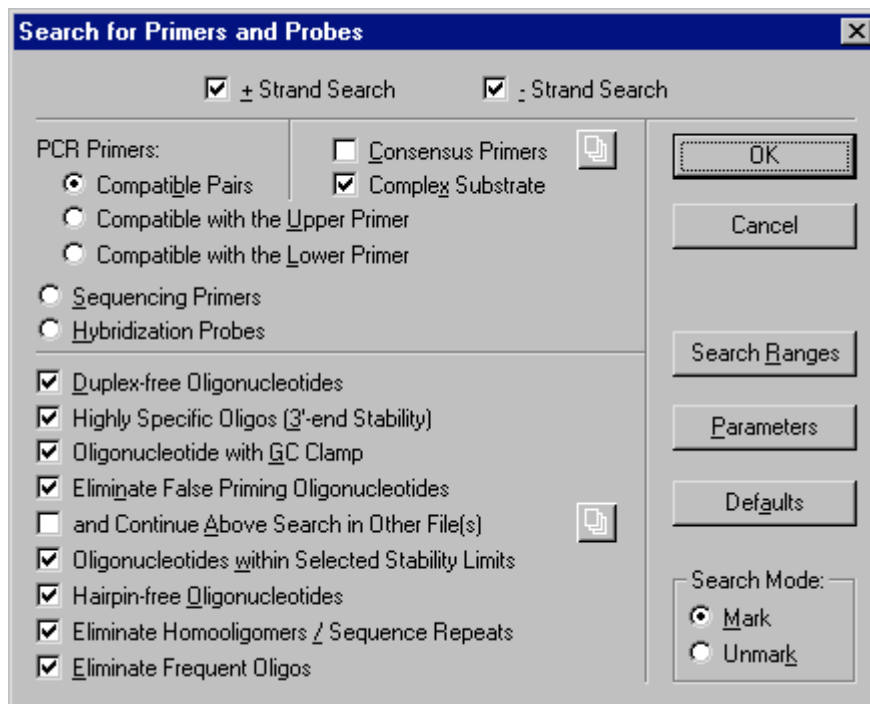


Figure 6.1 The Search for Primers and Probes dialog box (Win).

The *Search Ranges* dialog box and *Search Parameters* dialog box may be accessed by clicking on the respective buttons in the "Search for Primers and Probes" dialog box. These options are also available from the *Change* menu.


6.1.1 Available Search Modes

6.1.1.1 + Strand Search/- Strand Search

You can search in the positive strand, negative strand, or both. If a search is deselected in both the positive and negative strands, OLIGO automatically assigns the search to the positive strand.

6.1.1.2 Consensus Primers

When this box is activated you may select other file(s), homologous to the active sequence, to find primers common to all files. Consensus primers are those which prime at least with the "minimum consensus priming efficiency", a variable set in the

search parameters dialog. Clicking the  icon calls up the "Select Files" dialog from which you may select homologous sequence files for consensus primer design.

6.1.1.3 Complex Substrate

When this box is checked, two additional sub-searches are performed: the "Highly Specific Oligos" sub-search, that eliminates primers with excessively stable 3'-ends, and the "Eliminate Frequent Oligos" sub-search, that eliminates primers having 3'-ends which occur frequently in a given database of sequences. If the substrate for your PCR or sequencing reaction is not complex (i.e. plasmid) checking this box is not typically necessary.

6.1.2 Composite Search Options

6.1.2.1 PCR Primers

Compatible Pairs

The "Compatible Pairs" option is the most comprehensive search in the OLIGO program. It is also typically the most frequently used because it generates optimal PCR primer pairs from a nucleic acid template. Compatible primer pairs can be selected across the entire active sequence, or they can be selected from specific regions on the file by setting search ranges. Setting search ranges also speeds up this and other OLIGO searches. The stringency of the search can be controlled using the search stringency settings (global) and/or individual parameter settings in the "Search Parameters" dialog box.

The "Search for Compatible Primer Pairs" proceeds as follows:

- Search for optimized positive strand primers for PCR
- Search for optimized negative strand primers for PCR
- Match cross-compatible primers as potential primer pairs

The primer pairs generated in this search have these characteristics (the characteristics depends on the chosen search stringency):

- Free of 3' dimers and hairpins, free of 3'-end homology with any sites on the active sequence (false priming), and free of 3' end homology with any sites on other checked files, including repetitive sequence files
- Closely matched T_m s within the set T_m range
- Stable (GC Clamp)
- Have 3'-ends with low false priming homology on the active file and other selected files
- Free of homooligomers and dinucleotide repeats
- Cross compatible

Additionally, with "Complex Substrate" option, OLIGO searches for:

- Specific — minimized for false priming on unknown sequence (moderately low 3' stability)
- Primers of which 3'-end sequences are not abundant in a selected database.

Primers Compatible with the Upper Primer

This option searches the negative strand for optimal Lower Primers that are compatible with a pre-selected Upper Primer. The Upper Primer may be selected from the active sequence or entered with the keyboard (see the Edit menu).

Note

You must have selected an Upper Primer for this option to be available.

When you choose this option with the default subsearches, OLIGO selects negative strand primers that:

- Are within the selected stability limits (T_m or ΔG)
- Are free of homooligomers and dinucleotide repeats
- Are free of duplex-forming (self-dimerizing or hairpinning) structures
- Have 3'-ends with low false priming homology on the active file and other selected files
- Are compatible (have no potential 3'-terminal dimer forming homology) with the Upper Primer
- Have a stable 5'-end (GC Clamp)

Additionally, with "Complex Substrate" option, OLIGO searches for:

- Are highly specific (tested for moderate stability on the six 3'-terminal bases)
- Primers of which 3'-end sequences are not abundant in a selected database.

Following a search, the position numbers in the R2 table represent the 3' terminal nucleotide positions of primers compatible with the Upper Primer. It may be more useful, however, to click on the "Primer Pairs" button at the bottom of the "Search Status" window.

Primers Compatible with the Lower Primer

This option searches the positive strand for optimal Upper Primers that are compatible with a pre-selected Lower Primer. The Lower Primer may be selected from the active sequence or entered with the keyboard in the *Edit* menu.

When OLIGO conducts this search, it looks for potential Upper Primers that:

- Are within the selected stability limits (T_m or ΔG)

- Are free of homooligomers and dinucleotide repeats
 - Are free of duplex-forming (self-dimerizing or hairpinning) structures
 - Have 3'-ends with low false priming homology on the active file
 - Are compatible (have no potential 3'-terminal dimer formation) with the Lower Primer
 - Have a stable 5'-end (GC Clamp)
- Additionally, with "Complex Substrate" option, OLIGO searches for:
- Are highly specific (tested for moderate stability on the six 3'-terminal bases)
 - Primers of which 3'-end sequences are not abundant in a selected database.

For consensus primers, it checks the priming efficiency number, not the homology.

Following a search, the position numbers in the R1 table represent the 3' terminal nucleotide positions of primers compatible with the Lower Primer. It may be more useful, however, to click on the "Primer Pairs" button at the bottom of the "Search Status" window.

6.1.2.2 Sequencing Primers

The "Sequencing Primers" search option searches for and selects optimal sequencing primers in either or both strands of the active sequence. The OLIGO program automatically adjusts and/or deactivates search parameters in order to emphasize those characteristics most desirable in a sequencing primer.

When a standard search for sequencing primers is conducted using the programs default settings, the following parameters are active:

- OLIGO selects for high T_m sequencing primers, therefore, the stability (T_m or ΔG) maximum is not limited — it is set to the value of the most stable oligonucleotide in the sequence, while the stability minimum threshold is set just below the mean temperature of all the oligos in the file.
- OLIGO selects for highly specific primers resistant to false priming. Thus, the 3' terminal stability threshold is set to -8.0 kcal/mol, which filters out most non-specific (excessively stable) oligos. This threshold value can vary if the temperature used by the program to calculate stability (ΔG) is changed from the default.

To run the search for sequencing primers from the "Search for Primers and Probes" dialog box:

1. Select *Primers and Probes* from the *Search* menu.
2. Click on the "Sequencing Primers" button.
3. Click in either or both boxes at the top of the window to select "+ Strand Search," "- Strand Search," or both.
4. Click on the "Search Ranges" button to check your search ranges. The default is to check the entire sequence.
5. Establish your subsearches by clicking on the subsearch boxes you want (optional).
6. Click on the "Search Parameters" button to check your search stringency and other search parameters (optional).
For most searches, a high or very high stringency setting is recommended, since the program automatically relaxes settings if no oligos are selected at higher settings. However, the "Automatically change stringency" box must be checked for OLIGO to relax the settings.

Note

Turn off the "Automatically change stringency" box (Search Parameters dialog) when you manually set search parameters. You may lock certain parameters by clicking at the "lock" button. When the parameter is unlocked, it may be changed automatically during the search. Stringencies are automatically changed in a "smart" way, such that a parameter that causes most oligos to be removed is relaxed first. When too many oligos are selected OLIGO may automatically increase the search stringency.

7. Return to the "Search for Primers and Probes" dialog box and click "OK" to start the search. During the search, the "Search Status" window displays the progress of each subsearch in each strand, and the number of oligonucleotides accepted and rejected.
8. At the conclusion of the search, you may check stringency settings using "Search-Primers & Probes Search" Data option. To review the selected sequencing primers, click on the "Selected Oligos" button at the bottom of the window to call up the "Selected Oligos" window.
9. After selecting the sequencing primers you want as Upper and/or Lower Primers, you can download them to the database or otherwise export them from the program.

6.1.2.3 Hybridization Probes

The "Hybridization Probes" search option searches for and selects optimal hybridization probes in either or both strands of the active sequence. The default stability setting (T_m or ΔG) is high for the hybridization probe search. The stability maximum is not limited (it is set to the value of the most stable oligonucleotide in the sequence) and the stability minimum for the search is set at the 83rd percentile between the least and most stable oligonucleotides in the active sequence.

When OLIGO conducts the hybridization probe search, it selects probes that are:

- Within stability limits
- Are free of homooligomers and dinucleotide repeats
- Hairpin free

For consensus probes, it checks the homology, not priming efficiency number.

6.1.3 Primers/Probes Subsearches

In the lower left corner of the "Search for Primers and Probes" dialog box are eight subsearches that are activated in various combinations in OLIGO's composite searches for primers and probes. By clicking on any subsearch box, however, you can add or subtract the subsearch from any composite primer/probe search. The subsearches filter oligonucleotides according to search parameter values. These search parameters are automatically set by the OLIGO program to values that depend on the specific search selected and the search stringency setting. These can be overwritten by values you enter.

Search parameters and search stringency settings are on the "Search Parameters" dialog box called up by clicking on the "Search Parameters" button.

6.1.3.1 Duplex-free Oligonucleotides Subsearch

The "Duplex-free Oligonucleotides" subsearch is included in the default composite PCR searches and sequencing primers searches. It is not included in the default search for hybridization probes.

This subsearch, when active, checks the designated search ranges of the active sequence for duplex-free oligos, applying the following search parameters:

- Acceptable 3' dimer ΔG
- Maximum length of acceptable dimers
- n 3'-terminal nucleotides checked for dimers

Oligos can be rejected, first, on the basis of an excessively stable 3' dimer involving the 3' terminus. This stability is determined by ΔG measurement. Second, oligos can be rejected (independent from the ΔG method) if they contain a dimer with a string of matching contiguous bases longer than the "maximum length of acceptable dimer" setting. This occurs, however, only if the dimer is within the "n 3'" terminal nucleotides checked for dimers" setting.

This subsearch checks oligonucleotides for hairpin stems, for self-dimerizing and, in the case of PCR primers, cross-compatibility between positive strand and negative strand primers. "Cross-compatibility" in the OLIGO program refers to the lack of 3' dimers between two primers.

6.1.3.2 Highly Specific Oligos Subsearch

The "Highly Specific Oligos" (3'-end Stability) subsearch checks the 3'-ends of potential primers, selecting only oligos with two 3'-terminal pentamers (six bases) that fall within the defined terminal stability ranges. The default setting selects for oligos with moderately unstable 3'-ends, as these oligos will typically be specific — not false prime — yet still prime efficiently on the intended target. This subsearch is designed to decrease false priming potential in genomic samples, or samples containing unknown sequences.

Oligonucleotides with unstable 3'-ends are more specific because a longer stretch of homologous 3' nucleotides are required to false prime than are oligos with stable 3'-ends. Thus, there are statistically fewer sites where an oligo with an unstable 3'-end can false prime. A very low stability at a primer's 3'-end, however, makes it less efficient; the optimal 3'-end pentamer stability is approximately -8.5 kcal/mol.

The selection filter for this subsearch is controlled by the "3'-terminal stability range" setting in the "Search Parameters" dialog box. The current stability ranges set for this parameter are displayed in the "Internal Stability" window as two dotted lines.

6.1.3.3 Oligonucleotides With GC Clamp Subsearch

The "Oligonucleotides With GC Clamp" subsearch selects for oligonucleotides that contain a stable segment of nucleotides anywhere along its length. Note that OLIGO selects also for less stable 3'-ends to improve specificity (oligos with 3'-terminal GC clamps may be removed).

To form a stronger bond with the intended target and offset the reduction of priming efficiency because of an unstable 3'-end, the program selects for stable segments over the rest of the oligo.

The selection filter for this search is controlled by the "GC Clamp Stability" setting in the "Search Parameters" dialog box.

6.1.3.4 Eliminate False Priming Oligonucleotides Subsearch

The "Eliminate False Priming Oligonucleotides" subsearch selects oligonucleotides that have no false priming sites on the active sequence. This subsearch uses a proprietary algorithm that measures the stability of a false priming site regardless of mismatches. The stability of a false priming site is expressed in a "priming efficiency" number.

The priming efficiency is calculated with a complex algorithm that considers the ΔG of duplexes, mismatches, and/or bulge loop size, and the distance of these elements from the 3' end. Priming is likely when this value is approximately 200 or more.

The selection filter for this subsearch is controlled by the "False Priming Efficiency" setting in the "Search Parameters" dialog box.

6.1.3.5 Continue False Priming Search In Other Files Subsearch

The "Continue False Priming Search in Other Files" subsearch uses the same algorithm as the "Eliminate False Priming Oligonucleotides" subsearch, except that it searches in files other than the active sequence file. This may be helpful if you have a sequencing project where many sequences are produced and are in separate files.

This is the only subsearch that is not active in the default configuration for PCR and sequencing primer searches.

Click on the subsearch box to call up the "Selected Files" dialog box. The dialog box lists the directory of sequence files stored in the OLIGO folder and provides access to other drives and folders where you may have files.

To add files to the list to search:

1. Click on the file name you want to add to the search.
2. Click on the "Add" button.
You can add as many files as you want to check. The files are checked sequentially (in a batch file search) for false priming sites.
3. When you are finished selecting files, click the "Done" button to return to the "Search for Primers and Probes" dialog box.

To remove files from the list:

1. Click on the file name you want to remove from the list.
2. Click on the "Remove" button.

Note

If you are conducting searches for PCR and sequencing primers that are to be used in genomic or other complex DNA samples, this subsearch has a special "FREQSEQ" folder (frequently occurring or repetitive sequences in various organisms), where files may be called up to filter out oligos in the active file containing repetitive sequence.

Click on the "FREQSEQ" folder to call up the individual organism files.

The "FREQSEQ" folder includes sequence files for human DNA, *Drosophila*, mouse, rat, wheat, and yeast. If you are working with other organisms, you can create

your own frequent or repetitive sequence file(s) by extracting them from GenBank.

Once the files are selected and the search is started, OLIGO rejects any oligonucleotide sequence that would false prime on any target in a repetitive sequence file.

This subsearch is controlled by the "False Priming Efficiency" setting in the "Search Parameters" dialog box.

6.1.3.6 Oligonucleotides Within Selected Stability Limits Subsearch

The "Oligonucleotides Within Selected Stability Limits" subsearch selects oligonucleotides with T_m s or (ΔG values) that fall within the default values set by OLIGO or values you have entered in the "Search Parameters" dialog box, (the "Oligo T_m Range" search parameter).

OLIGO settings are based on the premise that optimal PCR primers should have moderate T_m s and optimal sequencing primers and hybridization probes should have high T_m s.

The program sets the following default stability values for the various primer/probe searches when the search stringency is set to "Very high."

PCR Primer Searches	Between the 25th percentile to the 75th percentile of the range between the lowest and the highest T_m oligos
Sequencing Primer Searches	Between the 50th percentile and the 100th percentile
Hybridization Probes	Between the 90th percentile and the 100th percentile

When the search stringency setting is reduced, these default ranges are progressively broadened. The actual upper and lower stability (T_m) thresholds are displayed as dotted lines on the T_m window graph.

6.1.3.7 Hairpin-free Oligonucleotides Subsearch

The "Hairpin-free Oligonucleotides" subsearch selects hairpin-free oligonucleotides from the active sequence. It is active in the default configuration for all the primer/probe automated searches.

This subsearch is similar to the "Duplex-free Oligonucleotides" subsearch in that both searches remove oligos with hairpins, but the "Hairpin-free Oligonucleotides" search differs in the following respects:

- It uses an algorithm that takes into account loop size in addition to the stability of the hairpin stem.
- It checks the entire oligonucleotide (as opposed to only the 3'-end).

This subsearch is controlled by the "Loop ΔG Threshold" setting in the "Search Parameters" dialog box.

6.1.3.8 Eliminate Homooligomers/ Sequence Repeats Subsearch

The "Eliminate Homooligomers/Sequence Repeats" subsearch checks for oligos that contain strings of the same nucleotide (AAAAA, for example), or strings of dinucleotide repeats (CGCGCGCG, for example) and eliminates these from the selection process. Oligos with nucleotide repeats may not align correctly on the intended target, causing subsequent deletions or insertions. Also, such sequences frequently occur, and thus, are more likely to false prime.

Oligonucleotides that have a string of six or more of the same nucleotide or three or more repeats are eliminated.

This is the only subsearch that is not controlled by the settings in the "Search Parameters" dialog box, so the selection parameters are never modified by the program and cannot be edited.

6.1.3.9 Eliminate Frequent Oligos Subsearch

This subsearch eliminates oligonucleotides having 3'-ends (the last 6 or 7 nucleotides.) which occur frequently in GenBank - specifically, the sequence subset of GenBank for the species you are sequencing , or amplifying with PC. The user-selected frequency tables (located in the Frequencies folder) contain normalized frequencies of either 6- or 7-mers of GenBank sequences. The header of each frequency file describes in detail how each table was created. The sequence frequency is a relative number and does not

represent absolute number of hits in a given database. It is normalized such that frequency of 1000 is the average frequency for a given 6- or 7-mer. Sequences below this number are less frequent. The actual formula is the following: $\# \text{ of Hits} \times 1000 \times 4096 (= \text{Table size}) / \text{number of analyzed 6-mers in a database}$.

By choosing oligos of which 3'-ends are infrequent in a given database you are decreasing chances of selecting a primer that primes in several sites on a complex substrate, such as genomic DNA, or in other words, you are decreasing chances of unspecific PCR.

6.1.4 Search Mode

The "Search Mode" is controlled by the "Mark" and "Unmark" buttons at the lower right corner of the "Search for Primers and Probes" dialog box.

Mark — The "Mark" search mode is standard for all composite searches and subsearches, wherein oligonucleotides that pass through all search filters are "marked" for selection. Oligos selected in a search have their 5' or 3'-terminal nucleotide marked (blue squares) on the memory arrays at the bottom of the "Internal Stability" window. The R1 array is for positive strand selections (5' is marked); the R2 array is for negative strand selections (3' is marked).

Unmark — The "Unmark" search mode is reserved for specialized applications where the user manually marks on the R1 or R2 array groups of oligos to be considered for selection. Oligos may be marked by a previous automatic search.

Setting upper and lower search ranges can be used to limit program selections, in a broad sense, but manual marking and unmarking gives you more flexibility.

After manually selecting oligonucleotide positions on a memory array, click "unmark," and run a standard composite search such as a PCR search. Oligos that fail to pass through the various subsearch filters are unmarked, leaving only oligos that have been both

marked by you (or by the program in an earlier search) and pass through all filters of the selected search.

6.1.5 Primers and Probes — Search Ranges

Using the "Search Ranges" dialog box, you may limit searches to specific segments of the active sequence, as opposed to the entire active sequence (default). PCR product length limits may also be set in this dialog box, in addition to the positive and negative strand search range limits for primer and probe selection.

The setting of search ranges are most frequently used to shorten search times and/or when a researcher has limited targets to which he can design primers. Setting search ranges can also be used to limit the total number of primer pairs selected in a given PCR primer search since the computer's memory limits the total number of selected primer pairs to approximately 3,000.

6.1.5.1 Positive Strand Primer Search Range

Establish the positive strand search range by setting the 5' and the 3' position limits. Note that the position limits refer to the 5'-end of an oligo, not the entire oligo — the 3'-end of a selected primer can extend beyond the 3' limit of the search range.

6.1.5.2 Negative Strand Primer Search Range

Establish the negative strand search range by setting the 5' and the 3' position limits. Note that the position limits refer to the 3'-end of an oligo, not the entire oligo — the 5'-end of a selected primer can extend beyond the 3' limit of the search range.

6.1.5.3 PCR Product Length

Establish the PCR product length by entering the maximum and minimum desired product lengths. Make sure that your search ranges on the positive and negative strand can accommodate the PCR product length you want.

The default minimum length is 150 nucleotides. The minimum allowable length is two times the Current

Oligo length (the length of the Upper and Lower Primers).

6.1.6 Primers and Probes — The Search Parameters Dialog Box

The *Search Parameters* are the numerical thresholds and windows that control the selection of the filters used in OLIGO searches. In this dialog box, most parameters may be adjusted from default values.

In addition, six discrete settings are available in the dialog box for global control of search stringency, while the following functions are "On/Off" functions:

- Inverse PCR
- Automatic Adjustment of Oligo Length to Match T_m 's or P.E. #.
- Automatic Adjustment of Search Stringency

For more information on "Search Parameters," including detailed descriptions of the functions in the "Search Parameters" window, refer to *Chapter 8*, page xx.

6.2 Search — Hairpin Loop Stems

The *Search for Hairpin Loop Stems* command searches the active sequence and marks the locations of hairpin loop stems within oligonucleotides of the selected length. This search may be limited to a specific region by using the *Search Ranges* command from the *Change* menu.

The position data of the hairpin stems found are stored in the active Memory Table. The positions marked represent all of the nucleotides comprising a hairpin stem, not just the 5'-terminal position of the stem. This search is particularly useful when designing the oligonucleotides to be used in a synthetic gene construction project.

6.3 Search — Sequence String

The *Search for Sequence String* command searches the positive or negative strand of the active sequence for a sequence string of nucleotides that you select.

The position data of the strings found are stored in the active Memory Table and displayed in the memory array on the "Internal Stability" window. A search in the positive strand marks the 5'-terminal bases of any sequence strings found, and a search in the negative strand marks the 3'-terminal bases.

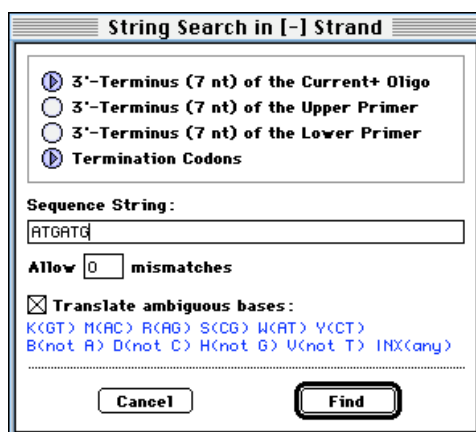


Figure 6.3 The Search in the [-] Strand dialog box (Mac).

Note

The reporting of found positions after a string search in the negative strand can be confusing. The reported position of a short string — six nucleotides, for example — will be 15 nt downstream from the actual location of the string, if the Current Oligo length is 21; the string is located at the 5'-end of the Current (-) Oligo, whereas the position number reported is the Current (-) Oligo's 3'-terminus.

Sequence strings that include ambiguous bases can be used in this search option. A search for AGN, for example, marks the positions of every AGA, AGC, AGG, and AGT string in the active sequence.

When you select *Search for Sequence String*, choose from the submenu a search in either the positive or negative strand.

After selecting the positive strand, the "String Search in [+] Strand" dialog box appears. After selecting the negative strand, the "String Search in [-] Strand" dialog box appears.

You may also search for pre-defined strings and/or define any number of mismatches in the string of interest.

6.4 Search — Palindromes

The *Search For Palindromes* command searches the active sequence for palindromes (nucleic acid sequence strings that are identical to their complements).

The position data of the palindromes found are stored in the active Memory Table and displayed on the memory array in the "Internal Stability" window. The positions marked represent all of the nucleotides within a palindrome, not just the terminal position. The position data may be confusing if palindromes overlap.

The search for palindromes lists all palindromic sequences greater than five nucleotides in the active sequence, regardless of the settings in the "Search Ranges" dialog box.

6.5 Search — Restriction Sites

The *Restriction Sites* function searches a nucleic acid active sequence for restriction enzyme recognition sites and displays the resulting data in a table of positions and fragment sizes, and/or on a position map. The nucleic acid sequence can be treated as circular or linear DNA. When circular, the first and last fragments

on the active sequence from a given enzyme cut are treated as one.

When you access this option, the "Search for Restriction Sites" dialog box appears where you set search range, circular/linear, and the desired output.

If you would like to update the restriction enzyme database, you may download OLIGO-compatible REBASE files from WWW.NCBI.NLM.NIH.GOV or from the New England Biolabs' computer (FTP:VENT.NEB.COM, directory: pub/rebase; the type 2 format is compatible. Contact NCBI for the details. The most common enzymes are in the NICE6&UP.ENZ file. You may open it with any word processor and change the list. The format is quite simple and easy to follow.

Search

To set the search:

1. Click on the "Entire" button or the "Portion" button.
2. If you select "Portion," enter the start and end position for the search range.
3. If your sequence is linear, click on the "linear" button. If it is circular, click on "circular."

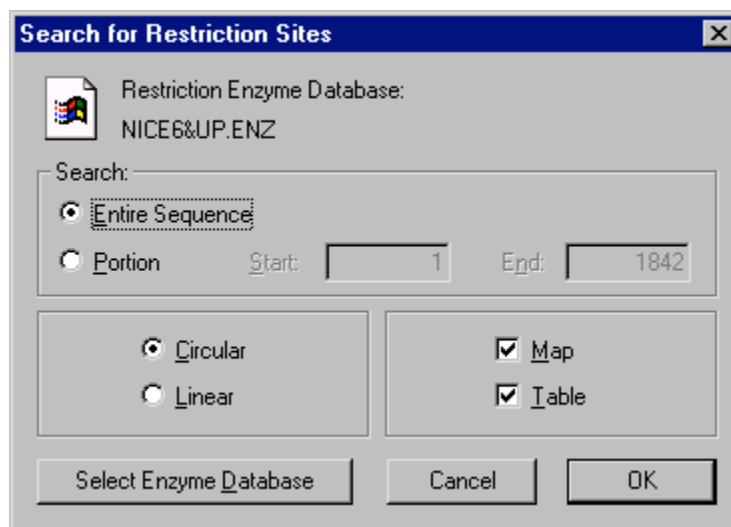


Figure 6.5.1 The Search for Restriction Enzymes dialog box (Win). The Start and End search positions are dimmed when the "Entire Sequence" button is ON. The default is "Portion", however, the entire sequence length is displayed.

Circular or Linear

The "Circular/Linear" button option selects for the correct calculation of the restriction fragment sizes upstream of the first restriction cut and downstream of the last cut on the active sequence. If "circular" is selected, these distal fragments are considered as one contiguous fragment in the fragment table. If "linear" is selected, they are considered two separate fragments.

Map And Table

You may have the data from a restriction site search displayed on a restriction site position map (see Fig. 6.5.2),

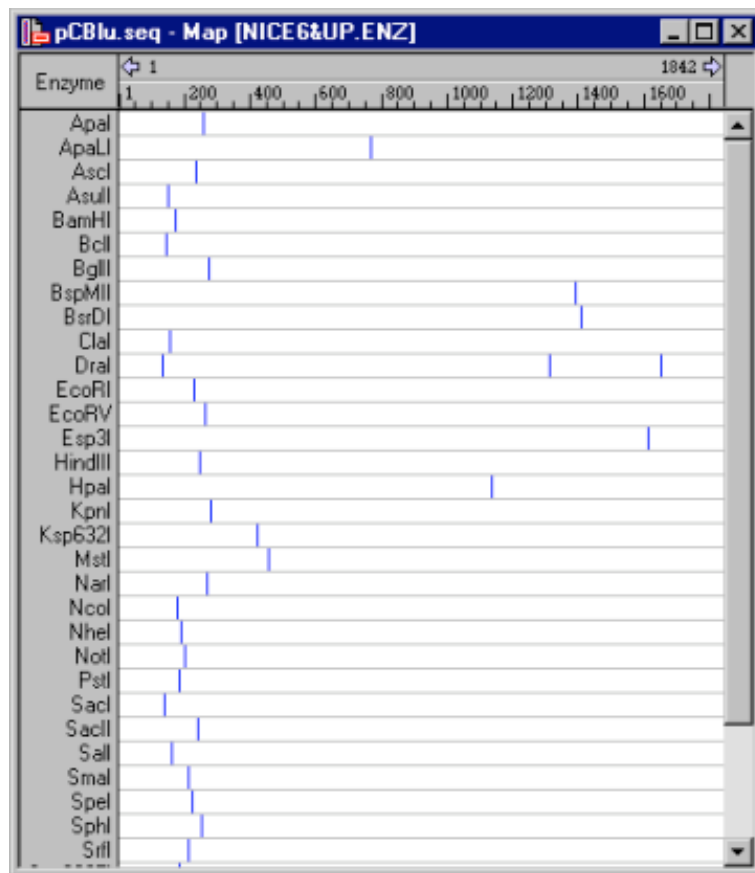


Figure 6.5.2 Results of the Search for Restriction Enzymes, graphical map (Win).

or in a table listing the number of cuts and fragment position/size (Fig. 6.5.3), or both.

Enzyme	Site	# Cuts	Positions & Fragment Sizes
NcoI	C [^] CATGG	1	180 1842
NheI	G [^] CTAGC	1	193 1842
NotI	GC [^] GGCCGC	1	200 1842
PstI	CTGCA [^] G	1	187 1842
SacI	GAGCT [^] C	1	140 1842
SacII	CCGC [^] GG	1	240 1842
Sall	G [^] TCGAC	1	164 1842
SmaI	CCC [^] GGG	1	215 1842
SpeI	A [^] CTAGT	1	222 1842
SphI	GCATG [^] C	1	252 1842
SrfI	GCCC [^] GGGC	1	214 1842
Sse8387I	CCTGCA [^] GG	1	186 1842
SspI	AAT [^] ATT	1	1706 1842
SwaI	ATTT [^] AAAT	1	131 1842
XbaI	T [^] CTAGA	1	208 1842
XhoI	C [^] TCGAG	1	169 1842
XmaII	C [^] GGCCG	1	201 1842

Non-cutting enzymes:
AatI AatII AflII AgeI AvrII BclI Bsp1407I BspHI Eco31I Eco47III MluI NaeI
PvuI PvuII SapI Scal SnaBI SphI VspI XcmI

Figure 6.5.3 Results of the Search for Restriction Enzymes, table (Win).

6.6 Search — Restriction Sites in Protein

This function searches the amino acid sequence currently displayed on the Melting Temperature window for potential restriction sites. The protein sequence is reverse translated using the degenerate method yielding all possible oligo sequence combinations before the sequence is searched. The search parameters and output are the same as for the restriction sites search.

OLIGO has 79 restriction enzymes that can be selected for the search. All are six-base cutters or longer, and none have degenerate bases. In the table displayed after the search, under the "Site" field, protein sites that are recognized during the search are listed. OLIGO uses its own symbols for various degenerate amino acids, see *Appendix E — Degenerate Amino Acid Symbols*. Standard amino acid symbols are listed in *Appendix E — Nondegenerate Amino Acid Symbols*.

6.7

Search — Memory Table Functions

6.7.1 Memory Tables

The *Memory Table* command opens the Memory Tables window. You may search manually for the selected positions and primers. You may move to any displayed position (select Current Oligo) by clicking on the displayed numbers.

6.7.2 Copy Memory Table

The *Copy Memory Table* command copies one Memory Table to another. This function is generally used to preserve position data stored in the R1 or R2 Memory Tables by copying them to the R3 table.

Caution

The R1 and R2 tables automatically receive data from new searches, and any data residing there from previous searches is overwritten. Use the Copy Memory Table option to avoid losing search data you want to save.

6.7.3 Delete Memory Table Data

The *Delete Memory Table Data* command clears all position data stored in the current Memory Table. The Memory Tables — R1, R2, and R3 — are marked in the lower left corner of the "Internal Stability" window.

Caution

There is no undo command available to reverse this deletion.

6.7.4 Combine Memory Tables

The *Combine Memory Tables* command selects data common to two selected Memory Tables and stores the results in the active Memory Table.

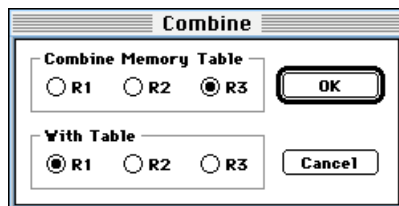


Figure 6.7.3. The "Combine Memory Tables" dialog box. After combining Tables R3 and R1, table R3 holds the results.

6.7.5 Invert Memory Table

The *Invert Memory Table* command marks all unmarked positions and unmarks all marked positions

in the active Memory Table. This function is typically used in association with specialized search applications, such as the marking of non-unique oligonucleotides.

6.8

Search — Primers and Probes Search Data

The *Primers and Probes Search Data* command from the *Search* menu records the search settings and raw data for the previous "primers and probes" search conducted. This data window saves all settings from the "Search for Primers and Probes" dialog box, the "Search Parameters" dialog box, the search ranges dialog box, and the search results listed in the "Search Status" window, including the oligos eliminated in each subsearch. If you want to save the results of a given search, save or print the "Primers and Probes Search Data" window before running another search because each new search overwrites the search data from the last search. This window is especially useful when searching for consensus primers: it is easy to spot non-homologous sequence files and eliminate them from consideration.

Primers & Probes Search Data		
House histone H4.seq		
General Info		
File Name:	House histone H4.seq	
Search Type:	Compatible Pairs	
Oligonucleotide Length:	21	
Adjust Length to Match T _m 's:	Yes	
Inverse PCR:	No	
Search Ranges		
+ Strand:	212 to 500	
- Strand:	400 to 778	
Product Length:	150 to 968	
Conditions for Upper and Lower Primers		
Minimum Acceptable 3' ΔG:	-3.7 kcal/mol	
Maximum Length of Acceptable Dimers:	6 base pairs	
# of 3' Terminal Nucleotides Checked for Dimers:	12 nucleotides	
3' Terminal Stability Range:	-4.5 to -10.2 kcal/mol	
GC Clamp Stability Minimum:	-9.0 kcal/mol	
Minimum Acceptable Loop ΔG:	-0.5 kcal/mol	
Oligo T _m Range:	61.5 to 87.9 °C	
Minimum Consensus Priming Efficiency:	320 points	
Maximum Acceptable False Priming Efficiency:	180 points	
Number of Acceptable Sequence Repeats:	5	
Frequency Threshold:	1150	
Maximum Degeneracy:	1	
Conditions for Primer Pairs Selection		
Minimum Acceptable 3' ΔG:	-3.5 kcal/mol	
Maximum Length of Acceptable Dimers:	4 base pairs	
# of 3' Terminal Nucleotides Checked for Dimers:	12 nucleotides	
Search Results		
Subsearches	Oligos Rejected	
	+ Strand	- Strand
Oligonucleotides within Selected Stability Limits:	27	46
Eliminate Ambiguous Bases:	0	0
Eliminate Homooligomers:	0	24
Oligonucleotides with GC Clamp:	3	11
Highly Specific Oligos:	63	69
Frequent Oligos:	92	78
Duplex-Free Oligonucleotides:	15	21
Hairpin-Free Oligonucleotides:	51	54
Select Consensus Primers:	34	62
Eliminate False Priming Oligonucleotides:	2	3
Files Searched for False Priming Oligonucleotides:	-	-
Total Oligos Rejected:	267	368
Total Oligos Accepted:	22	11
Total Pairs Accepted:	24	
Frequency Database		
GBPRI.FR6		
Files Searched for Consensus Primers		
Drosophila histone H4.seq		

Figure 6.8 The Primers and Probes Search Data window (Mac).

7

The OLIGO Select Menu

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7 The OLIGO Select Menu

From the Select menu you can select Upper and Lower Primers, a New Current Oligo Position, a new active Memory Table, and a new Current Oligo from the Memory Tables.

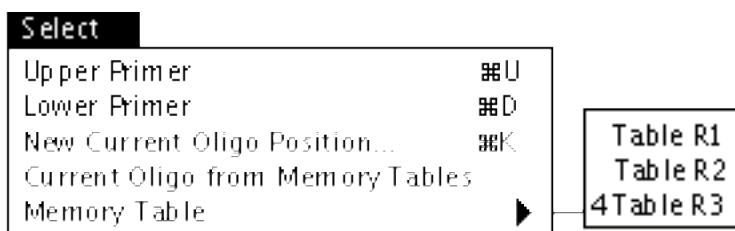


Figure 7.0 The OLIGO Select submenu (Mac). In Windows the same items are displayed, but the short-cut keys are different:

7.1 Select — Upper Primer

The *Upper Primer* command from the *Select* menu selects the positive strand Current Oligo as the Upper Primer. Once selected, the Upper Primer is displayed in red on the "Melting Temperature" window, one line above the Current Oligo and followed by a "primer extension" arrow pointing toward the 3'-end of the sequence. The Upper Primer is displayed in upper case letters, except for nucleotides mismatched to the active sequence, which are displayed in lower case letters.

You can also select the Upper Primer with the "Upper" button at the left of the "Melting Temperature" window, or enter and/or modify it via the keyboard (see the *Edit* menu). The short cut key: Mac: <⌘U>, Win: <Ctrl-U>.

7.2

Select — Lower Primer

The *Lower Primer* command from the *Select* menu selects the negative strand Current Oligo as the Lower Primer. Once selected, the Lower Primer is displayed in blue on the "Melting Temperature" window, one line below the Current Oligo and followed by a "primer extension" arrow pointing toward the 5'-end of the sequence. The Lower Primer is displayed in upper case letters, except for nucleotides mismatched to the active sequence, which are displayed in lower case letters.


You can also select the Lower Primer with the "Lower" button at the left of the "Melting Temperature" window, or enter and/or modify it via the keyboard (see the *Edit* menu). The short cut key: Mac: <⌘L>, Win: <Ctrl-L>.

7.3

Select — New Current Oligo Position

The *New Current Oligo Position* command calls up the "Current Oligo Position" dialog box where you can choose a new Current Oligo by entering a new 5' position number (upper strand). After entering a new position, click "OK."

The Current Oligo position also can be changed by using one of the following methods:

1. Move the scroll bar on the bottom of either window to position the graph approximately where you want to move in the sequence. Using the mouse, position the cross-hair cursor on the position you want and click. OLIGO automatically shifts the graph on both windows and displays updated information.
2. Click on the far left icon  at the bottom of the "Melting Temperature" window to call up the "Current Oligo Position" number. Enter the position number you want in the dialog box — the 5'-end of the positive strand Current Oligo or the 3'-end of the negative strand Current Oligo.

OLIGO automatically updates all relevant windows.

3. Use short cut key: Mac: <⌘K>, Win: <F10>.
4. Click on any row in the "Selected Oligos" table.
5. Click on any number in the "Memory Tables" window, that is, use the *Select-Current Oligo from Memory Tables* option.
6. Export a primer from a Database.

When browsing the active sequence for a *New Current Oligo*, it is helpful to first call up *Current Oligo* from the *Analyze-Key Info* menu, or relevant analysis windows.

Click and drag the *Current Oligo* window title bar to position it near the bottom of the screen. With the data visible, in the "Melting Temperature" window, you can analyze each potential oligonucleotide.

7.4 **Select — Current Oligo From Memory Tables**

This command calls up the "Memory Tables" window from which you can select a new Current Oligo position.

Clicking on a given position number in the "Memory Tables" window selects that position as the Current Oligo. The number of positions listed in each Memory Table is displayed at the bottom of the table. Use the adjoining scroll box to view all of the position numbers in a given table. Click on the title bar of the Memory Table to display a small window that lists the source of the data in the table.

The Current Oligo position is highlighted. Positions of the Upper and Lower Primers, if present, are marked with the letters U and L, respectively.

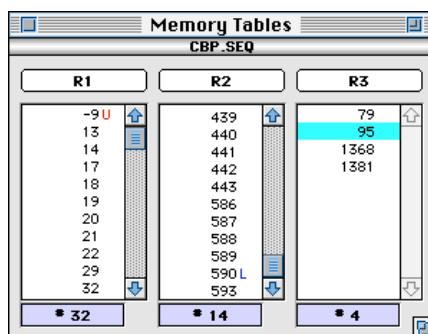


Figure 7.4 The Memory Tables window.

In Figure 7.4, the R2 table scroll box has been moved. The letters "U" and "L" following position numbers -9 and 590 in the R1 and R2 tables denote these oligonucleotides as the Upper and Lower Primers. The Current Oligo position in the Memory Tables is designated by the highlighted position number displayed (#95 in the R3 table). Clicking on any position number in a Memory Table selects it as the new Current Oligo.

7.5 Select — Memory Table

The *Select — Memory Table* command selects a new active Memory Table from the R1, R2, and R3 Memory Tables. When a given Memory Table is activated, it can accept data from a manual OLIGO search or by selecting specific positions on the "Memory Array" at the bottom of the "Internal Stability" window.

Caution

When a manual search is run that saves position data in the active Memory Table, previous data is erased. All searches for primers & probes use only R1 and R2 table.

Most of the composite searches automatically activate specific Memory Tables in which to store data from specific subsearches. In general, all positive strand searches store data in the R1 table and negative strand searches store data in the R2 table. Most other

searches (palindromes, hairpin loop stems, and sequence string) store data in the active table.

8

The OLIGO Change Menu

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8 The OLIGO Change Menu

The *Change* menu is used to change various parameters in the OLIGO program. These include changing the oligonucleotide length to be searched or analyzed, DNA active sequences to RNA and vice versa, search ranges and parameters, parameters used to calculate T_m and ΔG (non-search parameters), and parameters involving OLIGO displays.

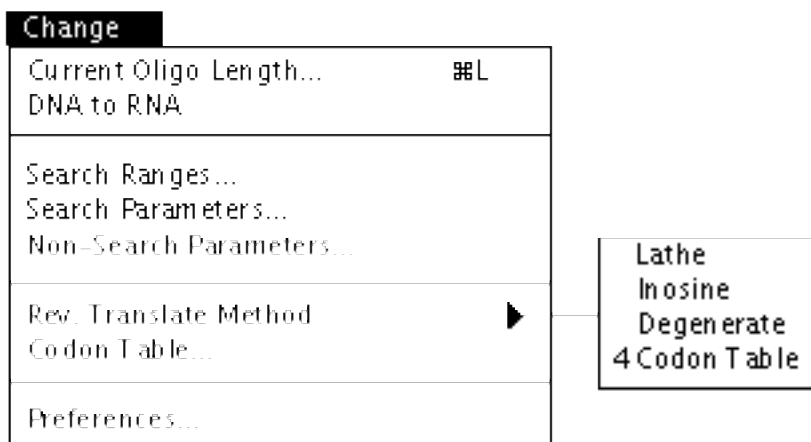


Figure 8.0 The OLIGO Change menu (Mac). In the Windows version the short cut <⌘L> key is replaced by <F9>.

8.1 Change — Current Oligo Length

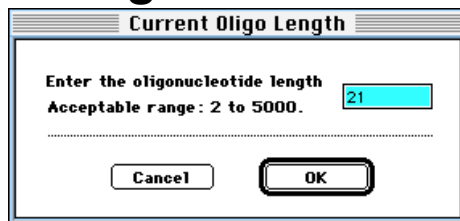


Figure 8.1 The Change-Current Oligo Length dialog box.

The *Current Oligo Length* command allows you to change the Current Oligo length for all searches and

analyses in the program. The default length is a 21-mer. To change it, enter a new oligonucleotide length within the allowable range of 2 to 5000 and click "OK." The Current Oligo length is displayed in the "Melting Temperature" window title bar.

This parameter sets the length of all oligonucleotides searched for and analyzed in the OLIGO program. However, if an oligonucleotide is saved as the Upper or Lower Primer, its length is not changed following a change in the oligo length setting. Therefore, oligonucleotides of different lengths can be searched for and analyzed.

Upper and Lower Primer lengths may be modified in the *Edit* menu.

Note

Several analysis features are inactive when the oligo length is set above 200 bases.

8.2 Change — DNA to RNA

The *DNA to RNA* command changes the active sequence from a DNA sequence to RNA and vice versa. All calculations, including molecular weight, melting temperatures, and absorbency values are changed accordingly. When the sequence is RNA, G-U pairings are permitted.

Note

This option changes the active sequence only in memory. There is no effect on the active sequence file unless it is saved after the change using the Save Sequence command from the File menu.

8.2.1 DNA to RNA — RNA Templates

To analyze a DNA oligonucleotide on an RNA template:

1. Save an oligo as an Upper or Lower Primer on a DNA active sequence.
2. Change the active sequence by selecting *DNA to RNA* under the *Change* menu.

DNA/RNA hybrid T_m s are calculated either as DNA/DNA or RNA/RNA in the "Composition & T_m " window.

8.3 Change — Search Ranges

Using the "Search Ranges" dialog box, you can set the range of searches to be run on the active sequence and the length of PCR products to be selected for in the various PCR searches. In the default configuration, searches are conducted over the entire length of the active sequence. However, you can set any beginning and/or ending nucleotide position, in either or both strands, to limit searches. Note that where a search is actually conducted (positive strand, negative strand, or both) is controlled by the check boxes at the top of the "Search for Primers and Probes" dialog box. The PCR product range setting limits the primer pairs selected to those that generate the correct product size. The default is 150 bp to the end of the active sequence, but shorter product sizes can be set. The minimum is the Upper Primer length plus the Lower Primer length.

The PCR product length may not always be compatible with search range. In such cases, the search range is the primary selection criteria. For example, if the positive strand primer search range is 1-100, the negative strand is 200-300, and PCR product length is 150-1,000, then possible product lengths are limited to 150-300 nt, plus the length of the Current Oligo.

Note

Setting search ranges can speed up searches considerably, plus eliminate unwanted data from search results. Also, low stringency settings typically yield many selected primer pairs. This may make further analysis difficult.

Sequence File: M13MP18 - [1 to 7251]

Positive Strand Primer Search Range: 1 to 7230

Negative Strand Primer Search Range: 1 to 7230

PCR Product Length: 150 to 7250

Parameters Cancel OK

Figure 8.3 The Change — Search Ranges dialog box.

8.3.1 Search Ranges — Positive Strand Primer Search Range

Establish the positive strand search range by entering the start and end position numbers as the limits for the intended search in the positive strand of the active sequence.

8.3.2 Search Ranges — Negative Strand Primer Search Range

Establish the negative strand search range by entering the start and end position numbers as the limits for the intended search in the negative strand of the active sequence.

8.3.3 Search Ranges — PCR Product Length

Establish the PCR product length by entering the upper and lower size limits of the desired product. Make sure that your search ranges on the positive and negative strand can accommodate the PCR Product Length you want.

Note

If you search for primers compatible with the Upper Primer whose position is close to the 3'-end, the search may not find any compatible primers, even if the search range is the whole active sequence.

8.4

Change — Search Parameters

The "Search Parameters" dialog box is accessed by clicking on the "Parameters" button on the "Search for Primers and Probes" dialog box. It contains the program default selection limits (parameters) for the various subsearches used by OLIGO's automated (composite) searches. It is also the only place in the program where you can customize a given search (or subsearch) by entering a search setting different than the default value. Searches can also be customized by adding or deleting subsearches on the "Search for Primers and Probes" dialog box.

8.4.1 Primers and Probes — Search Parameters Dialog Box Overview

The "Search Parameters" dialog box also includes search stringency settings for setting global search parameters, access to the "Search Ranges" dialog box through the "Search Ranges" button, and "OK" and "Cancel" buttons for accepting or rejecting changes. The "Search Parameters" dialog box can be accessed from the *Change* menu or from the *Search* menu via the "Search for Primers and Probes" dialog box.

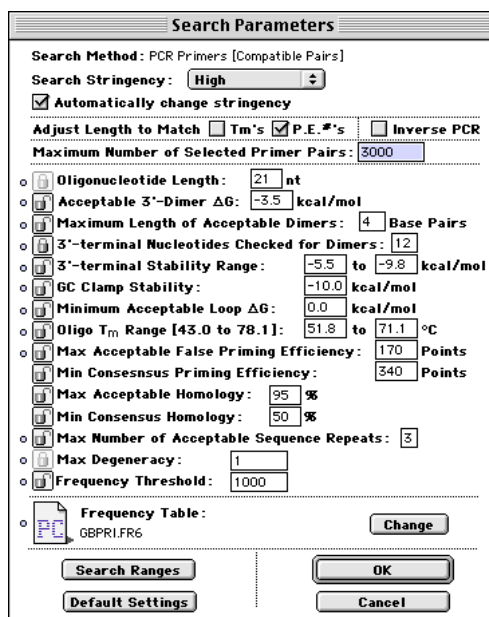


Figure 8.4.1a The Search Parameters dialog box (Mac).

In the Windows version this dialog box is replaced by 3 separate dialog windows, shown on Fig. 8.4.1b-d.

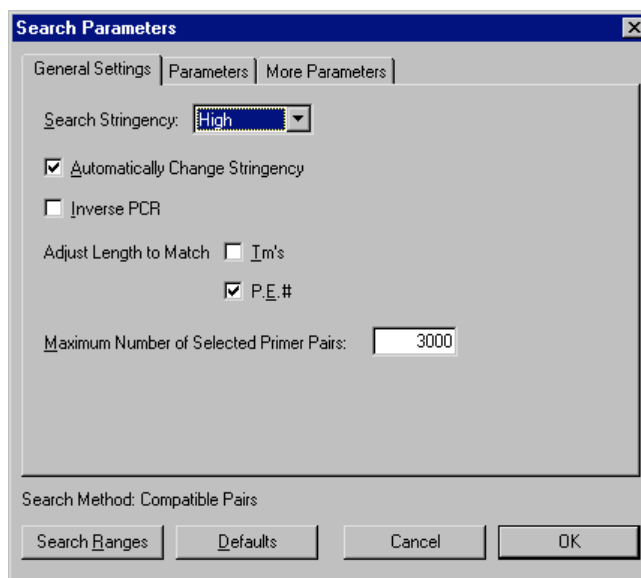


Figure 8.4.1b The Search Parameters dialog box: General Settings screen (Win).

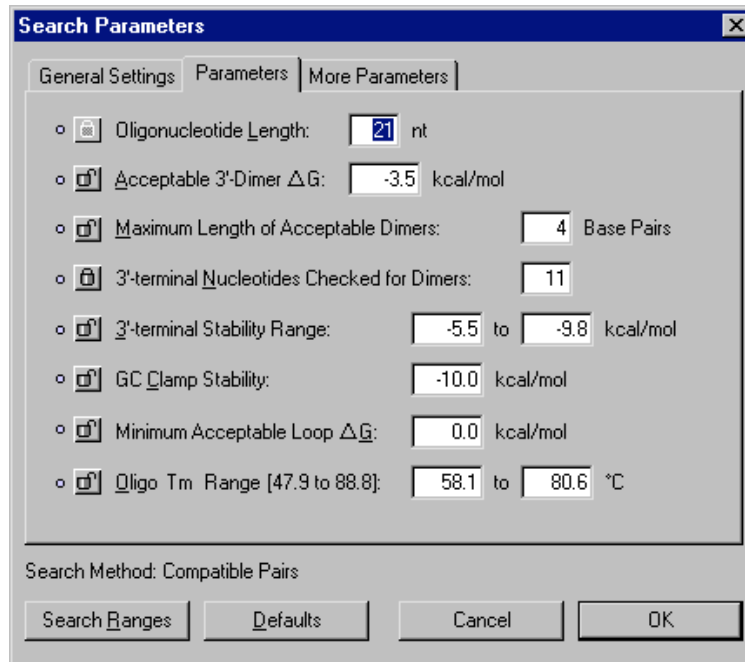


Figure 8.4.1c The Search Parameters dialog box: Parameters screen (Win).

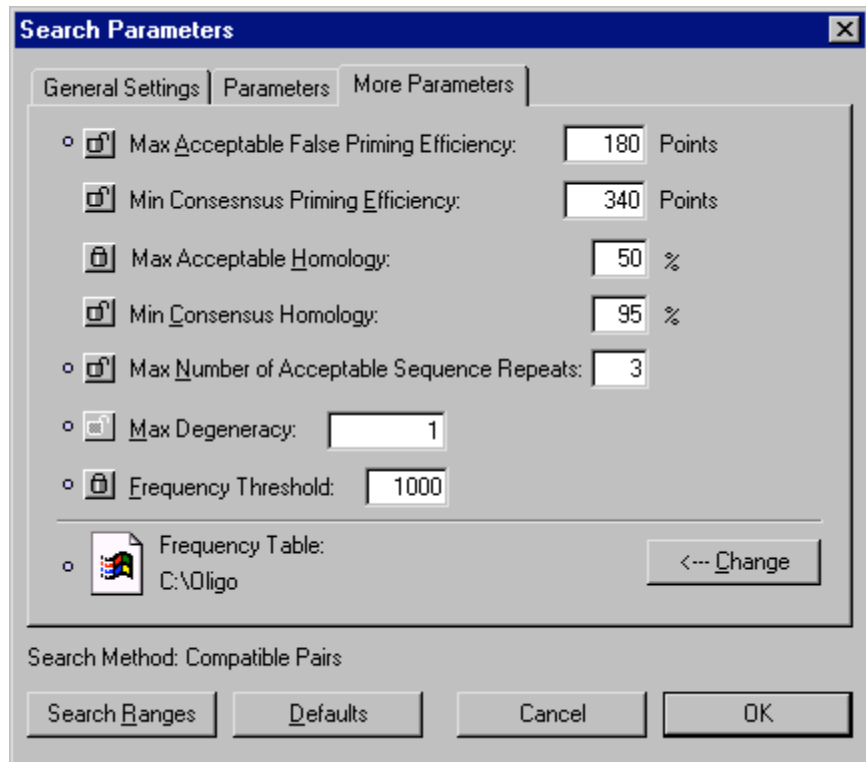


Figure 8.4.1d The Search Parameters dialog box: More Parameters screen (Win).

8.4.1.1 Search Stringency

The "Search Stringency" option provides you with the ability to change globally all search parameters in discrete increments preset in the OLIGO program using a single operation. Using this option, you can make "expert" decisions about your oligonucleotide selection process without knowing the optimal setting of the various search parameters.

OLIGO has pre-determined settings for the six search stringency options for each search type: the search for PCR primers, for sequencing primers, and for hybridization probes. They are: Very High, High, Moderate, Fair, Low, Very Low. Each stringency has its own set of parameters. Primers and probes are useful even when selected at the lowest stringency setting.

Note

A search stringency you enter may be automatically lowered by OLIGO if no oligonucleotides (or primer pairs) are found that meet or exceed the search stringency and the "Automatically change stringency" box is checked. Your search stringency setting is then automatically reduced by the program.


8.4.1.2 Automatically Change Stringency

The "Automatically Change Stringency" option on the "Search Parameters" dialog box — when active — enables the OLIGO program to automatically reduce the selected search stringency setting to the next lower stringency level if no primers, primer pairs, or probes pass through all the selection filters of the search. Initially, OLIGO changes only those parameters which eliminate most potential primers. When primers are found this way the stringency setting is called "Customized".

If no primers are found, the message "No matches found" appears. To find primers, perform one of the following:

- Broaden the search range settings
- Select a different region of the active sequence to search

- Deactivate one or more of the subsearches
- Change specific search parameters that eliminate oligos; review the search data and check the statistics using the *Primers and Probes Search Data* command from the *Search* menu

The Automatically Change Stringency option can be disabled for any individual search parameter by using the search parameter locking feature. Clicking the locking icon  adjacent a given search parameter, keeps that value unchanged throughout the search. This gives you more control over the search process in oligonucleotide selection.

The "Automatically Change Stringency" option ensures maximum optimization of oligos or oligo pairs for a given set of search ranges on a given active sequence. When this option is active, the program proceeds until primers or probes are selected at the highest possible stringency for a given search condition.

Note

This option overwrites any unlocked search parameter values you enter, if and when stringency is automatically reduced.

8.4.1.3 Adjust Length to Match T_m s / P.E. #'s

With the "Adjust Length to Match T_m s / P.E. #'s" option, OLIGO brings the melting temperatures of selected primer pairs into close agreement to optimize primer performance and to prevent possible false priming of the higher T_m (or higher P.E. #) primer — if Upper and Lower Primer T_m s (P.E. #'s) are disparate.

The "Adjust Length to Match T_m s / P.E. #'s" option — when active — removes nucleotides from the 5'-end of the more stable primer until T_m s, or P.E. #'s, match as closely as possible. This applies to all primer pairs following any PCR search.

Note

If it is important to keep the length of oligo pairs constant, deactivate this feature. Also, this feature is inactive in the Multiplexing command under the Analyze menu.

The default option is "Adjust Length to Match P.E. #'s", because matching primers with their priming efficiency numbers provides more balanced primer pairs (for more info check Chapters 5.5 and 8.4.2.10).

8.4.1.4 Maximum Number of Selected Primer Pairs

This field controls the max. number of selected PCR primer pairs. If the search finds more pairs it stops and asks whether to proceed with further search. Usually 3000 pairs (default) is sufficient.

8.4.1.5 Change [Frequency Database]

The Eliminate Frequent Oligos sub-search uses a table of oligonucleotide frequencies (6- or 7-mers). The selected Frequency table should correspond to the species on which you are working. The list of frequency tables is given in Chapter 1.5.3. If you need a frequency table not included in the list, please contact an authorized distributor of OLIGO software.

8.4.1.6 Search Ranges

This button opens the "Search Ranges" dialog, described in detail in Chapter 8.3.

8.4.1.7 Default Settings

This button changes all customized parameters to default settings for a given search stringency.

8.4.2 Primers and Probes — Search Parameters Dialog Box Options

In addition to the search stringency options on the "Search Parameters" dialog box are several other search features.

8.4.2.1 Inverse PCR

When the "Inverse PCR" box is checked, OLIGO orients selected primers in the opposite direction — Upper Primers downstream, Lower Primers upstream — permitting PCR searches to be useful for circular templates where an opposite-oriented PCR product is desired. The PCR window displays the inverse PCR product and predicts experimental conditions.

8.4.2.2 Oligonucleotide Length

Using this option, you may select oligos of up to 200 nt for searches. This search parameter can also be set

in the *Change* menu. This menu lets you change Current Oligo length to a greater value than 200 nt, however, in such a case the search for Primers & Probes is disabled.

Note

Any length setting entered in the "Oligonucleotide Length" option from the "Search Parameters" dialog box changes Current Oligo length globally.

8.4.2.3 Acceptable 3' Dimer ΔG

The "Acceptable 3' Dimer ΔG " option sets the stringency of the filter that selects or rejects oligos or oligo pairs on the basis of the stability of any 3' terminal dimer structure (hybridized to itself or to another oligo).

This feature operates on the basis of dimer stability only. It operates separately from, but complements, the "Maximal Length of Acceptable Dimers" search parameter.

8.4.2.4 Maximum Length of Acceptable Dimers

The "Maximum Length of Acceptable Dimers" option sets the stringency of a dimer filter that accepts or rejects oligos strictly on the basis of the length of contiguous matching nucleotides that comprise a dimer.

This feature checks "X" (the specified) number of 3'-terminal nucleotides for dimers. The dimer does not need to be at the 3'-terminus position.

8.4.2.5 3'-terminal Nucleotides Checked for Dimers

This option limits the action of the "Maximal Length of Acceptable Dimers" search parameter to various 3' segments of the oligos being checked. If the nucleotide position in this parameter is set the same as in the "Oligonucleotide Length" parameter, then the entire oligo is checked for dimers, including the 5'-end.

The default setting for this option excludes the 5'-end of oligos from dimer checks, since 5' dimers have little negative effect on priming.

For a given 3' segment to be checked for dimers, the entire dimer must fall within this segment to be eliminated. (It cannot span the boundary.)

8.4.2.6 3'-terminal Stability Range

The "3'-terminal Stability Range" search parameter sets the range of the 3' stability window of tested oligonucleotides. This search parameter selects only oligonucleotides that have a 3'-end sufficiently unstable to resist false priming in complex DNA samples, yet stable enough to prime on the intended target efficiently.

8.4.2.7 GC Clamp Stability

The "GC Clamp Stability" search parameter sets the minimum stability of a GC clamp that must reside along each selected oligo's length, excepting the 3'-end.

This feature counterbalances the effects of unstable 3'-ends. The net effect of the "3'-terminal Stability Range" and the "GC Clamp Stability" search parameters is oligonucleotides that are specific, yet prime efficiently on their intended targets.

8.4.2.8 Minimum Acceptable Loop ΔG

OLIGO eliminates oligonucleotides with hairpin loop stability values greater than or equal to this value.

8.4.2.9 Oligo T_m Range

OLIGO limits the oligonucleotide selection to this melting temperature range.

8.4.2.10 Max Acceptable False Priming Efficiency

OLIGO eliminates primers that have a priming efficiency value (P.E. #) at the potential false priming site that is greater than or equal to the selected priming efficiency value. Not used in hybridization probes search.

The priming efficiency value is calculated with a complex algorithm that considers the ΔG of duplexes, mismatches, bulge loop sizes, and the distance of these elements from the 3'-end. Priming is likely when this value is over 200 points.

8.4.2.11 Min Consensus Priming Efficiency

When the "Consensus Primers" box is checked, OLIGO will search for primers common to all selected files. Primers with a higher or equal priming efficiency number than this parameter will be accepted as consensus primers. Not used in hybridization probes search.

8.4.2.12 Max Acceptable Homology

OLIGO eliminates probes that have a homology value at the potential false hybridization site that is greater than or equal to this selected value. This parameter plays a role only in the search for hybridization probes.

8.4.2.13 Min Consensus Homology

When the "Consensus Probes" box is checked, OLIGO will search for probes common to all selected files. Hybridization probes with a higher or equal homology than this parameter will be accepted. This parameter plays a role only in the search for hybridization probes.

8.4.2.14 Max Number of Acceptable Sequence Repeats

Set the maximum number of sequence repeats. The homooligomer/sequence repeat default value is three. Repeats can be either homooligomer, such as "GGG," or dinucleotide repeats such as "ACACAC." Potential oligonucleotides containing trinucleotide repeats and higher are not removed by this parameter.

8.4.2.15 Max Degeneracy

This option filters out potential primers and probes containing degeneracies. The default degeneracy setting is "1," indicating that the oligo under consideration has only one unique sequence. Any degeneracy threshold setting may be entered.

If you are working with sequences containing degeneracies and you do not want the program to filter out any oligos using this parameter, you can

"deactivate" it by setting the maximum degeneracy to a very high number, such as 10,000.

Note

This parameter is not automatically reset to the default value when the program automatically changes search stringency settings.

8.4.2.16 Frequency Threshold

This variable is used by "Eliminate Frequent Oligos" sub-search. If the frequency of all sub-sequences (6- or 7-mers) would be identical, then each sub-sequence would have frequency of 1000. Sub-sequences less abundant than average have lower numbers than 1000. More on this subject is described in Chapter 5.13.

8.4.2.17 Frequency Table

In order to eliminate "Frequent Oligos", an oligonucleotide frequency table needs to be specified. This table lists the frequencies of all oligonucleotide (6- or 7-mers) combinations in various subsets of GenBank and is used by the "Frequency Threshold" parameter. To choose a frequency table click on the "Change" button and select a table from the "Frequencies" folder.

8.5

Change — Non-search Parameters

The *Change — Non-search Parameters* options set the concentrations of various salts and nucleic acids used in the nearest neighbor calculations of melting temperature and the temperature used in calculating ΔG (free energy) values.

If your experimental conditions do not match these values, you should reset them here.

The formula OLIGO uses for calculation of sodium equivalent of magnesium concentration is:

$$Na^+ = 4 \times [Mg^{2+}]^{0.5}$$

Caution

These parameters affect the stringencies of all searches for primers and probes and the data calculations for several items.

The bivalent cation concentration has a profound effect in T_m calculations.

The following windows are affected by salt and nucleic acid concentration changes in this dialog box:

- Analyze — Duplex Formation
- Analyze — Hairpin Formation
- Analyze — LCR
- Analyze — T_m Graph
- Analyze - Key Info — Current Oligo
- Analyze - Key Info — Selected Primers

Note

Non-search parameters are not used to make the "Edit" window and "PCR" window calculations. Nucleic acid and salt concentrations affecting calculations in the PCR window are entered within the "PCR" window. In the "Edit" window, conditions are set to 1M salt and 100 pM oligo.

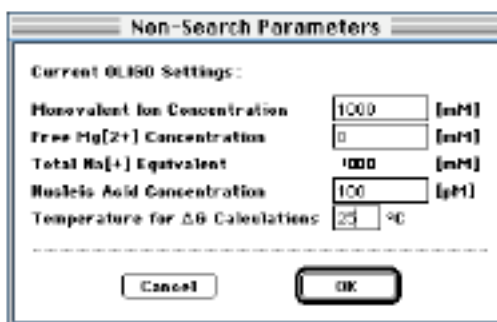


Figure 8.5. The Change — Non-search Parameters window (Mac).

8.6

Change — Rev. Translate Method

8.6.1 Reverse Translate — Lathe

The Lathe method is based on the most probable codons in mammalian mRNA. (Ref. 14.)

8.6.2 Reverse Translate — Inosine

When the Inosine method is selected, an inosine (universal substitute nucleotide) is inserted at the third base position in certain degenerate codons. (Ref. 11.)

8.6.3 Reverse Translate — Degenerate

This method gives all possible oligonucleotide combinations using the standard biochemical symbols for degenerate codons — N, Y, R, etc.; see Appendix D, Table 7. (Ref. 12.)

8.6.4 Reverse Translate — Codon Table

This back translation method creates a non-degenerate guess-mer sequence from the most frequent codons for a given organism. This is the default reverse translation method used in OLIGO.

OLIGO includes these codon tables:

- | | |
|----------------------------|--------------------------|
| • Arabidopsis | • Plasmodium |
| • Bacillus | • Pseudomonas |
| • Barley | • Rabbit |
| • Caenorhabditis elegans | • Rat |
| • Chicken | • Rhizobium |
| • Clostridium | • Rice chloroplast |
| • Cow | • Salmonella typhimurium |
| • Distyostelium discoideum | • Soybean |
| • Drosophila | • Staphylococcus |
| • E. coli | • Streptomyces |
| • HIV | • Tobacco |
| • Human | • Tomato |
| • Influenza virus A | • Trout |
| • Mouse | • Vaccinia virus |
| • Neurospora crossa | • Wheat |
| • Pea | • Wheat chloroplast |
| • Pig | • Xenopus laevis |
| | • Yeast (S. cerevisiae) |

8.7 Change — Codon Table

The *Codon Table* command selects the codon frequency table for a given organism. To add a new codon frequency table to the list, modify the codon table file "Codon Usage Tables."

Note

When you change the codon table, the reverse translation method automatically sets to "Codon Table".

There are four lines in each codon table file:

1. The name of the organism
2. The codon frequencies for that organism as described in *Nucleic Acid Research* (Ref. 18.)
3. The codons listed in order of frequency in the organism
4. The amino acids

A time-saving way to add a new user-specified codon table is to open the "Codon Usage Tables" file with a word processor, copy the last codon table record, change the organism name and codon frequencies accordingly, and save the file. The new updates of codon usage tables can be found on the web site: www.dna.affrc.go.jp/~nakamura/CUTG.html.

8.8 Change — Preferences

When OLIGO is started up for the first time, the file "OLIGO Preferences" is created in the "Preferences Folder." This file contains several OLIGO parameters. When OLIGO is started again, these parameters are read from this file.

8.8.1 Apply Changes To:

This *Preferences* command allows you to apply oligo search parameters to either the active sequence file or to all open sequence files.

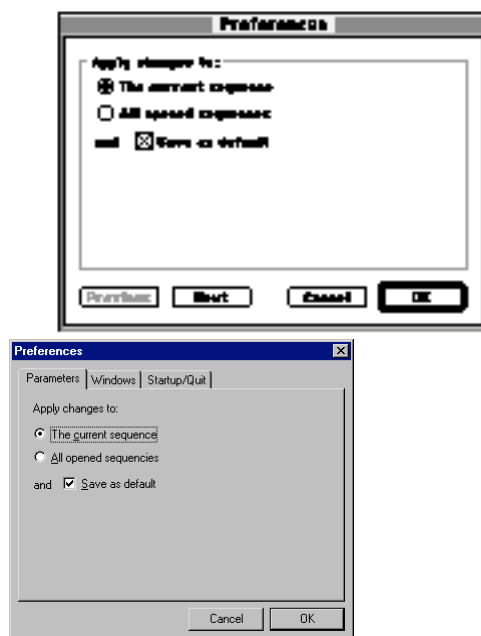


Figure 8.8.1. The Preferences dialog 1 (Mac on left, Windows on right).

8.8.2 Windows

The Windows dialog permits you to save size and position of selected OLIGO windows. When the "Save size and position" box is checked, the positions and sizes of all windows are "memorized" during program exit, and recalled in subsequent OLIGO sessions. Default windows that are displayed at the program start up may be changed using the "Add" button (see Fig. 8.8.2) or by using the Remove and Select/Deselect buttons, after highlighting the listed window name .

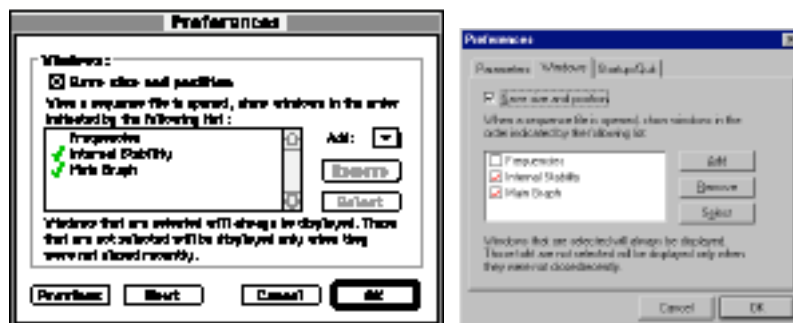


Figure 8.8.2. The Preferences window 2 (Mac on left, Windows on right).

8.8.3 Viewing/Editing/Printing Saved Data

This option, available in Macintosh version only, lets you choose your favorite text editor (word processor) to view, edit and print saved data. After clicking the "Select Editor" button choose the application from a list.

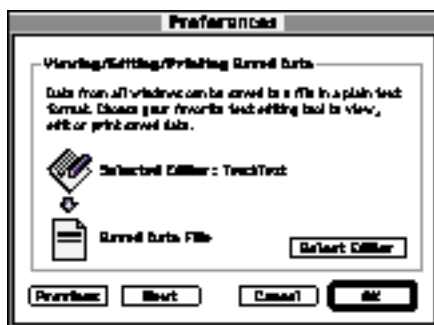


Figure 8.8.3 The Preferences window 3 (Mac).

8.8.4 Startup and Quit Options

OLIGO is able to memorize not only the size and positions of its windows, but also the primers, search results and parameters within the windows. This may significantly speed up your work, particularly when several researchers are using the same computer. Oligo windows and data are saved into a "Saved Work" file or other user-defined file. To recall the windows and data to the previously saved status, open "Your Work" file from the File-Open-Work File menu. The preference window lets you choose to save "Your Work", and to automatically (or not) open "Your Work" file on start-up.

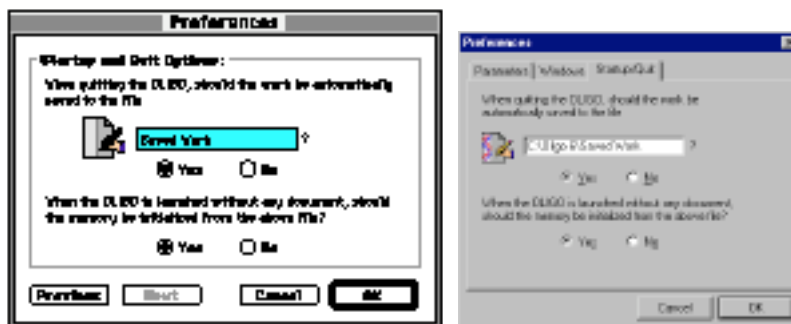


Figure 8.8.4. The Preferences window 4 (Mac on left, Windows on right).

When you open a sequence file, the parameters saved during your last OLIGO session are applied to that sequence. These parameters, in addition, are applied to all open sequences when you select "All opened sequences."

When you select "Save as default", the set parameters will be applied to every sequence that will be opened or created.

You may change preferences even when no sequence file is open. When you do this, the changes you make are saved as the default.

9

The OLIGO Window Menu

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9 The OLIGO Window Menu

While working with OLIGO, many windows may be open at one time. You can move each window manually, by clicking on and dragging its title bar, so that the main window may be seen. However, some windows may remain obstructed. The *Window* menu options help you view all opened windows.

There are different menu items for the Macintosh and Windows versions, so they need to be described separately.

9.1 Macintosh Window Menu

You can also use the *Window* menu to keep track of open windows, take a snapshot, and bring to front any previously opened window.

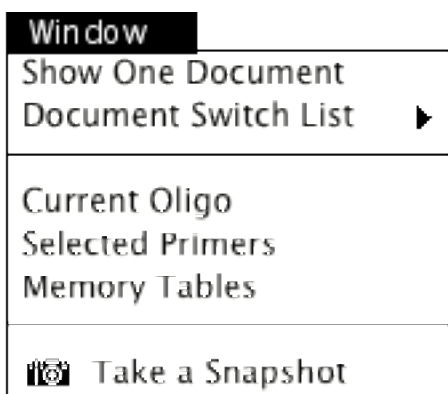


Figure 9.1 The OLIGO Window menu (Mac).

9.1.1 Window — Show One Document

This option displays only windows for the currently analyzed sequence file. You may have other open sequence files and this option allows you to choose

windows belonging to one of these open sequence files. In this case, a document is any file you have opened and that can be saved by OLIGO.

9.1.2 Window — Document Switch List

This option allows you to switch between documents. It displays a list of open files and allows you to select the one you want to view.

9.1.3 Window — Take a Snapshot

The *Take a Snapshot* command copies the screen or any part of the screen to the Clipboard.

To take a snapshot and copy it to the Clipboard:

1. Select the *Take a Snapshot* command from the *Window* menu. The cursor will change to a the cross-hairs tool icon.
2. Click one of the corners of the area you want to copy and drag the cursor until the box contains the portion of the screen that you want to copy.
3. Release the mouse button.

You can paste this image into another application document once the snapshot has been copied to the Clipboard.

9.2 PC Window Menu

You can use the *Window* menu to arrange open windows and bring to front any previously opened window.

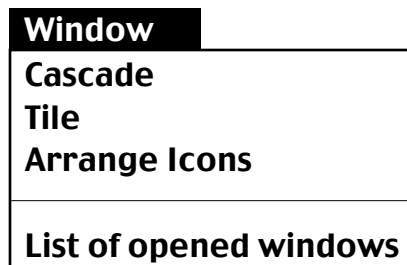


Figure 9.2 The OLIGO Window menu (Win).

9.2.1 Window — Cascade

The *Cascade* command arranges all opened Oligo windows one over another such that for all covered windows only the title bars are visible.

9.2.2 Window — Tile

The *Tile* command arranges and re-sizes all opened Oligo windows such that all of them are visible and non-overlapping. It is most useful when only a few windows are opened and each of them takes up a reasonable screen area.

9.2.3 Window — Arrange Icons

The *Arrange Icons* command arranges icons of the minimized Oligo windows. It is especially useful after resizing the Oligo application window such that those icons are not visible. They will become visible after using this command.

9.2.4 Window — List of opened windows

The remaining items in the *Window* menu are the window titles of all opened windows. To activate a given window, select it from the menu.

10

PRIMEFORM

Oligonucleotide Ordering Software

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10

PRIMEFORM Oligonucleotide Ordering Software

PRIMEFORM is an oligonucleotide ordering feature that allows you to transfer oligonucleotides from the OLIGO program to the PRIMEFORM ordering form for synthesis. PRIMEFORM is, in essence, a program within the OLIGO program; it interacts with the OLIGO program to retrieve data.

With the PRIMEFORM feature in OLIGO, each oligonucleotide sequence can be transferred from an OLIGO database, from another program on the computer, or entered directly with the keyboard to an order form. Once a sequence is uploaded or entered, it can be edited, and the quantity, scale, grade, and any end-labeling information designated.

PRIMEFORM also contains dialog boxes where you can enter accounting and shipping information and a notes section that supplies the synthesis facility with detailed notes pertaining to a specific synthesis order.

To use PRIMEFORM with OLIGO, you must have selected an oligonucleotide and have selected *Order Form* from *Export Functions* of the database feature.

Once you access PRIMEFORM, you are in a different program and you have a main menu that is different from the OLIGO main menu.

10.1

The PRIMEFORM File Menu

The *File* menu allows you to create, open, print, and save order forms.

File	
New	⌘N
Open...	⌘O
<hr/>	
Save	⌘S
Save As...	⌘A
Save As E-mail...	⌘E
<hr/>	
Page Setup...	
Print...	⌘P
<hr/>	
Quit	⌘Q

Figure 10.1 The PRIMEFORM File menu, Macintosh. The Windows version has additional Print Preview and Recent File items.

10.1.1 PRIMEFORM File — New

The *New* command under the *File* menu is where you can create a new order form with your specific information.

The *New* command calls up a blank template the first time you access the program. After you use the program, this command calls up a template that includes the shipping and accounting information from your last order form, as well as the "header" and "footer" information entered under *Order Information*. PRIMEFORM assumes that this information rarely changes, except for the PO number that you change through the *Edit — Shipping Information* command.

10.1.2 PRIMEFORM File — Open

The *File — Open* command allows you to open an existing file. You can edit the information in this order form file by selecting the appropriate options under the *Edit* menu.

10.1.3 PRIMEFORM File — Save

The *Save* command saves the order form. You can access this order form later using the *File — Open*

command. When you access *File — Save* with a new order form, PRIMEFORM automatically calls up the "Save As" dialog box to enter a file name for your new order form.

10.1.4 PRIMEFORM File — Save As

The *File — Save As* command allows you to save an existing order form under a new name. The *Save As* command saves this information:

- Shipping Information
- Accounting Information
- Order Information (the header and footer information on your form)

Save As does not save this information:

- Oligonucleotide Sequences
- Synthesis Specifications

To save the oligonucleotide sequences, use the "Save As" button from the "Oligonucleotide Sequences List" window. Synthesis specifications are most likely changed for each order form.

10.1.5 PRIMEFORM File — Save As E-mail

The *Save As E-mail* command allows you to save an order form as a plain text file so you can send the form via E-mail to your synthesis facility.

Note

This is the only place in the program where you can save everything from an order form.

The E-mail file has a text format that allows you to load it in any word processing software and modify it for your purposes.

10.1.6 PRIMEFORM File — Print

The *Print* command allows you to print your order form. PRIMEFORM uses a standard Mac interface.

10.1.7 PRIMEFORM File — Print Preview

The *Print Preview* command allows you to see a printout of the order form before it is printed.

10.1.8 PRIMEFORM File — Page Setup (Print Setup)

You can access the *Page Setup* command from the *File* menu.

The *Page Setup* (Mac) or *Print Setup* (Win) command allows you to set the specifications for your printouts. Within *Page Setup*, you establish what printer the order form should print to, the orientation of the paper and paper size, and other parameters.

10.1.9 PRIMEFORM File — Recent File

This option allows you to open an order form file that has been created and saved recently.

10.1.10 PRIMEFORM File — Quit

The *Quit* command returns you to the database feature of the OLIGO program.

10.2

The PRIMEFORM Edit Menu

Use the *Edit* menu to enter and edit shipping, accounting, sequence, and synthesis specifications, and customize your order form for your synthesis facility.

PRIMEFORM's *Edit* menu provides flexibility for synthesis orders. You can save separate templates for order, shipping, accounting, and sequence information, or you can combine these to form a single order form template.

Edit	
Cut	⌘X
Copy	⌘C
Paste	⌘V
Clear	
Select All	⌘A
Order Information	⌘1
Shipping Information	⌘2
Accounting Information	⌘3
Oligonucleotide Sequence	⌘4
Synthesis Specifications	⌘5

Figure 10.2 The PRIMEFORM Edit menu, Mac. The Windows version has only the last 5 items.

10.2.1 PRIMEFORM Edit — Order Information

The "Order Information" window is for setting the top and bottom messages to appear on your order form. In the example form, the top, or title, is "Synthesis Order Form" and the bottom message is "Please fax this form to..."

Depending on your needs, you may want to customize these fields for your synthesis facility.

There is one default file in PRIMEFORM that calls up pre-determined information for the "Order Information" window.

Generic.inf

This template permits you to add names, phone numbers and other information specific to your ordering style, so that you can save it in a file.

Entering Order Information

To enter data in the "Order Information" window:

1. Using the mouse, place the cursor in the field you want to change and begin entering your own

- data. Like any word processor, you will need to either delete or type over the existing data.
2. Use the <TAB> key (or mouse) to move to the different fields. If you press <RETURN>, PRIMEFORM assumes you are finished and returns to the main menu. To get back to the "Order Information" window, access it from the menu.

To use a template you have previously saved:

1. Click on the "Load" button on the bottom of the screen.
2. Select the file you want.
3. Edit the data, if necessary.

10.2.2 PRIMEFORM Edit — Shipping Information

Use the "Shipping Information" window to enter information pertinent to the shipping process. When using this option for the first time, open a new order form and enter your information. On subsequent orders, you can either open a new form or an existing form and edit it.

Shipping information includes date, purchase order number, the principal investigator's name, address, phone, and other information directly related to shipping.

The current date is automatically entered each time you open a shipping information window; however, you can edit it.



Figure 10.2.2.2 The Shipping Information window.

Entering Shipping Information

To enter data in the "Shipping Information" window:

1. Using the mouse, place the cursor in the field you want to change and begin entering your data. Like any word processor, you will need to either delete or type over the existing data.
2. Use the <TAB> key (or mouse) to move to the different fields. If you press <RETURN>, PRIMEFORM assumes you are finished and returns to the main menu. To get back to the "Shipping Information" window, access it from the menu.

To use a template you have previously saved:

1. Click on the "Load" button on the bottom of the screen.
2. Select the file you want.
3. Edit the data, if necessary.

To clear the fields of data:

1. Click on the "Clear All" button on the bottom of the screen.
2. Enter your new data.

You can save shipping information by clicking on the "Save" button and entering a name for this template. You can upload this template later by using the "Load" button.

10.2.3 PRIMEFORM Edit — Accounting Information

Use the "Accounting Information" window to enter your purchasing or accounting department information. When using this option for the first time, open a new order form and enter your "Bill To" information. On subsequent orders, you can either open a new form or an existing form and edit it.

Accounting Information

Name:

Institution:

Department:

Address:

City: State: ZIP:

Phone: Fax:

E-mail:

(Fax required for FAXBACK Order Confirmation and Analysis)

Load Save As Clear All Cancel OK

Figure 10.2.3 The Accounting Information window.

Entering Accounting Information

To enter data in the "Accounting Information" window:

1. Using the mouse, place the cursor in the field you want to change and begin entering your own data. Like any word processor, you will need to either delete or type over the existing data.
2. Use the <TAB> key (or mouse) to move to the different fields. If you press <RETURN>, PRIMEFORM assumes you are finished and returns to the main menu. To get back to the "Accounting Information" window, access it from the menu.

To use a template you have previously saved:

1. Click on the "Load" button on the bottom of the screen.
2. Select the file you want.
3. Edit the data, if necessary.

To clear the fields of the data that is in the fields displayed:

1. Click on the "Clear All" button on the bottom of the screen.
2. Enter your new data.

You can save accounting information by clicking on the "Save" button and entering a name for this template. You can upload this template later by using the "Load" button.

10.2.4 PRIMEFORM Edit — Oligonucleotide Sequence

Use the "Oligonucleotide Sequences List" window to load your oligonucleotide sequence and make edits to the sequence.

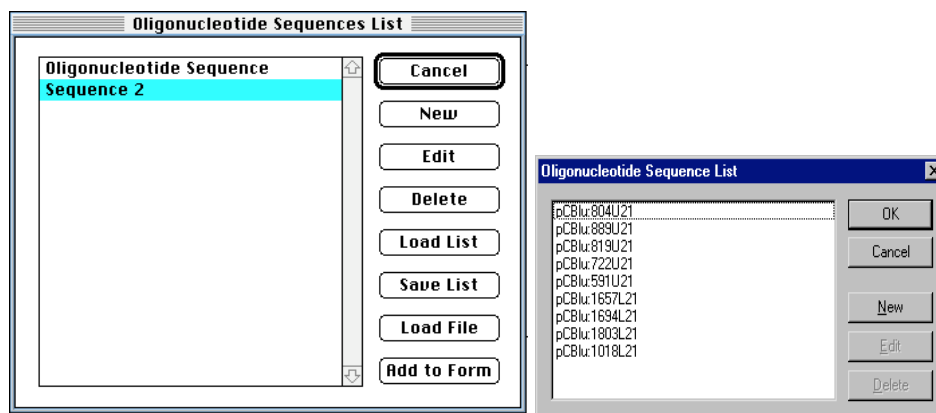


Figure 10.2.4.1 The Oligonucleotide Sequences List windows, Mac and Win.

Once you have a sequence loaded, or are in the "Oligonucleotide Sequence" window to enter or edit a sequence, add other sequence-specific information, such as 5' and 3' modifications, wobbles, minor bases, etc. Sequence "entry" is typically an automatic import from a record in the OLIGO database.

From this window, you may also create a new sequence, delete a sequence, edit a sequence, and save a sequence.

Note

The Oligonucleotide Sequences List is not automatically saved when you select Save or Save As from the File menu. To save your sequences lists, you need to click on the "Save As" button in the "Oligonucleotide Sequences List" window. Later, you can upload the list using the "Load" button.

New

The "New" button calls up the "Oligonucleotide Sequence" window where you can enter your oligo sequences and establish synthesis specifications, such as labeling.

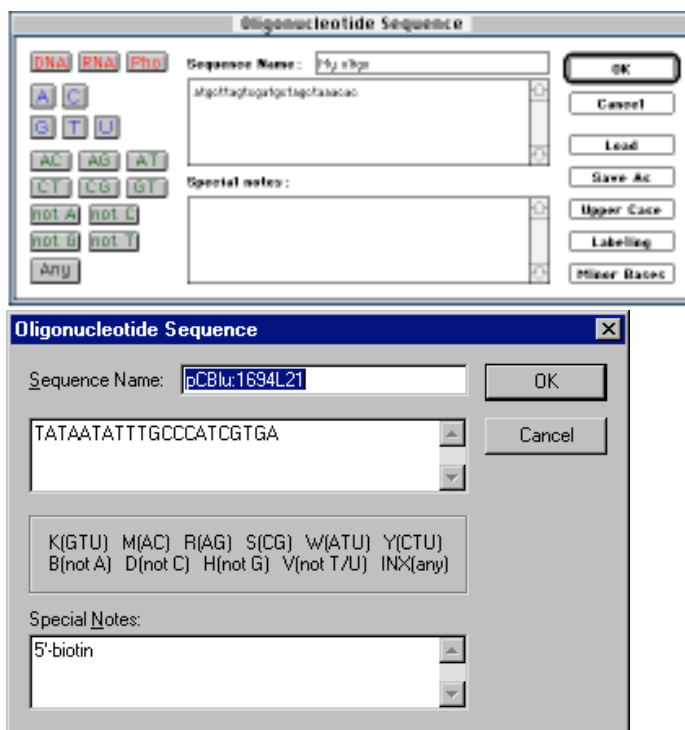


Figure 10.2.4.2 The Oligonucleotide Sequence windows, Mac & Win.

Sequence Name — Enter the name of the new sequence.

Sequence Type — To select your sequence type (DNA, RNA, or Phosphorothioate), click on the service in the upper left corner. The default is "DNA." In the Windows version the sequence type can be altered in the Synthesis Specification window.

Sequence Entry — To enter a sequence, use the base keypad on the left of the screen. For mixed bases, click on the mixed base buttons, below the standard bases on the left of the screen. You may also enter your sequence using the keyboard. Symbols for the degenerate nucleotides are listed in *Appendix E — Amino Acid Symbols*.

Load Button — (Mac only) Within the "New" option, you can load an existing sequence in text format to the sequence window by selecting it from the disk.

Save As Button — (Mac only) The "Save As" option allows you to save an existing sequence as a text file. You can load this file later by using the "Load" button.

Upper Case/Lower Case Button — (Mac only) The "Upper Case/Lower Case" button toggles between upper and lower case letters for your sequence entry. This button applies to entries you make using the mouse and the on-screen bases.

Note

The upper case/lower case button does not apply to keyboard entry. Use the shift or "caps lock" on the keyboard if you want upper case letters.

Labeling Button — (Mac only) The "Labeling" button allows you to select from several labeling options for both the 5' and 3'-ends. In the Windows version use the "Special Notes" field.

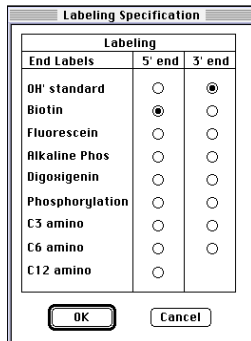


Figure 10.2.4.3 The Labeling window (Mac). In the Windows version use the "Special Notes" field.

PRIMEFORM gives you all available labeling and labeling combinations available at the time the product was released. When you select a label, the labeling information is automatically displayed in the "Special Notes" window. This is also where you can edit labeling information. If your end label of interest is not listed in the labeling specifications window, enter it into the "Special Notes" window via the keyboard.

Minor Bases Button — (Mac only) Using the "Minor Bases" option, you can select from a list of minor bases to add to your sequence. In the Windows version use the "Special Notes" field.

PRIMEFORM gives you all available minor bases at the time the software was released. Make sure the cursor is set on the position in the sequence where you want the minor base. When you select a minor base, the minor base information is automatically displayed in

the "Special Notes" window and an "X" appears in the position where the cursor is placed.

Special Notes — The "Special Notes" window displays minor base and other nucleotide-specific information selected for your sequence. In addition, you can click in this window and add notes such as position, special synthesis specifications, and other research data.

Edit

When you select "Edit" from the "Oligonucleotide Sequences List" window, you can edit the sequence. You have the same features available in "Edit" as you do in "New."

Delete

The "Delete" option deletes the selected sequence. You will be prompted to confirm your deletion.

Load List

"Load" allows you to load a previously saved oligonucleotide sequences list to the order form.

Save List

The "Save As" option allows you to save an existing sequences list as a separate file. This file can be loaded later for a different order form.

10.2.5 PRIMEFORM Edit — Synthesis Specifications

Use the "Synthesis Specifications" window to enter the service you want for your custom synthesis (DNA, RNA, S-Oligo, hybrid), grade (desalted, RP cartridge, HPLC, or P.A.G.E. purified), and scale/quantity.

You also specify shipping instructions — turnaround and carrier — in this window.

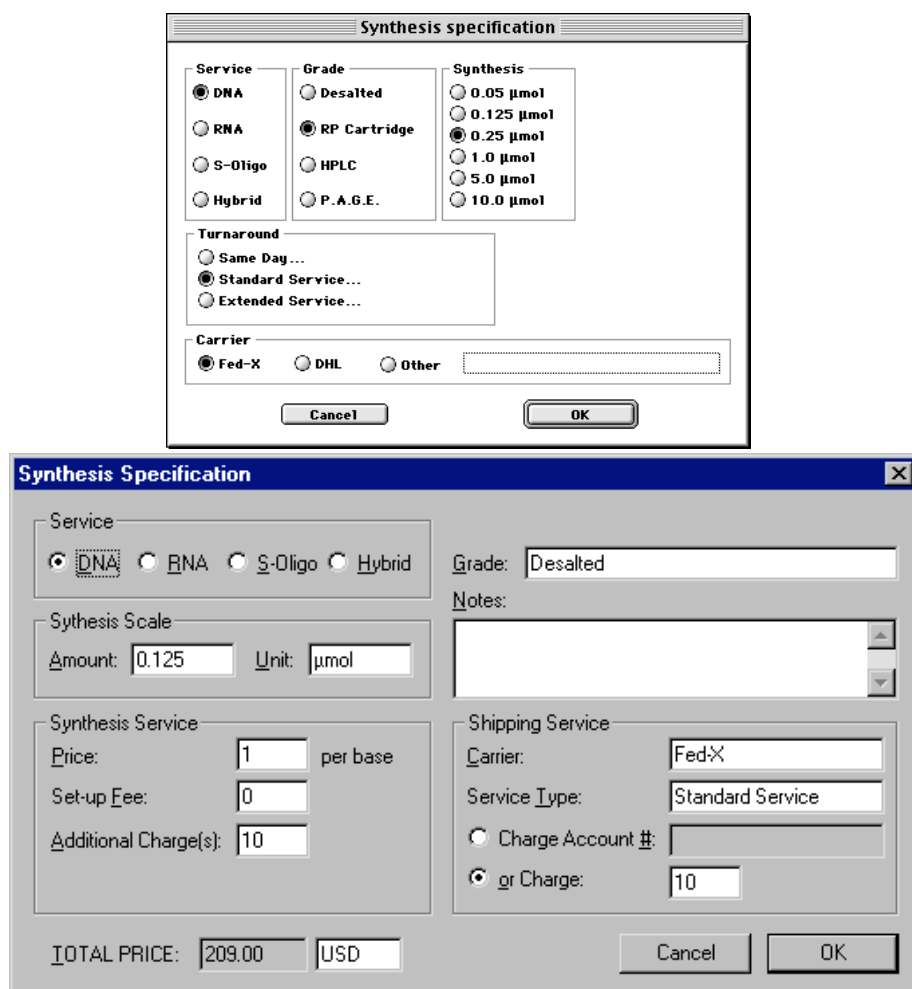


Figure 10.2.5 The Synthesis Specifications windows, Mac & Win.

Service

Select which synthesis service you want — DNA, RNA, S-Oligo, or hybrid. The PRIMEFORM default is "DNA." If you need more than one oligo service (DNA, RNA, S-Oligo), you may not combine them on one order form. Use a separate order form for each.

Grade

Select the grade — purification level — you require for each oligo. Options include desalted (unpurified), RP (reverse phase) cartridge purified, HPLC purified, and P.A.G.E. purified. The PRIMEFORM default is RP

cartridge purified. In the Windows version simply type the grade name.

Synthesis Scale/Quantity

Select the scale of synthesis you need.

Turnaround or Shipping Service- Service Type

Select the turnaround time for your synthesis order (Mac only). Check that the turnaround time and other options on the order form are consistent with the offerings of your synthesis facility. In the Windows version print the shipping service type (could be "Priority" or "Next Afternoon").

Carrier or Shipping Service- Carrier

Select the method by which you want your synthesis order shipped. The PRIMEFORM default is "Fed-X" (Federal Express). In the Win version, type the name of your shipping service provider.

Notes (Win)

Enter comments applicable to the entire order. For example the turnaround time may. Use "Special Notes" field in the Oligonucleotide Sequence window if the note is specific to a particular single oligonucleotide.

Synthesis Service (Win)

Enter the price per base, setup fee and additional fees (such as minor bases charges). The TOTAL PRICE will be calculated. You may change the currency name.

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11.1 Navigating in OLIGO ver. 6

The tutorials in this section give you an opportunity to learn how the features of the OLIGO program can assist you in adapting the program many capabilities to your applications of interest. Some of the tutorial examples are intended to provide a general guide from which you can construct your specific application. Others are specific, so that you can duplicate, if you wish, similar results in the manual (similar, not exact, because future revisions of the program may alter results).

Note if you want to duplicate the results in the search examples, open CBP.SEQ for your sequence file.

11.1.1 Changing Positions on the Sequence Template

Depending on the monitor resolution, OLIGO displays only 80-150 nucleotides of the active sequence in the "Melting Temperature" window at a time. You may need to move along the sequence to select or analyze oligonucleotides. There are four ways to select a new Current Oligo in the program, each of which moves you to a new position on the active sequence.

11.1.1.1 Scrolling and Clicking

To use this method to change position on the sequence:

1. Move the scroll bar on the bottom of either the "Melting Temperature", "Internal Stability" "Memory Tables", or the "Selected Oligos" window to position the graph approximately where you want to move on the sequence.
2. Using the mouse, position the cross-hairs tool on the position you want and click.

11.1.1.2 Choosing a Specific Position From the Menu

To use this method to change position on the sequence:

1. Pull down the *Select* menu from the main menu bar.
2. Choose the *New Current Oligo Position* function from the *Select* menu.

3. Enter the nucleotide position number in the dialog box (the 5'-end of the positive strand Current Oligo or 3'-end of the negative strand Current Oligo).

OLIGO automatically updates all relevant windows.

11.1.1.3 Using the Icons

To use this method to change position on the sequence:

1. Click on the positioning icon (circle with cross-hairs) at the bottom left of either window.
2. Enter the position number in the dialog box; 5'-end of the Upper Primer or 3'-end of the Lower Primer.

OLIGO automatically updates all relevant windows.

When browsing the active sequence for a New Current Oligo, it is helpful to select the "Current Oligo" window from the *Analyze-Key Info* menu.

Click and drag on the "Current Oligo" window title bar to position the window below the "T_m" window. With the Current Oligo data visible simultaneously with the "Melting Temperature" graph, you can analyze each potential oligonucleotide.

11.1.1.4 Using the Zoom Submenu

To use this method to change position on the sequence:

1. Pull down the Zoom from the "Melting Temperature" or "Internal Stability" window bar (Mac) or click on the right mouse button (Win) and choose "2" or "3."
2. Click on the desired position.
3. To see the T_m graph in detail choose "1" from the Zoom submenu.

11.1.1.5 Using the Short Cut Keys

To select new Current Oligo position press the ⌘K (Mac) or F10 function key (Win).

11.2 File Saving, Printing & Reset Operations

11.2.1 Loading a DNA / RNA Sequence File

To load a DNA or RNA sequence file:

1. Choose *Open* from the *File* menu on the main screen.
2. Enter the sequence file name or click on one of the file names shown in the list box.
3. Click "OK."

The sequence file is loaded, displayed on the main screen, and the 5'-end of the Current Oligo is set on the first nucleotide.

You must load a sequence file before most Edit, Analyze, Search, or Select functions can be accessed.

If you don't have a sequence file on disk, cancel the "File List" box and use *New* from the *File* menu and type the sequence or paste it from the Clipboard.

11.2.2 Loading a Protein Sequence File

To load a protein sequence file:

1. Choose *Open* from the *File* menu on the main screen.
2. Choose *Protein* from the *File type* menu and click on one of the file names shown in the list box. If your file is not displayed, uncheck the "Show OLIGO Files Only" box.
3. Click "OK."

The sequence file is loaded, reverse-translated according to the current reverse translation method, and DNA is displayed on the main screen. In order to choose a different reverse-translation method use *Change - Rev. Translate Method* option.

If you don't have a sequence file on disk, cancel the "File List" box and use *New* from the *File* menu, from the Edit submenu choose *Protein* and type the sequence or paste it from the Clipboard.

11.2.3 Printing Windows in OLIGO

11.2.3.1 Printing Text

OLIGO can print the current window, pre-selected windows, and/or all open windows. In order to choose the window to be printed use "Print/Save Options" from the "File" menu. When you choose "Selected" windows, you need to check the appropriate boxes indicating which windows you want to be printed. When printing a database you may choose to print full analysis of oligos.

One of the more important functions of *Page Setup* (Macintosh *File* menu) is the "Reduce or Enlarge" dialog. Printing at 50% will fit twice more text on one page.

1. Select *Print* from the *File* menu and click the "Print" button.

11.2.3.2 Printing Graphics

To copy window contents to Clipboard use the <Alt-Print Screen> key combination in Windows or use Take a Snap Shot function in Macintosh.

1. Press <Alt-Print Screen> key (Win) or select *Take a Snapshot* from the *Window* menu (Mac).
2. (Mac only) Click on top left rectangle image to be copied and drag the mouse to the bottom right, release the mouse button. The image is copied into the Clipboard
3. Paste the Clipboard image into your favorite application screen.

11.2.4 Using the Automatic Reset Functions

OLIGO has two automatic reset functions to reset previously set parameters and data.

11.2.4.1 Using Reset to Clear Data

To clear all results from a search (memory tables data), reset search ranges, and remove any Upper or Lower Primers that have been selected:

1. Select *Reset* from the *File* menu and choose *Data*.

11.2.4.2 Using Reset to Clear Parameters and Return to Original Defaults

To reset the program and all its parameters to the original defaults without changing data:

1. Select *Reset* from the *File* menu and choose *Original Defaults*.

You may want to reset both data and original defaults each time you proceed with an example to get the same results described in the example.

11.2.5 Using the "Save Your Work" Function

File - Save - Your Work option saves positions and contents of all OLIGO windows.

1. Use *Change - Preferences* menu to check the status of startup and quit options (the last "Preferences" window). Both "Yes" buttons should be activated to save your work automatically, with no questions asked. Default file name for this kind of a file is "Saved Work" that can be changed.
2. Use *File - Save - Your Work* to save the status of the program at any given point of your work with OLIGO. You may give any name to such a file.
3. Quit OLIGO.
4. Re-start OLIGO. All windows should look exactly like those before quitting.

11.3 PCR & Multiplex PCR Applications

11.3.1 Searching for PCR Primers (A Basic Search)

This example takes you through a quick search for a pair of optimal PCR primers. To run this search:

1. Choose the Primers and Probes option from the Search menu.
2. Verify that the "Compatible Pairs" button under "PCR Primers" in the dialog box is checked.
3. (Optional) Click on the "Search Ranges" button and set the search range to **-18-200** for the positive strand and **400-600** for the negative strand.
4. (Optional) Set the PCR product length to **200-400** and click "OK."
5. Click "OK" to start the search. The "Search Status" progress window appears, and the message

"Search Completed" appears when the search is complete.

6. When the search is complete, check the "Search Status" window to make sure that the final search stringency is acceptable. OLIGO automatically reduces search stringency if it does not find any compatible pairs.
7. Click "OK" with "Show: Primer Pairs" displayed at the bottom of the "Search Status" window.
8. There are several sort options on the "Primer Pairs" window. For this example, click on the circle above "Prod. Len" to sort by product length.
9. Click on a primer pair to view the "PCR" data for the selected pair.
10. Choose Selected Primers from the *Analyze - Key Info* menu to view the primer sequence and related data.

11.3.2 Searching for PCR Primers to Generate a Specific Product Length (A Detailed Search)

This example takes you through a comprehensive search for a pair of optimal PCR primers that amplify a product of approximately 525 bp and include the nucleotide subsequence between positions 600 and 800. To run this search:

1. Choose *Reset* from the *File* menu and select *Data* and then *Original Defaults*.
2. Choose the Primers and Probes options from the Search menu on the main menu bar.
3. Click on the button next to "Compatible Pairs," if it isn't marked already.
4. Click the "Continue False Priming Search in Other Files" box in the lower left portion of the screen to call up the "Select Files" window.
5. Click the "Add" button and then open the "OLIGO" folder and then the "FreqSeq" folder.
6. Click on the "humanfr.seq" file and then click on the "Add" button to select it. Click the "Done" button.
7. With the "humanfr.seq" file displayed in the "Select Files" window, click "OK." By adding this file to the false priming subsearch, any oligo from the active sequence file (cbp.seq) that contains humanfr.seq sequence will be eliminated as a potential primer.
8. Click the "Search Ranges" button.

9. Set the ranges for the desired PCR product size and location:
 - a. Enter **350** to **600** for the positive strand primer search range and **800** to **1000** for the negative strand primer search range.
 - b. Enter **400** to **650** for the PCR product length to bracket the desired 525 bp product.
 - c. Click "OK."
 10. Click on the "Parameters" button.
 11. Check that the "Search Stringency" is set to "Moderate."
 12. Make sure that "Adjust Length to Match P.E. #'s" box and the "Automatically change stringency" box is marked.
 13. Click "OK" to exit the "Parameters" window.
 14. Click "OK" to start the search.
- The "Search Status" progress window appears, and the message "Search Completed" appears when the search is complete.
15. Click "OK" with "Show: Primer Pairs" displayed at the bottom of the "Search Status" window.
 16. In the "Primer Pairs" window, click on the "Prod. Len" "Sort" button to sort primer pairs by the product length they'd make.
 17. Use the scroll bar and/or arrows to find the desired product length of 525 or closest to 525.
 18. Click on this primer pair to select it and open the "PCR" window.
 19. From the *Analyze-Key Info* menu, choose "Selected Primers" to review primer data for synthesis.
 20. Review search results by selecting *Primers and Probes Search Data* from the *Search* menu.
 21. Check the selected oligos under the *Analyze* menu.

11.3.3 Selecting a Lower PCR Primer Compatible With a Specific (Pre-synthesized) Upper Primer

1. Intend to select the Lower Primer.
2. Select *Edit - Upper Primer* and enter your sequence into the red box reserved for nucleic acid entry.
3. In the upper part of the "Edit Upper Primer" window, adjacent to the red "5'," enter the primer's position relative to the active sequence. For example, enter -200 if the 5' position if the primer is 200 nt upstream from the nucleotide at position 1 on the active sequence.
4. Choose the *Accept & Quit* option from the *Accept/Discard* menu.
5. Select *Primers and Probes* from the *Search* menu.

6. Activate the "Compatible With the Upper Primer" button on the "Search for Primers and Probes" dialog box.
7. After setting search ranges, search stringency, and other parameters, click "OK" to start the search.
8. When the search is complete, click on the "Selected Oligos" button and then click on the primer you want, based on T_m , position, or both. Clicking on a primer selects it as the Current Oligo.
9. Make "Melting Temperature" the active window and then click the "Lower" icon on the left of the "Melting Temperature" window to select this primer as the Lower Primer. This prompts you to enter the "%GC Content."
10. With the Lower Primer selected, choose PCR from the Analyze menu to review the PCR data for the primer pair.

11.3.4 Analyzing (Previously Synthesized) PCR Primers without a Template

To analyze two PCR primers not on a sequence file template you can access with OLIGO:

1. Choose *New* from the *File* menu.
2. Type the sequence of the positive-strand primer.
3. If the position of this primer is different than 1, type the new 5' position number adjacent to the red "5" at the top of the "Edit" window.
4. Choose Accept & Close from the Accept/Discard menu.
5. If the primer sequence length is different from that of the Current Oligo, change the length of the Current Oligo from the "Change" menu.
6. Select the sequence as the Upper Primer by clicking on the "Upper" icon.
7. Choose Lower Primer from the *Edit* menu and type in the sequence of the negative strand primer in the "Edit Lower Primer" window.
8. At the upper left corner of the "Edit" window, enter the 5' position number, relative to the Upper Primer.
9. Choose Accept & Close from the Accept/Discard menu.

In order to get an approximate optimal annealing temperature, the length and GC content of the PCR product are needed.

A dialog box is displayed for the entry of the GC content of the amplified product. (The average GC percentage for mammalian mRNA is 44%.)

10. Enter the expected GC content, or choose the default, and click "OK."

Use the *PCR* and *Selected Primers* commands from the *Analyze* menu, or any "Analyze-Dimer Formation" window to analyze the primers and their resulting PCR data.

You may use existing oligonucleotide database and "export" any of its records to any opened sequence file (if the parental sequence file does not exist you must open any available on disk sequence file).

11.3.5 Analyzing Previously Synthesized PCR Primers with a Template

To analyze two PCR primers that can be found on a sequence file you can access with OLIGO:

1. Load the sequence file from which you designed the primers, using the *File - Open* command.
2. Set the Current Oligo Length to match your primer length.
3. Find your primer sequence by using *Search - for a Sequence String*, or, if you know the position of the primer move the Current Oligo to this position using *Select - New Current Oligo Position*.
4. When the Current Oligo overlaps your primer, select the primer by clicking "Upper" or "Lower" button in the T_m window.
5. Repeat steps 2-4 to select the second primer.
6. Use *Analyze* options to get info on the selected primers.

If the primers were saved into a database, open this database file, select the primer by clicking on it, and use Export function to align primers on the sequence.

11.3.6 Selecting Primers for Multiplex PCR using a Single Template

This application permits the automatic selection of two or more sets of cross-compatible (multiplex) primer pairs from one sequence file (template). Multiplex selection always requires a PCR primer search before the multiplex primer selection. To select a set of two or more multiplexed primer pairs:

1. Load the sequence file from which you wish to design the multiplex primers, using the *File - Open* command.
2. Choose the *Primers and Probes* option from the *Search* menu.
3. See that all the subsearch boxes are checked except "Continue False Priming Search in Other Files" (unless you have sequence files accessible to the OLIGO program which may contain false priming sites, and the DNA represented by these sequence files will be in your PCR reaction mix).
4. Activate the "PCR Primer Search" function by clicking on the circle next to "Compatible Pairs."
5. Click on the "Search Ranges" button.
6. Enter **-18** to **200** for "Positive Strand Primer Search Range." Select **400** to **700** for "Negative Strand Primer Search Range." Leave default values (150-1868) for "PCR Product Length."
7. Click on the "Parameters" button on the "Search Ranges" dialog box and choose "Moderate" for the "Search Stringency."
8. Check to be sure the "Inverse PCR" box is not checked and that the "Adjust Length to Match T_m's" box is checked. Click "OK" to return to the "Search Ranges" dialog box. Click "OK" again to return to the "Search for Primers and Probes" dialog box.
9. Click "OK" to start the search; several hundred pairs will be accepted.
10. Click "OK" with "Show: Primer Pairs" displayed at the bottom of the "Search Status" window.
11. Choose *Multiplexing* from the *Analyze* menu.
12. Choose "Zoom" from the "Multiplexing" window, and select level 5 to zoom in.
13. Select your multiplex primers by clicking directly on the squares representing the primers. The position number of each square appears along the top right of the window.
14. Once you have selected the desired number of multiplex primers, click on the "Pairs" button to display the "Primer Pairs -Multiplexing" window.
15. Click on any or all of the multiplex pairs to analyze PCR conditions for them.
16. Multiplexed primers may be downloaded as a group to an OLIGO database (use "Multiplexed Primers" under the "Import" submenu of any database window).

11.3.7 Selecting Primers for Multiplex PCR using Multiple Templates (Exons in Complex Genes/ Multiple Gene Amplifications)

This application takes you through the selection of two or more pairs of cross-compatible PCR primers from two or more sequence files (templates). Use this selection method when you wish to amplify multiple exons in a complex gene, (when the exon sequence s are in different files), or you wish to amplify more than one gene in the same PCR reaction. To select multiplex primer pairs from multiple sequence templates:

1. Using *File-Open*, load the DNA/RNA sequence file (template) for the first exon/gene you wish to design multiplex primers for.
2. Choose *Search for Primers and Probes* from the *Search* Menu. Then click "PCR Primers: Compatible Pairs".
3. Click the "Search Ranges" button at the right margin of the "Primers and Probes" dialog box. Set the search ranges for the upper and lower primers consistent with the subsequence you wish to amplify from this template. Set the PCR product size limits (optional) you require consistent with the constraints of the search ranges.
4. Click the "Parameters" button at the bottom of the "Search Ranges" dialog box. Then, in the "Search Parameters" dialog box, select the appropriate search stringency for this search. Typically, you will need 5-10 upper primers and 5-10 lower primers per search for searches of 2 or 3 templates. For multiplexing more templates, you may need to start with more upper and lower primers per template (>10).
5. In order to find the desired number of upper and lower primers per template, your search should yield approximately 50 primer pairs, depending on the cross-compatibility of the primer group. To determine the actual number of primers, count them in the Primer Pairs Window. Note that there are usually more "Oligos Accepted" in the "Search Status" Window than there are primers in the "Primer Pairs" window, because some primers (1) cannot be paired with other primers, or (2) they do not amplify a PCR product of the correct size (set in "Search Ranges").

6. Check that the "Adjust size to match T_{ms}" check box and the "Automatically Change Stringency" check box are checked "on", and that the "Inverse PCR" check box is checked off.
7. Under the *File* menu, select either *New Database* or *Open - Database* depending on whether you wish to load your multiplex primers to a new or existing database.
8. In the "Database" window, click "Multiplexed Primers" under the *Import* Submenu. This will download all the optimal primers listed in the "Primer Pairs" window. The primers downloaded have been selected using all of OLIGO PCR search selection criteria, (including the elimination of self-dimerizing oligos). Cross-compatibility, (for multiplex pairing), however, is performed in the database after downloading, using the database multiplexing feature.
9. With the optimal primers from the first template PCR primer search loaded in your database, load the second DNA(RNA) template into OLIGO using "File - Open".
10. Repeat steps #2 - #8 above. Note that you must use the same database (step #7) into which you downloaded primers from the first template. If you have other templates (exons/genes) that you wish to include in the multiplex, wait on the primer selection and downloading until you have completed the multiplexing operation in your database with the first two templates.
11. Once you have downloaded the primer sets from templates one and two, you are ready to have OLIGO select cross - compatible primers from these templates. Select an Upper Primer from the first template as the starting point for your multiplex PCR. Do this by clicking on the primer and then *Multiplex* under the *Analyze* submenu in the database window.
12. Check the results of the multiplexing operation; you will see that the selected primer will be indicated by a green "M" in the "3' dim.ΔG" database column and all primers that are cross-compatible with that primer will display a blue "C" (compatible) in that column. Primers that are not compatible with this first primer will display a "NC" in the column.
13. Continue multiplex primer selection by choosing a cross-compatible ("C") Lower Primer from the same template. Click on the primer and then *Analyze - Multiplex* again. Now, this second primer will also display an "M" and the remaining

- primers displaying a "C" are compatible with both the first and second primer.
14. Once all multiplex primers are selected from the first and second templates, load the third template, select and download primers from this template, (steps 2 - 8 above) and then multiplex them (steps 9 - 14). Note: If a particular primer appears to be incompatible with many primers (i.e. it eliminates many "C" primers), *Deselect* it under the *Analyze* submenu and choose another one or lower the Search Parameters stringency.
 15. With all of the multiplex primers selected for all your exon/gene templates, you may want to erase the unwanted primers from this database.

11.3.8 Conducting an Inverse PCR Search

When a DNA template is circular, it can be useful to search for inverse PCR primers, where the position of the Upper Primer is higher than that of the Lower Primer.

An Inverse PCR search is set in the "Search Parameters" dialog box. To do an Inverse PCR search on CBP.SEQ file:

1. Choose *Reset — Data* from the *File* menu.
2. Choose *Primers and Probes* from the *Search* menu.
3. Click the circle next to "Compatible Pairs," if it isn't marked already.
4. Click on the "Search Ranges" button.
5. Set the ranges for the search:
 - a. Enter **1300** to **1830** for the positive strand primer search range and **-18** to **400** for the negative strand primer search range.
 - b. Enter **150** to **1868** for the PCR product length.
 - c. Click "OK."
6. Click on the "Parameters" button.
7. Activate "Inverse PCR" by clicking the box.
8. Click on "High" for your "Search Stringency."
9. Make sure that "Adjust Length to Match T_m's" is marked.
10. Click "OK" to exit the "Search Parameters" dialog box and then click "OK" again on the "Primers and Probes" dialog box to start the search.
11. Click on "Primer Pairs" to view the primers and click on any set of selected primers to view the PCR data.

12. From the *Analyze-Key Info* menu, choose *Selected Primers* to see sequence data for synthesis.

11.3.9 Designing TaqMan Primers & Probes

TaqMan is a molecular diagnostic system that detects the presence and the quantity of a PCR amplicon. The direct detection is provided by the release of a fluorescent reporter molecule during PCR. The system consists of:

- An oligonucleotide probe with a 5' "reporter dye," a 3' "quencher dye," and a 3' blocking phosphate
- Two oligonucleotide PCR primers that bracket the hybridization site of the probe

The 5' - 3' endonucleolytic activity of the TaqMan polymerase enzyme cleaves the probe between the 5' reporter dye and the 3' quencher dye, creating the reporter signal. The probe cleavage can only occur, however, when the primers and probe anneal to their target sequences and the TaqMan polymerase extends from the primers through the probe. The amount of reporter signal is directly proportional to the quantity of PCR product generated.

TaqMan Primer/Probe Design Criteria

- The T_m of the primers should be $\sim 10^\circ$ less than the T_m of the probe. It is advisable to select primers with consistent T_m ($\sim 65^\circ$) or rather P.E. # (~ 450), such that several primer pairs can be tried in one run of a cycler.
- Primers should be selected to create an amplicon between 70 and 400 nt in length.
- Select the upper or lower strand for the probe, such that the probe will contain more C's than G's, if possible.
- Avoid primers and probes with a 5' G.
- The probes should be designed to hybridize only a few nucleotides downstream from the primer of the same orientation. The primer and probe, however, must not overlap.
- Probes should be designed to be between 20 and 30 nt in length.
- Avoid primers and probes that contain three or more sequence repeats, particularly G's.

- Avoid secondary structure (hairpins) in primers, probes, and their target sequences.

To design TaqMan primers and probes:

1. Using *File — Open*, load a sequence file of the gene for which you are creating the TaqMan system.
2. Using the *Change* menu, set the oligonucleotide length to 18-21 nt.
3. Select *Search for Primers and Probes* from the *Search* menu.
4. Click on the "Compatible Pairs" button to select PCR primers.
5. Click on the "Search Ranges" button and set the search ranges for upper and lower primers in the "Search Ranges" window. If the size of the gene and your requirements permit it, select a wide range for the selection of the upper and lower primers. If possible, use the entire sequence file because this will improve your chances of finding optimal primers and probes that meet the TaqMan criteria. Make sure you set the PCR product size to 70 - 400 nt.
6. Click on the "Parameters" button and set the search stringency in the "Search Parameters" window to "High" or "Very High."
7. Make sure that the "automatically change stringency" option is checked, or "on."
8. In the "Search for Primers and Probes" dialog box, click "OK" to start the search.
9. When the search is complete, click on "primer pairs." Test various primer pairs until you find a pair that meets the selection criteria. These criteria are best checked for in a "trial and error" fashion. If any primer under consideration does not meet any of the criteria, look for a new set and start again.

There should be another primer within 15 - 100 nt downstream of the upper (or lower) primer that can be designated the probe. The designated probe should be no closer than 15 nt because nucleotides will need to be added to the 5' of the probe to bring its T_m to 10° above the T_m of the primers. The designated probe should also contain more C's than G's.

Any nucleotide other than G will be on the 5' end of the primers.

Add nucleotides to the 5' end of the probe in order to increase its T_m to 10° above the primer T_m s or choose a probe of the same length, with T_m 10° higher than the primers. After the correct number of nucleotides is added, check that the

probe is between 20 -30 nt in length and that there is no "G" on the new 5' end.

Note

You should use the T_m s in the "Melting Temperature" window as a rough gage of T_m only, for PCR. Using standard PCR conditions, the T_m s in the PCR window should be the final check of the T_m difference between the primers and probe.

10. Select *Analyze — Duplex Formation* to check the primers, probes, and their targets for secondary structure.
11. Select *Search — for Hairpin Loop Stems* to check the primer and probe targets for potential secondary structure.
12. Select the two primers and the probe as the upper and lower primer so that they can be downloaded to an OLIGO database.
13. Open a database and load the selected primers and probe by selecting *Import* from within the database for each primer and probe.
14. Check the probe against the primers for 3' end cross compatibility using the database multiplex feature. See *Section 5.7 — Analyze — Multiplexing*.

11.4 Sequencing Primers & Hybridization Probe Selection Applications

11.4.1 Finding and Selecting an Optimal Sequencing Primer

1. Choose *Reset* from the *File* menu and select "Data" and then "Original Defaults."
2. Choose *Primers and Probes* from the *Search* menu.
3. If you are selecting sequencing primers, click the "Sequencing Primers" button on the "Search for Primers and Probes" dialog box.
4. At the top of the dialog box, select the + or - strand. (If you want to select a primer from the + strand that will hybridize to the - strand, check the "+ strand" box.) Note that both boxes may be checked; you will need to click in one to "deselect" it.
5. Click on the "Parameters" button.
6. Select "High" or "Very High" for the "Search Stringency."
7. Set the length of the primers to be selected in the search using the "Oligonucleotide Length" parameter in the "Search Parameters" window.

8. Click "OK" to return to the "Search for Primers and Probes" dialog box.
9. Set the ranges for the desired location of the primer on your template by clicking the "Search Ranges" button and enter your range in the strand you wish to search.
10. Click "OK" to return to the "Search for Primers and Probes" dialog box.
11. Click "OK" to start the search.
12. Click "OK" when the "Search Completed . . ." message appears and "Show: Selected Oligos" is displayed at the bottom of the window.
13. Sort the oligos selected by T_m , 3' ΔG , or GC clamp, and click on the desired oligo, selecting this oligo as the "Current Oligo" in the "Melting Temperature" window.
The most optimal primers are those with the highest T_m and highest negative ΔG value for GC clamp and moderate ΔG value for the 3'-end. You may check the oligos by selecting any of the *Analyze* options. Usually, the negative strand (blue) is displayed below the positive strand (red) in the "Melting Temperature" window, unless the strands have been changed under the *Change — Strands* option while editing the sequence, or if the original active sequence was reversed.
14. If it is a + strand primer, select it as an upper primer by clicking on the "Upper" button on the left side of the "Melting Temperature" window (for a - strand primer, click "Lower"). You may need to click into the "Melting Temperature" window first.

11.4.2 Finding and Selecting an Optimal Hybridization Probe

1. Choose *Reset* from the *File* menu and select *Data* and then *Original Defaults*.
2. Choose *Primers and Probes* from the *Search* menu.
3. If you are selecting sequencing primers, click the "Hybridization Probes" button on the "Search for Primers and Probes" dialog box.
4. At the top of the dialog box, select the + or - strand. (If you want to select a probe from the + strand that will hybridize to the - strand, check the "+ strand" box.) Note that both boxes may be checked, however the search for hybridization probes is 'apolar', so that the probes for + and -

- strands cover the same DNA areas; you may click in any strand to "deselect" it.
5. Click on the "Parameters" button.
 6. Select "Very High" for the "Search Stringency."
 7. Set the length of the probes to be selected in the search using the "Oligonucleotide Length" parameter in the "Search Parameters" window, usually 21 - 28-mers.
 8. Click "OK" to return to the "Search for Primers and Probes" dialog box.
 9. Set the ranges for the desired location of the probe on your template by clicking the "Search Ranges" button and enter your range in the strand you wish to search.
 10. Click "OK" to return to the "Search for Primers and Probes" dialog box.
 11. Click "OK" to start the search.
 12. Click "OK" when the "Search Completed . . ." message appears and "Show: Selected Oligos" is displayed at the bottom of the window.
 13. Sort the oligos selected by T_m , and click on the desired oligo, selecting this oligo as the "Current Oligo" in the "Melting Temperature" window. The most optimal probes are those with the highest T_m and highest negative ΔG value for GC clamp with no significant hairpin loops ($T_m < 50^\circ$). You may check the oligos by selecting any of the *Analyze* options.
 14. If it is a + strand primer, select it as an upper primer by clicking on the "Upper" button on the left side of the "Melting Temperature" window (for a - strand primer, click "Lower"). You may need to click into the "Melting Temperature" window first.

11.5 Finding Motifs, Palindromes & Secondary Structure

11.5.1 Searching for a DNA Pattern or Motif using the Search for a Sequence String

To search for a specific sequence string in the positive strand:

1. From the *Search* menu, choose a positive strand in the *Sequence String* command.
2. Enter the desired sequence string.

3. Click "OK."

The 5' positions of any and all matches are stored and displayed in the active Memory Table and marked in red on the "Internal Stability" window. To view these oligos, scroll through the "Melting Temperature" window, or click on their numbers in the "Memory Table" window.

11.5.2 Searching for Palindromes

The search for palindromes marks the recognition sites of most restriction enzymes and certain regulatory elements. It may be useful in localizing polylinker cloning sites in DNA vectors. To search for palindromes:

1. Select *Palindromes* from the *Search* menu.

All nucleotides which comprise a given palindrome are stored in the active Memory Table and marked in the active "Memory Array." To view palindromes, scroll through the graph, or click on their numbers in the "Memory Table" window.

11.6 Database & Order Form Applications

11.6.1 Saving Primers to an Oligonucleotide Database

With OLIGO, you can create oligonucleotide databases to which you can save selected oligonucleotides and related information. To save a primer to the database:

1. Select an Upper and Lower Primer by either clicking on those buttons in the "Melting Temperature" window, or by choosing them from the "Select" menu.
2. Select *New Database* from the *File* menu.
3. Click "Upper Primer" and "Lower Primer" under Import from the "Database" window menu. If you click "Multiplexed Primers" just after the search for primers, all found oligos will be imported into the database.
4. Choose *Save* from the *File* menu.
5. Select *Database* from the *Save* submenu.

6. Name the database or accept the default, NEW DATABASE.
7. Click "OK."

11.6.2 Creating, Loading, and Using the Oligonucleotide Database and the OligoNucleotide Order Form

The OLIGO program and the PRIMEFORM Oligonucleotide Ordering Software give you the ability to save your oligo sequences to a database and send them to a synthesis ordering form. To review these features:

1. From the *Select* menu or using the "Upper" or "Lower" icons, choose an Upper and Lower primer, if not already selected. (Move the Current Oligo downstream from the Upper Primer to select a Lower Primer.)
2. From the *File* menu, choose *New Database*. If more than one sequence file is opened, use the *Link* menu to choose the appropriate sequence to link with this database.
3. Once the database is loaded, under *Import*, choose "Upper Primer" and then "Lower Primer," adding these primers to the database as records.
4. Examine the "Priming Efficiency" feature by choosing "Priming Efficiency — All Oligonucleotides" from the *Analyze* menu. This checks all the records (oligos) in the database against the active sequence and within the search ranges set for this active sequence. Two numbers are calculated — the priming efficiency of the most stable site on the active sequence is listed first, followed by the highest theoretical P.E. (perfect homology). In this example, the numbers will match if the database oligos are from the active sequence and within the set search ranges. This feature may make it possible to reuse previously synthesized oligos on a new active sequence. In order to have a reasonable chance of finding an oligo with a high enough P.E. (280+) to prime effectively on an average 2kb template, however, a 500+ record database of previously synthesized oligos to run against the active sequence is recommended.
5. To send database primers to PRIMEFORM Oligonucleotide Ordering Software, click on the

- oligonucleotide record to export and then use *Select/Deselect* from the *Export* menu (alternatively use the <space bar> key). Do the same for additional primers. Notice this primer is marked in the first column, under the "O." This indicates that the record is selected.
6. Choose *Order Form* from the *Export* menu to call up PRIMEFORM and an order form containing the selected oligonucleotide sequences.
 7. From the *Edit* menu of PRIMEFORM, select *Shipping Information*.
 8. You will see header and footer information for your order form that you may edit. Click "OK."
 9. From the *Edit* menu, select *Accounting Information*.
 10. Enter the information for your accounting department by clicking in the fields. To move from field to field, press <Tab> or use the mouse. When you are finished, click "OK" to return to the main menu.
 11. From the *Edit* menu, select *Shipping Information*.
 12. Enter the information by clicking in the fields. To move from field to field, press <Tab> or use the mouse. When you are finished, click OK to return to the main menu.
 13. From the *Edit* menu, click on *Synthesis Specifications* to select the specific service, grade, scale and turnaround for your order. When you are finished, click "OK" to return to the main menu.
 14. Select *File — Print* to get a printout of your completed order form.
 15. Quit PRIMEFORM.

11.7 Restriction Enzyme Applications

11.7.1 Creating a Restriction Site Map and Fragment Table For a DNA Template

1. With a sequence file loaded, select the *Restriction Sites* option from the *Search* menu to call up the "Search for Restriction Sites" window.
2. Click the "Entire" button to generate restriction data for the entire sequence file.

3. Click on the "Linear" button to get fragment sizes appropriate for a non-circular DNA sample, if applicable.
4. Check both the "Map" and "Table" boxes to display search results in both formats.
5. Click on "Select Enzyme Table" to open the OLIGO folder (optional), double click on "Tables" to open the "Tables" folder, click on "NICE6&UP.ENZ" or any other ".ENZ" file and then "Select" to select this database.
6. Click on "OK" to start the search.

11.7.2 Finding Restriction Sites on a Template Using "Search for a String"

1. To find a specific restriction site on a sequence file (template), you may wish to use the "search for a sequence string" option, rather than the "search for restriction sites" - particularly if you wish to incorporate the site into a PCR or sequencing primer. Select "search for a sequence string" under the "search" menu, and then choose the + or - strand.
2. In the "String Search in (+) or (-) Strand", enter your restriction site into the entry box, including any ambiguities. Click the "Find" button. The position numbers of any restriction sites found are listed in the "R3" register of the "Memory Table" window. Click on any position number in the register to move the "Current Oligo" to that position.

11.7.3 Adding Restriction Sites to the 5' ends of PCR Primers

Many PCR applications call for the addition of restriction sites to the 5' ends of PCR primers so that these restriction sites will be incorporated into the PCR product by DNA Polymerase. Typically, you will search for optimal PCR primers with OLIGO and then add restriction sites using the EDIT functions.

Alternatively, you may go directly to EDIT and type in the oligo sequences with 5' restriction sites and then analyze them using the OLIGO *Analyze* functions. NOTE: Adding a restriction site to the 5' end of an Upper Primer will change the position number of the primer on the sequence file. You must adjust this in the

“Edit - Upper Primer” window. This is not required with Lower Primers, because their position numbers are determined in the OLIGO program at their 3' ends.

The example below uses the automatic search for Primers followed by the addition of restriction sites in the “Edit” windows:

1. Select the *Search for Primers and Probes* under the *Search* menu, and then “PCR Primers: Compatible Pairs”.
2. Set the “Search Ranges” and “Search Parameters” as appropriate. Return to the “Search for Primers and Probes” dialog box and click “OK” to start the search.
3. When the search is complete, select the PCR primer pair of your choice in the “Primer Pairs” window. Print out, or otherwise note the PCR conditions in the PCR window. These should guide your thermocycler setting during the first few cycles, prior the incorporation of the restriction sites into the PCR product.
4. Add the restriction site to the Upper Primer you have selected as follows: a.) Select “Edit Upper Primer”. b.) In the “Edit Upper Primer” window, check that “insert” is on and type the restriction site plus any buffer nucleotides at the 5' end of the upper primer (Appendix D Table 8 shows the guidelines) c.) Decrease the 5' position number of the primer (displayed in the upper left corner of the window) by the number of nucleotides you have added.
5. Choose *Accept & Close* from *Accept/Discard* in the *Edit* window submenu. On the Melting Temperature window, check your Upper Primer with the restriction site. The primer itself should be complementary to its target and, therefore, in upper case letters. If it isn't, it is probably misaligned because the wrong 5' position number is set in the “Edit - Upper Primer” window.
6. Add your restriction site to the 5' end of the Lower Primer as in #4 and #5 above. However, do not change the position number of the Lower Primer, because it is the 3' end of the Lower Primer which marks its position.
7. The addition of the restriction sites should slightly improve the PCR performance of the primers, (which were selected as optimal by the OLIGO program) unless the added restriction sites form

a hairpin with a primer's 3' end or a dimer with the 3' end of its primer pair. Check that there are no significant dimers or hairpins by using *Analyze - Duplex Formation - Upper Primer*, *Analyze - Duplex Formation - Lower Primer*, and *Analyze - Duplex Formation - Upper/Lower*.

11.8 Site Specific Mutagenesis

11.8.1 Planning a Site Specific Mutagenesis Experiment Using OLIGO ver. 6

1. Load the sequence file for the mutagenesis experiment using *File - Open*.
2. Make sure that the codon table set in OLIGO is the correct one for your organism of interest. Check it using the *Codon Table* option under the *Change* menu.
3. Check that your sequence is in the correct reading frame. Change reading frame using *Options* from the "Melting Temperature" window submenu.
4. In the "Melting Temperature" window, choose an oligonucleotide length of about 24 nt, using *Oligonucleotide Length* under the *Change* menu. Select an "Upper Primer" and a "Lower Primer" so the mutation is located near the 5' end of either primer. Use the "Upper" & "Lower" buttons at the left of the window to select them. The 5' end of the Upper Primer should be adjacent the 5' end of the Lower Primer unless your mutagenesis involves a deletion.
5. Use *Analyze - Duplex Formation* to ensure that there are no "primer-dimers" in your primers. If there is a significant dimer in either primer (stronger than - 0.5 kcal/mol), move the primer(s) slightly in either direction to eliminate it (them).
6. Select *Upper Primer* under the *Edit* menu. Check reading frame again. Change it, if necessary, using *Change - Reading Frame* under the *Edit* submenu.
7. Replace the nucleotide to be mutated using the keyboard. Check that the resulting codon has the highest possible relative frequency for your organism, assuming no problem hairpins, particularly on your primer's 3' end. OLIGO colors the amino acids according to the relative frequency of their codons.

8. Choose the *Accept & Close* option from *Accept/Discard* under the *Edit* submenu, to save your mutated primer and return to the “Melting Temperature” window.
9. Check your mutated primer on the “Melting Temperature” window. The mutation should be the only lower case nucleotide in your primer. Check for dimers and hairpins, once again, using *Analyze - Duplex Formation*.
10. Check for false priming sites using *Analyze - False Priming Sites*. Your mutated primer should retain a priming efficiency (PE) above 400. No other false priming site should have a PE higher than 200. If so, you will need to modify your primer design and try again.
11. Your primers should now be ready for synthesis and use in your inverted PCR experiment. If you are using a mutagenesis method that requires only one primer, you will find that hairpins will be even more detrimental to your mutagenesis than methods requiring two. Primers with hairpins having T_{ms} at or above 40 degrees should be redesigned.

11.9 Consensus Primer & Unique Primer Applications

11.9.1 Finding a Consensus Primer Pair to Amplify Each of Several Homologous Templates

In this example, we will design PCR primers in the human cap binding protein gene sequence file (CBP.SEQ) that will also prime and amplify two homologous genes in other organisms: rabbit eIF-4E and mouse eIF-4E.

1. Load the CBP.seq file using *File - Open*.
2. Open the *Search for Primers and Probes* dialog box under the *Search* menu and click “Compatible Pairs: PCR Primers”.
3. Click the “Consensus Primers” button. You should now see the consensus primers “sheets of paper” icon. Click this icon to bring up the “Select Files” dialog box.
4. In this dialog box, you will select the files homologous to CBP.seq you wish to find consensus primers for. : in this example, rabbit and mouse eIF-4E.

- Click "Add" in the dialog box to include these files in the consensus primer search. You may wish to save the consensus primer file set for later use, by clicking on the "Save Set" button.
5. After selecting your consensus files by clicking "OK" in the "Select Files" window, click the "Parameters" button in the "Search for Primers and Probes" dialog box., calling up the "Search Parameters" dialog box.
 6. Set the "search stringency" to "high" and the oligo length to 21 nt.
 7. Open the "Search Ranges" dialog box and set the positive strand search range to "-18 - 150" and the negative search range to "300 - 500". Click "OK" to return to the "Search for Primers and Probes" dialog box.
 8. Click "OK" to start the search. At the conclusion of the search, click the "Primer Pairs" button in the "Search Status" window, to call up the "Primer Pairs" window.

Note

When the search fails use the "Search Status" window to find a non-homologous file (the one with too small number of accepted primers). Then, exclude such a file from the list and start the search again.

9. All the primers present in the "Primer Pairs" window, should have a priming efficiency (PE) of 340 or higher in all of the consensus files. If you wish to confirm this, move the primers into a database, load each consensus file (as the active sequence) and link it to the database. PEs can then be confirmed and the priming position of each primer determined.

11.9.2 Finding a Unique Primer Pair to Amplify Only One of Several Homologous Templates

Finding PCR primers which will selectively prime and amplify a specific target while not amplifying closely related targets can be accomplished using the OLIGO program. This application is useful when trying to amplify and detect a particular strain of an organism but not amplifying closely related strains.

Strategy: the high degree of homology between similar organisms means that there should be a considerable reduction in the sequence target suitable for selective priming. Accordingly, we recommend that you set search stringency to automatically change to a

lower setting when suitable primers are not found (at the top of the “Search Parameters” window. This application focuses on the use of the “Continue False Priming Search in Other Files” subsearch.

In this example, we use the same files we used in the “Consensus Primers” search above: CBP.seq, Rabbit eIF-4E and Mouse eIF-4E.

1. Load the template you wish to selectively amplify with PCR (CBP.SEQ). The closely related templates you wish not to amplify (rabbit & mouse eIF-4E) should be in sequence files accessible to OLIGO.
2. Select the *Search for Primers and Probes* dialog box under the *Search* menu, and then click the “PCR Primers: Compatible pairs” button.
3. Click the “Eliminate False Priming” subsearch box and then the “Continue False Priming in Other Files” subsearch box. This will call up the “Select Files” dialog box.
4. Highlight the Rabbit eIF-4E , and then click the “Add” button and then highlight Mouse eIF-4E followed by “Add”. If you wish to save these files as a group, click “Save Set”.
5. Click “OK” to select the rabbit and mouse “4E” files for the “continue false priming in other files” subsearch.
6. Click the “Parameters” button in the “Search for Primers and Probes” dialog box., calling up the “Search Parameters” dialog box.
6. Set the “search stringency” to “high” and the oligo length to 21 nt.
7. Open the “Search Ranges” dialog box and set the positive strand search range to “-18 - 150” and the negative search range to “300 - 500”. Click “OK” to return to the “Search for Primers and Probes” dialog box.
8. Click “OK” to start the search. At the conclusion of the search, click the “Primer Pairs” button in the “Search Status” window, to call up the “Primer Pairs” window.
9. Select the primer pair of your choice. Each primer pair should prime efficiently on CBP.Seq, but have priming efficiency numbers of 170 or below for any site on rabbit or mouse eIF-4E.

11.10 Back Translation/ Degeneracy Operations

11.10.1 Loading a Protein Sequence File and Selecting a Low Degeneracy Probe

1. Select *File — Reset*, choose Original Defaults and then Data.
2. Load your protein sequence file. Using *File — Open*, choose Protein for file type and then select your protein file from the appropriate folder. For this example, select CBP.AMI from the OLIGO folder.
3. From the *Edit* menu, select Entire Sequence. This will load the CBP.AMI file in the "Edit Sequence" window.
4. Choose Rev. Translate Method from the *Change* menu. Then choose Degenerate for the reverse translation method.
5. Select "Entire Sequence" from the *Rev. Translate* menu. This performs the reverse translation.

Note

Reverse translation is automatic in the OLIGO program, so be certain not to select Entire Sequence from the Rev. Translate menu unless you have just changed reverse translation methods. If you do, you may inadvertently "double-reverse translate a sequence."

6. Confirm that the "Edit Sequence" window now contains a degenerate nucleic acid sequence. You should see "DNA" in the bottom right corner of the window. Choose "Accept & Quit" under the *Accept/Discard* menu.
7. You should see the degenerate nucleic acid sequence from CBP.AMI in the "Melting Temperature" window.
8. Check that the desired oligonucleotide length is listed at the top of the "Melting Temperature" window. If it is not correct, change it using *Current Oligo Length* under the *Change* menu.
9. Choose "Degeneracy" under *Graph* menu on the "Melting Temperature" window submenu (Mac) or pop-up menu (use right mouse button) in PC. The window name should now be "Degeneracy [oligo length]."
10. Scroll along the sequence file using the scroll box at the bottom of the window until you find an oligo of suitable low degeneracy.

11. After choosing the desired degenerate probe, select it as the current oligo (or upper/lower primer) and then analyze it for other desirable probe characteristics under the *Analyze* menu.

11.10.2 Reverse Translating a Peptide Using the Codon Table Method

The OLIGO program provides for reverse translation of any given peptide/protein. The program designs oligos via the Degenerate method, the Lathe method (the guess-mer method for human probes), the Inosine method, or via a codon table method. A list of reverse-translation tables for various organisms is included in the codon table method. You may also enter your own codon table data. For this example, use the codon table for "mouse."

The T_m extremes are calculated from all the possible sequence combinations for a given degenerate oligo.

1. Choose *Upper Primer* from the *Edit* function.
2. Select the reverse translation method from the *Edit Change* submenu and select *Codon Table*.
3. In the "Codon Tables" window, choose "Select File" and then "Tables" in the OLIGO folder and click on "Open."
4. With "Codon Usage Tables" highlighted in the "Tables" folder, click on "Select."
5. Choose the organism of interest (mouse) for reverse translation by clicking on it, and then click "OK."
6. Choose *Rev. Translation Method — Codon Table* under the *Change* menu.
7. Click on the protein sequence box just below the red border of the DNA box.
8. Enter the amino acid sequence of interest using the keyboard. Choose *Accept & Close* from the *Accept/Discard* menu.
9. To reverse translate the amino acid sequence using the selected codon table method, choose *Entire Sequence* from the *Rev. Translate* menu.
10. Click on any amino acid in the sequence to view the codon preferences for the selected organism (mouse) for that amino acid.
11. Choose *Accept & Close* from the *Accept/Discard* menu.

11.11 OLIGO Analysis Functions

11.11.1 Finding False Priming Sites

1. Select an *Upper* and *Lower Primer* using the *Select* menu.
2. Choose *False Priming Sites* from the *Analyze* menu.

Priming or false priming sites with priming efficiency of 200 may prime in PCR, and sites with priming efficiency of 160 may prime in low temperature sequencing reactions.

11.11.2 Determining the Concentration of an Upper Primer

The calculations may be performed in two modes, "Constant Concentration" and "Constant Volume". In the first case, changing amounts of a primer effects the volume of a solvent you need to use, in the latter case, the volume is constant and the primer concentration changes. Assume you've got 10 OD of a primer and you need to make 100 μ M solution. What volume of a buffer you need to use to dissolve it?

1. Choose an Upper Primer.
2. Choose *Concentrations* from the *Analyze* menu.
3. Click the "Constant Concentration" button, and in the bottom box type **100**, so it reads "yields 100 μ M".
4. Click the "Upper Primer" button.
5. Type **10** in the second box, so it reads "or 10.0 OD (260)".
6. Read the required volume from the 4th box from the top.

11.11.3 Designing Probes for LCR

The LCR function designs four oligos optimized for LCR to detect the presence or absence of a specific mutation. To select LCR primers designed to test for the presence of a mutation at position 50:

1. Click on the far left icon (circle with cross-hairs) at the bottom of the "Melting Temperature" window.
2. Enter **50** for the base number in the dialog box.
3. Choose *LCR* under the *Analyze* menu. (You can scroll through the sequence examining length

and melting temperature in this window using the mouse.)

4. Click on the "Select" button. The original and common oligos for both + and - strands appear.
5. Click on one or more of the remaining three nucleotides to select the appropriate mutation probes. OLIGO displays the mutation-detecting probe along with the original four probes.
6. LCR primers may be downloaded as a group to an OLIGO database (use "LCR Oligos" under the *Import* submenu of any database window).

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12 Troubleshooting

Your OLIGO distributor provides thorough technical support for all of its products. In order to provide you with the best support, however, please review these sections before calling. You may find your solution here, or will be able to provide more information to our technical support staff should you need to call.

12.1 OLIGO Messages

In this section, OLIGO messages you may receive, and possible solutions to the situation, are listed in alphabetical order.

3'-end dimer in both primers.

This condition may cause very low efficiency priming in PCR and sequencing. Choose different primers, if possible.

3'-end Lower Primer dimer.

This condition may cause very low efficiency priming in PCR and sequencing. Choose a different primer, if possible.

3'-end Upper Primer dimer.

This condition may cause very low efficiency priming in PCR and sequencing. Choose a different primer, if possible.

3'-end stability range has been incorrectly set.

Check your stability range. It may be out of the allowable limits, or it may not be consistent with the PCR product size.

Can't open file: (file name).

Your sequence file may have some incompatible characters. Sequence files interrupted with periods, semicolons, or other characters are truncated and can't

be loaded. Remove the characters using any standard word processing package and save the file as plain text.

Database is empty.

There are no oligo records in the database to export. Choose a different database or go back to this database and import Upper and Lower Primers, LCR oligos, and/or multiplexed primers.

Error

Try your last OLIGO operation again. You may have inadvertently tried a function that is not operable in combination with the features you are using.

Error encountered during print.

Check your printer and its connections.

Excessive difference between product and primer melting temperatures.

This difference may contribute to false priming of the higher T_m primer. Remove nucleotides from the 5'-end to reduce and match T_m s, or choose different primers.

Expecting a float number.

Check your last entry and re-enter it. There may be a decimal point, a letter, or an illegal character in your entry.

Expecting an integer number.

It's possible that there is a decimal in your entry. Remove the decimal and try again.

Expecting a negative number.

A positive value may have been entered. Make sure the entry is a negative number and try again.

Expecting a positive number.

A negative value may have been entered. Make sure the entry is a positive number and try again.

Hairpin loop in the Lower Primer.

Check hairpin stability. Consider redesigning the oligo if the hairpin is stable, particularly a 3' hairpin.

Hairpin loop in the Upper Primer.

Check hairpin stability. Consider redesigning the oligo if the hairpin is stable, particularly a 3' hairpin.

Invalid sequence.

The sequence file may contain illegal characters or it may be an invalid sequence format. Refer to *Troubleshooting Situations — Getting Started* for suggestions on solving this.

Manually modified by the user.

You have modified the active sequence file.

No info saved for this window.

You saved a window that does not have "savable" data in it, so OLIGO saved a blank window.

No match found.

No nucleotide string of this sequence was found in this file.

No primer pairs.

No cross-compatible primers were found in this search. You may want to change your search parameters and/or search ranges and try again.

No stems longer than (X) bp.

All hairpin stems are shorter than the minimum display setting that is selected in the Upper Primer/Lower Primer/ Current Oligo hairpin stems window.

Not accepted.

The entry is not recognized by OLIGO. Check your entry and try again. You may be trying to enter information that cannot be accepted by a particular field.

OLIGO cannot determine whether the sequence is DNA or RNA. Is it DNA?

The sequence file may contain illegal characters or it may be an invalid sequence format. Refer to *Troubleshooting Situations — Getting Started* for suggestions on solving this.

Oligo T_m range has been incorrectly set.

Check your T_m range settings. Your entry is probably outside the allowable range.

Out of disk space.

This operation has exceeded the space available on your hard drive. If you're trying to save a file, you may need to erase unwanted items on your drive or reduce the size of the file you are saving.

Out of memory.

This operation has exceeded the memory of your computer. You will need to reduce the search size or quit some functions and try again.

Overlapping primers.

The positions and lengths of these primers have produced an overlap. You will need to change the position of one or both primers and try again.

PCR product length has been incorrectly set.

Check your PCR product length setting. It may be outside the allowable limit for the selected search range settings.

Print error.

Check your printer connections and settings.

Printer initialization failed.

Check your printer connections and settings.

Printing canceled.

Check your printer connections and settings.

Printing unavailable for this window.

This window cannot be printed from OLIGO. If you need to print it, take a snapshot (Mac version, under the *Window* menu) and then print it from the Clipboard. In the PC-Windows version use "Alt-Print screen" key combination to copy contents of a front window to the clipboard.

Search ranges are incorrectly set.

Reset your search ranges and try again.

Sequence is too short.

The minimum sequence size accepted by OLIGO is nine nucleotides.

Terminal stability of the Lower Primer is too high.

As PCR or sequencing primers, these oligos may be susceptible to false priming in a complex nucleic acid sample. Select oligos with a less stable 3'-end if your sample is complex, unless your application does not involve priming.

Terminal stability of the Upper and Lower Primers is too high.

As PCR or sequencing primers, these oligos may be susceptible to false priming in a complex nucleic acid sample. Select oligos with a less stable 3'-end if your sample is complex, unless your application does not involve priming.

Terminal stability of the Upper Primer is too high.

As PCR or sequencing primers, these oligos may be susceptible to false priming in a complex nucleic acid sample. Select oligos with a less stable 3'-end if your sample is complex, unless your application does not involve priming.

That access code is incorrect.

Check your access code and re-enter it. If it is still incorrect, call, fax or E-mail for technical support.

The computer's memory has been exceeded:
must exit OLIGO.

Quit the program and restart it with fewer windows and/or applications open.

The number is not within the allowable range:
(range specified).

Check the parameters again. You may have entered something outside of the allowable range.

The position numbers you wish to read may
be unrelated to the current sequence.

Check your sequence file and/or the source of the position data.

This file does not contain valid
oligonucleotide data.

This is probably not a Memory Table data file. Select a Memory Table data file and try again.

This file does not contain valid
oligonucleotide database.

This is probably not an oligonucleotide database file. Select a database file and try again.

This file does not contain valid sequence
data.

Check your sequence file. It may have some characters OLIGO does not recognize. Sequence files interrupted with periods, semicolons, or other characters are truncated and can't be loaded. Remove the characters using any standard word processing package and save the file as plain text.

Too many potential primers — not all pairs analyzed.

The number of primer pairs found exceed the program's memory. Increase your search stringency and/or reduce your search ranges and try again.

Two-step PCR recommended: (70/94).

The two-step (two-temperature cycling) PCR procedure for these primers run at these temperatures should yield a product with less background. A typical two-step PCR for these temperatures is: Denaturation at 94°C for 30 seconds, followed by annealing and extension at 70°C for 60 seconds, repeated (x) times.

Two-step PCR recommended: (68/94).

The two-step (two-temperature) PCR procedure for these primers run at these temperatures should yield a product with less background. Denaturation at 94°C for 30 seconds, followed by annealing and extension at 68°C for 60 seconds, repeated (x) times.

12.2 Troubleshooting Situations

This section describes specific problems and solutions you may have while working with the OLIGO program. They are loosely organized into "Getting Started," "System Problems," "Working With the Windows on the Screen," "Searching," and "Printing." You may find that an explanation applies to other functions, as well.

12.2.1 Getting Started

I can't start the OLIGO program.

When you install the OLIGO program, you need to go through a registration process that identifies a "workstation code" for your computer. Each computer has a separate workstation code. For each workstation code, you will need to contact NBI or one of our affiliates for an access code in order to start up OLIGO.

Sometimes the "Saved Work" file gets corrupted. This may prevent normal software operation and cause crash. Delete this file before you start Oligo. This will usually correct the problem. You may clean-start Oligo

by clicking on the Oligo icon and then hold the Shift key while double clicking the Oligo application icon (newest versions only).

If you attempt to load a long sequence file Oligo may run out of memory. In Macintosh, OS 9 or below, increase the memory for Oligo by clicking once on the Oligo application icon and choose "Get Info" by clicking on ⌘I key. Add a few more megabytes to the application and close this window. Because Oligo

had already crashed for this reason, you must remove the "Saved Work" file before you attempt to start Oligo as described above.

I can't load a sequence file.

Check that your file is in a format that OLIGO can read. Acceptable file formats include GenBank, EMBL, text, and the formats included in most sequence analysis packages (typically text).

If your sequence analysis file won't open in OLIGO, it probably contains incompatible characters. A sequence interrupted with periods, semicolons, or other characters such as ">" or "^" are truncated and can't be loaded. Remove these characters using any standard word processing package and save the file as plain text.

Note

The character ">" begins a comment line. See the CPB.SEQ file for an example of the proper format. Comments should precede the sequence.

If your sequence file was created in a word processing program and won't open, it may not have been saved as a text file. Files saved as standard word processing files appear to have no illegal characters, but may have hidden characters that prevent OLIGO from reading the file properly. Try resaving the file as a text file.

I'd like to use the reset functions, but don't know the difference.

There are two levels of reset in the OLIGO program.

The *Reset - Data* command resets all user-generated results, calculations, and accumulated data generated since opening the active sequence. This

erases all data in the Memory Tables, erases any selected primers, and resets the temperatures for ΔG calculations to 25°. It does not, however, change search parameters, except search ranges, which are reset to the full sequence length.

The *Reset - Original Defaults* command changes all parameters back to the original program defaults and closes all but the "T_m" and "Internal Stability" windows, but leaves data and search ranges unaffected.

Make sure you selected the correct reset option for your application.

The examples don't work the way the manual says they should.

Make sure that all parameters and search ranges are set correctly. Use the appropriate *Reset* option from the *File* menu and try the example again. MBI may have changed OLIGO parameters and algorithms after the manual has been printed and this way the results won't exactly match.

12.2.2 System Problems

The program works slowly.

Your search may take longer than expected, especially when you are checking a large number of oligos for false priming sites. The false priming sites subsearch checks oligos against the entire sequence file, resulting in longer search times.

Check your search stringency and increase it if possible. More stringent searches are faster than less stringent searches.

System crashes when starting OLIGO.

This is most likely due to loading a large sequence file OLIGO could not handle due to too small memory allocation. After restarting the computer, click on the OLIGO icon and press the <⌘> and <I> keys simultaneously (Mac). Increase the memory used by OLIGO. You also must delete the recent "Saved work" file, usually located in the Oligo folder, before starting OLIGO. There are many reasons for crashes on

Windows system. Please contact the technical support and describe the circumstances. Some of them are due to bugs in Oligo that can be fixed.

It is always a good idea to check for the recent version update number & date, posted on www.oligo.net, updates page. If your version has a different number, download and install it on your computer according to the provided instructions.

12.2.3 Working with the Windows on the Screen

There are too many windows on the screen and you can't find the window you want to work with.

Use the Window menu to see all the open windows (listed at the bottom of this menu).

Use the title bar (click and drag) to move each window to its optimal place so that you can view the windows better, and close those you do not need.

12.2.4 Searching

The search is complete, but doesn't produce any results.

Check that the "Automatically change stringency" box in the "Search Parameters" dialog box is checked. This relaxes the search stringency setting and searches again if nothing is found at the original setting.

Check to see that your search ranges are sufficiently wide and that the PCR product size is consistent with the search ranges.

Search ranges should be at least 100 nt whenever possible (particularly for PCR primer searches).

Shift your positive strand or negative strand search ranges, particularly if the program has selected positive and negative strand primers but no cross-compatible pairs.

If you want to keep the search stringency high, but the search yields nothing, turn off the "Automatically change stringency" button and select *Primers and*

Probes Search Data from the *Search* menu. Review the individual subsearch statistics. If one subsearch has removed most of the oligos (typically the GC Clamp), consider relaxing that single parameter and try the search again.

12.2.5 Printing

The program doesn't print.

OLIGO uses a standard system printer driver. If the program is not printing for you, first check your printer connections. If they are OK, check to see if your printer driver was installed correctly. Refer to your printer manual.

The printout doesn't look right.

OLIGO uses a non-proportional font for all its printouts. Make sure you have a font (like Courier) available on your system. If the text is too large or too small, use Macintosh *Page Setup* (*File* menu) and type a new value into "Reduce or Enlarge" box. If you need a specially formatted document, save the data in a text file, open it with your favorite word processor, reformat and print it.

I can't print OLIGO windows.

Check *Print/Save* options from the *File* menu. In Macintosh, check the *Chooser* from the *Apple* menu. Make sure that your printer is active.

12.3 Design Problems and Possible Solutions

OLIGO Oligonucleotide Search Selection Criteria			
Performance Problem	Application	Design Remedy	Selection Feature
<i>Background</i> due to false priming in genomic or other DNA samples with much unknown sequence.	PCR, Sequencing	Select oligonucleotides with high specificity.	<i>3' Stability Window</i> — Determine stability (ΔG) of the 3' ends of oligonucleotides; select only those with low or moderate stability.
Multiple PCR product bands due to false priming within and near the intended amplification region.	PCR, Sequencing	Select only oligonucleotides that will not false prime within and near the PCR product on the active sequence.	<i>False Priming Check</i> — Determine the propensity of false priming using stability (ΔG) calculations; select oligos with no strong priming affinity to any region on the active sequence.
Background due to false priming and much of the sample is known but scattered over many active sequences.	Sequencing, PCR	Select oligonucleotides which will not false prime on known sequence outside the active sequence.	<i>False Priming Check Against Other Sequence Files</i> — Check for false priming sites in all the sequence files selected by the user.
High background due to false priming in repetitive active sequences, such as ALU.	PCR, Sequencing	Select oligonucleotides which will not false prime in repetitive active sequences.	<i>False Priming Check Against Repetitive Sequence Database File</i> — Check for false priming sites in repetitive active sequences selected by the user.
Very low efficiency PCR or sequencing reactions because of 3' dimerizing and hairpinning of oligonucleotide primers.	PCR, Sequencing	Select oligonucleotides with low dimer or hairpin formation potential at their 3' end.	<i>Duplex Formation Check</i> — Check the 3' ends of all oligos, using stability algorithms, eliminating those with dimer or hairpin potential.

OLIGO 6 Oligonucleotide Search Selection Criteria			
Performance Problem	Application	Design Remedy	Selection Feature
Reduced efficiency PCR or sequencing reactions due to low primer T_m .	PCR, Sequencing	Select oligonucleotides with high or moderately high stability along their lengths, except for the 3' end.	<i>GC Clamp</i> — Determine stability (ΔG) of the 5' end and center segments of oligonucleotides; select only those with high or moderately high stability.
Very low efficiency PCR reactions because of 3' dimerizing between the Upper (upstream) and Lower (downstream) primers.	PCR	Select cross-compatible Upper and Lower Primers (primers with low 3' dimer forming potential between them).	<i>Cross-compatibility Check</i> — Check the 3' ends of Lower Primers against the 3' ends of Upper Primers, using stability algorithms, eliminating those pairings with dimer potential.
Background due to false priming of the higher T_m primer in a PCR reaction.	PCR	Match the T_m s of the Upper and Lower Primer.	<i>T_m Matching</i> — Add nucleotides to the 3' end of the Lower T_m primer until its T_m most closely matches the higher T_m primer.
Reduced efficiency due to T_m s which are too low or too high for the application.	Sequencing, PCR, Hybridization	Select oligonucleotides with optimal T_m s.	<i>T_m Window</i> — Select only oligonucleotides with T_m s within Upper and Lower T_m thresholds.
Mutations in the primer area, in the form of insertions or deletions.	Sequencing, PCR, Hybridization	Select oligonucleotides that cannot misalign on the active sequence.	<i>Homooligomer and Sequence Repeat Checks</i> — Select only oligonucleotides which do not contain strings of the same nucleotide or sequence repeats.

12.4

OLIGO Technical Support

Your OLIGO software supplier provides technical support for all its software products and research services. In order to obtain technical support for software, you must be the licensed user, or work for the licensed user.

Complete the OLIGO installation and customer registration procedures to register your OLIGO program.

13.4.1 Seeking Help

Use the standard Windows help feature or Macintosh balloon help (up to OS 9 only). If you still need assistance, contact your dealer. When contacting for technical support, please have the following information available, or include it on your fax or E-mail:

- Your name
- Name of the licensed end user, if different
- OLIGO license number
- Phone or the E-mail address

If the technical help from your dealer is not available, use the web site oligo.net to obtain the updates or other support.

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Appendix A: Glossary of Terms

- Active Reading Frame* — The reading frame currently displayed in the Edit window set to Mutagenesis mode.
- Active Sequence* — The nucleic acid sequence currently analyzed. OLIGO has the capability of loading several sequence files into memory. Only the front window, or most recently selected file however, is the active sequence.
- Ambiguous Base* — A degenerate or unknown base (wobble). Symbols are listed in Appendix D — Theories and Formulas Used in the OLIGO Program.
- Check Box* — A small box next to selectable options in a dialog box. The options can be turned on (marked with an X) or off (blank box), by clicking directly on them.
- Click and Drag* — A mouse technique used to move icons, highlight text, reposition windows, and move other options on the screen. Position the pointer on the target, click and hold the left mouse button, and drag the target across the screen. Release the button to position the target.
- Codon Display* — In a single line edit window, this display shows the relative frequency of each codon present in a given organism.
- Codon Table Method* — This reverse translation method creates a guess-mer made of the most frequent codons for a given organism.
- Current Oligo* — The presently selected oligonucleotide, capitalized and underlined on the main screen, available for analysis or Upper and Lower Primer selection.
- Degeneracy* — The total number of base combinations possible in a sequence. The list of ambiguous (degenerate) bases is in Appendix D — Theories and Formulas Used in the OLIGO Program.
- Degenerate Method* — Reverse translates a protein sequence into all possible combinations of a nucleic acid sequence.
- Delta G (ΔG)* — Free energy, a measurement of nucleic acid duplex stability. A DNA duplex is more stable when its ΔG value is more negative. See Appendix D Theories and Formulas Used in the OLIGO Program for the formula.
- Dialog Box* — A window that requests information from the user before a function or command can be carried out.

- Dimer — Two nucleic acid molecules attached to each other with hydrogen bonds.
- Duplex — A double-stranded nucleic acid. It may be contained in one molecule (hairpin stem), in two molecules (dimer), or many molecules.
- False Priming — The initiation of a nucleic acid synthesis from an unintended site. This occurs when the 3'-end of a primer has significant homology with more than one site on an active sequence. See Priming Efficiency Number (P.E. #).
- Filter Dissociation Temperature (T_d) — T_d is the temperature at which 50% of the nucleic acid probe is retained on a hybridization filter after five minutes of incubation using 1 M NaCl. To arrive at correct T_d values, OLIGO uses 100 pM oligonucleotide concentration; however, during filter washing, the concentration of the oligo in the washing solution is variable but always low relative to the concentration used during hybridization.
- Hairpin — A nucleic acid strand forming stable hydrogen bonds (with $\Delta G < \text{zero}$). The hydrogen bonded region is referred to as a stem and the single-stranded region between the two stem regions is called referred to as a loop.
- Hairpin Loop — The single-stranded region in a nucleic acid hairpin between the double-stranded stem.
- Hairpin Stem — The double stranded region of a hairpin.
- Hybridization — Process of hydrogen bond formation (or duplex formation) between nucleic acid molecules.
- Hybridization Probe — an oligonucleotide used to detect a specific nucleic acid sequence using a hybridization method.
- Inosine Method — This reverse translation method substitutes the Inosine base (universal substitution nucleotide) for a certain degenerate base. This method is described in Appendix D — Theories and Formulas Used in the OLIGO Program.
- Inverse PCR — DNA amplification of unknown sequences flanking a known sequence. When using OLIGO, 3'-ends of primers should point to the outside of a linear active sequence. The actual DNA template, however, must be a circular DNA sequence for the inverse PCR application.
- Internal Stability — This refers to the stability of subsequences within a nucleic acid. OLIGO displays internal stability as the ΔG of pentamers. Optimal primers are usually very stable on their 5'-ends and somewhat unstable

- on their 3'-ends. The variable stability within an oligo is determined by measuring the ΔG of the various overlapping pentamers within it.
- Lathe Method** — This reverse translation method is based on the most probable codons on mammalian mRNAs and the codon bias. (Ref. 14.) For more information, see Appendix D — Theories and Formulas Used in the OLIGO Program.
- LCR (Ligase Chain Reaction)** — Is a method of detecting small concentrations of target DNA molecules, usually at mutation sites, originally described by Barany, et al. (Ref. 17.) Currently, there are four basic variations of this technique: blunt end ligation, single base "sticky" end ligation, fill-in ligation, and deblock and ligation. OLIGO automatically selects probes for the single base sticky end ligation.
- Loop ΔG (Hairpin)** — The stability of the hairpin stem plus the destabilizing effects of the hairpin loop, expressed in kcal/mol.
- Lower Primer** — The Lower Primer is a negative strand oligonucleotide that has been selected for analysis, generally as a PCR or sequencing primer. The Lower Primer can be manually selected by the user or automatically selected by OLIGO.
- Melting Temperature (T_m)** — The temperature at which 50% of oligonucleotides are in duplex (and 50% single stranded), or 50% of a long molecule is in duplex and 50% partially melted. For the default T_m calculations, 1 M salt (Na^+ or K^+ , neutral pH) and 100 pM nucleic acid concentrations are used. The OLIGO program provides T_m values for oligonucleotides based on the "nearest neighbor" thermodynamic method that are in line with, but approximately four times more accurate, than the "2xAT+4xGC" method. Melting temperature calculations for longer nucleic acid molecules (50-100 nt and longer), however, are most accurately determined by the %GC method.
- Memory Array** — The oligo position displayed at the bottom of the "Internal Stability" window. The individual squares indicate positions on the active sequence. A marked nucleotide appears as a blue square and represents a position in the R1-R3 memory tables. It usually represents the 5'-end of the selected positive strand primers (top sequence = R1) and 3'-end of the selected negative strand primers (sequence in the middle

- = R2) in searches for oligonucleotides. In hairpin and palindrome searches, the entire hairpin and/or palindrome is marked by blue squares. The Memory Array may be used to manually mark positions by clicking on the grid.
- Memory Table** — A table where the results (numerical position data) of a search are stored. There are three Memory Tables — R1, R2, and R3. R1 is the active table for positive strand searches and R2 for negative strand searches.
- Multiplex Primers** — Primers that do not form 3'-terminal dimers with each other that are used for PCR in one incubation mixture. The dimer stability threshold can be modified by changing search parameters.
- Nearest Neighbor** — The most accurate method for calculating T_m of oligonucleotides; see Appendix D for the formulas.
- Negative Strand** — A nucleic acid sequence complementary to the sequence loaded from a sequence file. In OLIGO, it is displayed in blue on the "Melting Temperature" window.
- Nested Primer Pair** — Two cross-compatible pairs, one positioned within the other, used to amplify a difficult template.
- OD Unit** — The quantity unit for nucleic acids (and other light-absorbing compounds) based on light absorption. One unit of nucleic acid dissolved in 1 ml gives an absorption of 1 using a cuvette with a 10 mm path length.
- Optimal Annealing Temperature (T_a^{OPT})** — The annealing temperature, calculated by OLIGO, that gives the highest product yield when no false priming and primer dimerization occurs. Usually, with complex templates, the optimal annealing temperature is higher due to the increased likelihood of false priming. In these situations, use T_a^{max} , which is three degrees higher than the T_m of the less stable primer. However, in certain circumstances (short primers, high GC template) using the T_a^{max} may yield no product.
- Positive Strand** — A nucleic acid sequence strand identical to the sequence loaded from a sequence file. It is displayed on the "Melting Temperature" window.
- Primer Extension Arrow** — An arrow extended from the 3'-end of the Upper/Lower Primer to show the potential polymerization direction.
- Priming Efficiency (P.E.)** — This number is a formulation unique to the OLIGO program that quantifies the likelihood that a given oligonucleotide will prime at

a given site on the active sequence. The priming efficiency calculation is derived from an algorithm that considers mismatches, duplex stability, bulge loops, and the distance of these elements from a primer's 3'-end.

Reverse Translation — Prediction of a DNA sequence from a protein sequence.

Salt Concentration — The concentration of monovalent ions — sodium or potassium, for example, (but not magnesium).

Search Stringency — The level at which the search parameters are set in the OLIGO program to select oligonucleotides optimized for the various search applications. OLIGO gives the user six global stringency options that automatically set the eight individual search parameters in the "Search Parameters" window to appropriate pre-set values. These stringency settings can be automatically relaxed to a lower global setting by the program in the event that no suitable oligo positions remain after a search.

SSC — Sodium Chloride/Citrate Buffer. 1x SSC is 0.15 M NaCl in 0.015 M Sodium Citrate, pH 7.0 (NaOH).

Terminal Stability — The free energy (ΔG) threshold for an oligonucleotide's 3'-terminal pentamer and adjacent pentamer.

Upper Primer — The Upper Primer is a positive strand oligonucleotide that has been selected for analysis, as a PCR primer, a sequencing primer, or a hybridization probe. The Upper Primer can be manually selected by the user or automatically selected by OLIGO.

Appendix B: PCR and Sequencing Primer Selection Criteria

There are three essential considerations in selecting PCR primers:

- The primer has the ability to form a stable duplex with the specific site on the target DNA
- The primer has no duplex formation with another primer molecule
- The primer has no false priming sites on the target DNA

Primer stability can be measured in terms of the length (base pairs), the GC/AT ratio (free energy expressed in kcal/mol), or in T_m ($^{\circ}\text{C}$) of a DNA duplex. The most accurate methods for computing helix stability are based on nearest neighbor thermodynamic parameters (1).

Calculations of melting temperature (T_m) according to the nearest neighbor method is complicated, and therefore not practically determined without computer software. A duplex stability measure of similar accuracy, however, may be achieved by a simpler method — by calculating the free energy of duplex formation. (See Table 1 in *Appendix D — Theories and Formulas Used in the OLIGO Program.*)

Internal Stability

Primers that are stable at their 5'-termini but somewhat unstable on their 3'-ends perform best in sequencing and PCR reactions. This primer structure greatly reduces false priming on unknown sample sequence. These findings, based on primer internal stability, are supported by the experimental data presented in *Figure B.1 — Internal Stability Graph*.

A primer with low stability on its 3'-end will function well in PCR because the base pairings at and near the 3'-end with non-target sites are not sufficiently stable to initiate synthesis (false priming). The 5'-end and the central part of the primer must also form a duplex with the target DNA site in order to prime efficiently. Conversely, oligonucleotides with stable, GC-rich, 3'-

termini need not anneal with the target along their entire length in order to efficiently prime, resulting often in non-specific product synthesis. Examples of highly specific primers (G1 and G2), non-specific primers (B1 and B2) for PCR and sequencing are presented in Figure B.1.

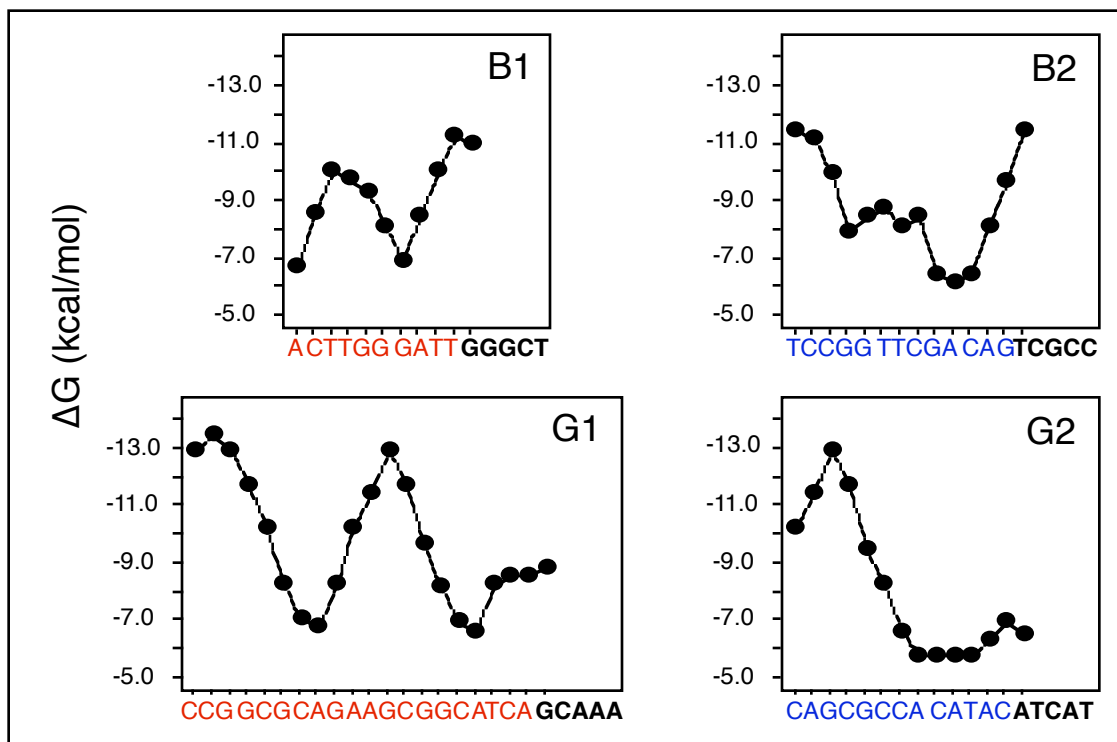


Figure B.1 Internal Stability Graph. Primers B1 and B2 are poorly functioning. Primers G1 and G2 are efficient sequencing primers. Primers G1 and G2 perform better than average (with almost any other compatible primer) in PCR. The ΔG values were calculated for all pentamers in each primer. The last symbol in each inset represents the ΔG value of the 3'-terminal pentamer.

Note the high 3'-end stability of the non-specific primers and the low stability of the specific primers. The optimal annealing temperature range is unusually broad when primers exhibiting low 3'-terminal stability are used. This improves the chances of running PCR at optimal conditions without preliminary optimization experiments. In addition to optimal primers, the quality of the PCR product is dependent on the enzyme and the reaction buffer composition, the active sequence (substrate complexity), the product length, the product

T_m , and on PCR program times and temperature settings (particularly annealing).

Under certain conditions, primers with high 3'-terminal stability perform satisfactorily in PCR. Nevertheless, oligonucleotides with 3'-terminal pentamers less stable than -9 kcal/mol are more likely to be specific PCR and sequencing primers, particularly in complex samples.

Appendix C: The Effect of Primer Duplex Formation on PCR and Sequencing

PCR primers should be free of significant complementarity at their 3'-termini, as this promotes the formation of primer-dimer artifacts that reduce product yield. Formation of primer-dimer artifacts may also cause more serious problems, such as non-specific DNA synthesis due to an unbalanced primer ratio — asymmetric PCRs fail more frequently than "standard" reactions. PCR yield dependence upon the ΔG of 3'-terminal duplexes is illustrated in *Figure C.1 — PCR Yields*.

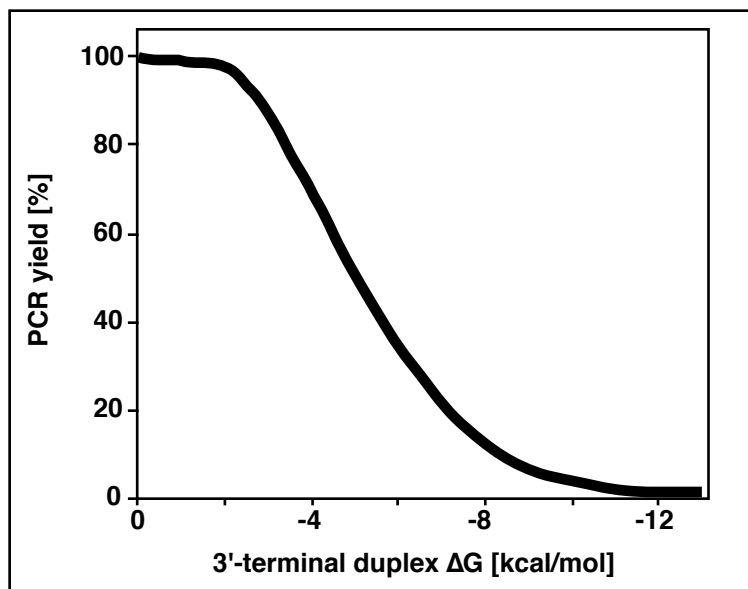


Figure C.1 PCR Yields. Dependence of PCR yield upon the ΔG of 3'-terminal primer duplexes. The ΔG values are calculated according to the equation VI (T set to 25°).

The values shown in Figure C.1 are approximate since the PCR yield also depends on the annealing temperature, the specificity of primers, and other parameters not considered here. The high dependence of yield on 3'-dimer formation tendency is the result of the very high processivity of thermostable polymerases. Duplexes need not be stable to prime DNA synthesis. Very little time is required for the enzyme to recognize a 3'-terminal duplex and start polymerization.

In general, oligonucleotides forming intramolecular duplexes with negative ΔG should be avoided. Although self-complementary PCR primers with hairpin stem ΔG approaching -3 kcal/mol (at 25°) are suitable in certain cases, a hairpin loop-forming primer is troublesome when its 3'-end is "tied up," since this can cause internal primer extension, eliminating a given primer from the reaction. Hairpins near the 5'-end, however, do not significantly affect PCR. Non-3'-end primer dimers are well tolerated in PCR. Internal or 5'-terminal dimer ΔG may approach -20 kcal/mol usually with only slight effects on PCR.

Appendix D: Theories and Formulae Used in the OLIGO Program

The OLIGO program algorithms take into account several formulae. Many of the formulae used in the OLIGO program are described here.

T_m — Melting Temperature, Nearest Neighbor Method

The melting temperature of an oligonucleotide duplex (T_m^{primer}), is calculated using nearest-neighbor thermodynamic values (Formula I). Since these values were obtained in 1 M Na⁺, it is necessary to add a factor to correct for salt concentration (3), resulting in the equation:

$$T_{m}^{\text{Primer}} = \frac{\Delta H}{\Delta S + R \times \ln(C/4)} + 16.6 \log \frac{[K^+]}{1 + 0.7 [K^+]} - 273.15 \quad (I)$$

Where ΔH and ΔS are the enthalpy and entropy for helix formation, respectively, R is the molar gas constant (1.987 cal/°C X mol), and C is the concentration of the probe. Values of ΔH and ΔS used by OLIGO are listed in Tables 3 and 4. For the T_m^{primer} calculations in the PCR window, the expression $C/4$ is replaced by C since the primer and the active sequence concentrations are different.

T_d — Filter Dissociation Temperature

For filter hybridization applications, the dimer dissociation temperature, T_d , is calculated:

$$T_d = T_m - k \quad (II)$$

Where k is a temperature correction for filter hybridization, and in Ref. 4 it is equal to 7.6°C. Recently, new thermodynamic values have been reported (5), eliminating the requirement for the k value ($k = 0$); therefore, $T_d = T_m$ (T_m calculated by nearest neighbor

method). For DNA calculations OLIGO 6 uses -15.1 e.u. for initiation ΔS (5), and other values from Ref. 1. Values obtained using this new formulation are more accurate — standard deviation from values obtained experimentally (4) for ten oligonucleotides was $\pm 0.4^\circ\text{C}$. The temperature for filter hybridization should be approximately 10°C lower than T_d .

2xAT+4xGC Method

Add 2° for each A and T and 4° for each G and C nucleotide. This is the simplest but the least accurate method for oligonucleotide T_m calculations. Note that the concentration of nucleic acid is ignored. To roughly match these T_m values with the nearest neighbor method values, the oligonucleotide and salt concentrations should be set (for the nearest neighbor method) at 100 pM and 1 M, respectively.

%GC Methods:

T_m^{product} (T_m of Long DNA Duplexes)

The T_m of long DNA duplexes (T_m^{product}), required in calculating the optimal annealing temperature for PCR (T_a^{OPT}), cannot be derived from equation I since the nearest-neighbor model is not applicable. Instead, the formula of Wetmur (3) is used:

$$T_m^{\text{Product}} = 81.5 + 16.6 \log \frac{[K^+]}{1 + 0.7 [K^+]} + 0.41 (\%G + \%C) - \frac{500}{\text{Len}} - \%mismatch \quad (\text{III})$$

Note: This formula has been changed since previous versions of OLIGO.

RNA T_m Calculation

OLIGO uses a similar equation for RNA T_m calculation (3):

$$T_m = 78 + 16.6 \log \frac{[K^+]}{1 + 0.7 [K^+]} + 0.7 (\%G + \%C) - \frac{500}{\text{Len}} - \%mismatch \quad (\text{IV})$$

DNA-RNA Hybrids

The DNA RNA Hybrid formula is calculated according to the following equation:

$$T_m = 67 + 16.6 \log \frac{[K^+]}{1 + 0.7 [K^+]} + 0.8 (\%G + \%C) - \frac{500}{Len} - \%mismatch \quad (V)$$

T_a^{OPT}

In order to calculate T_a^{OPT}, a modified empirical formulation is used (6):

$$T_a^{OPT} = 0.3 \times T_m^{Primer} + 0.7 \times T_m^{Product} - 25 \quad (VI)$$

in which T_m^{primer} is the calculated T_m of the less stable primer-template pair and T_m^{product} is the T_m of the PCR product (in 0% formamide and 0% mismatch; for primer T_m calculations its actual concentration should be used).

OLIGO calculates free energy (ΔG) values according to the equation:

$$\Delta G = \Delta H - T\Delta S \quad (VII)$$

Where H, S, and T are the enthalpy, entropy, and temperature, respectively. The default temperature used by OLIGO is 25°C.

Table 1 Free energy values of a nearest neighbor nucleotide used by OLIGO (in kcal/mol X (-1); from Ref. 1 and 2; adjusted to 25°).

Second Nucleotide →	dA	dC	dG	dT	A	C	G	U
First Nucleotide ↓								
dA or A	1.9	1.3	1.6	1.5	1.1	2.4	1.9	1.1
dC or C	1.9	3.1	3.6	1.6	2.2	3.3	2.2	1.9
dG or G	1.6	3.1	3.1	1.3	2.7	3.8	3.3	2.4
dT ¹ or U	1.0	1.6	1.9	1.9	1.4	2.6	2.2	1.1
Initiation ²	-3				-5			

¹Not listed nucleotides are treated as dT.

²Initiation ΔG value is not included OLIGO ΔG calculations.

Although Oligo does not calculate T_ms of PNA, you may find important guidelines in (7, 21).

For example, pentamer d(AACTG) ΔG is:

$$\Delta G_{AACTG} = \Delta G_{AA} + \Delta G_{AC} + \Delta G_{CT} + \Delta G_{TG} = - (1.9+1.3+1.6+1.9) = -6.7 \text{ kcal/mol}$$

Table 2 Free energy values of hairpin loop formation used by OLIGO (in kcal/mol; from Ref. 2 and 9; extrapolated).

Size	ΔG	Size	ΔG	Size	ΔG	Size	ΔG
3	5.2	13	5.2	23	7.7	>40	10.2
4	4.5	14	5.6	24	7.9	>45	10.6
5	4.4	15	5.8	25	8.1	>50	11.0
6	4.3	16	6.1	26	8.3	>55	11.3
7	4.1	17	6.4	27	8.4	>60	11.5
8	4.1	18	6.7	28	8.6	>65	11.7
9	4.2	19	6.9	29	8.8	>70	11.9
10	4.3	20	7.1	30	8.9	>80	12.1
11	4.5	21	7.3	31	9.1	>90	12.2
12	4.9	22	7.5	>35	9.7	>100	12.3

Hairpin Loop T_m

Hairpin loop T_m is calculated according to the equation:

$$\text{Hairpin loop } T_m = T_{G0} + 2.1 \times \log[\text{salt}] \times (n-1) \quad (\text{VIII})$$

Where T_{G0} is the temperature at which ΔG of the structure is equal to 0, and n is the hairpin loop stem length. This equation is based on data of Groebe and Uhlenbeck (9). Inosine is known to destabilize duplex formation. Although the thermodynamic data are not complete, OLIGO partially corrects loop ΔG calculations (based on Ref. 10): for T-I, G-I, and I-I base pairs values of 0.6, 0.8, and 0.9 kcal/mol are added to the total hairpin ΔG .

Hybridization Time

Hybridization Time is calculated according to the formula:

$$t_{1/2} = \frac{N \ln 2}{3.5 \times 10^5 \times \sqrt{C} \times C_N} \quad (\text{IX})$$

Where N is the total number of base pairs in a non-repeating sequence (molecular complexity), L is the probe strand length, and C_N is the probe concentration (mol nucleotides/liter; 14).

For simplicity, $N = L$, therefore,

$$t_{1/2} = \frac{\sqrt{L} \times \ln 2}{3.5 \times 10^5 \times C} \quad (x)$$

Where C is probe concentration (moles/liter).

Table 3 Entropy values of a nearest neighbor nucleotide (in negative entropy units, -cal/°K/mol; from Ref. 1, 2, and 5).

Second Nucleotide →	dA	dC	dG	dT	A	C	G	U
First Nucleotide ↓								
dA or A	24.0	17.3	20.8	23.9	18.4	26.2	19.2	15.5
dC or C	12.9	26.6	27.8	20.8	27.8	29.7	19.4	19.2
dG or G	13.5	26.7	26.6	17.3	35.5	34.9	29.7	26.2
dT ¹ or U	16.9	13.5	12.9	24.0	22.6	35.5	27.8	18.4
Initiation	15.1				10.8			

¹ Not listed nucleotides are treated as dT.

Table 4 Enthalpy values of a nearest neighbor nucleotide (in -kcal/mol; from Ref. 1 and 2).

Second Nucleotide →	dA	dC	dG	dT	A	C	G	U
First Nucleotide ↓								
dA or A	9.1	6.5	7.8	8.6	6.6	10.2	17.6	5.7
dC or C	5.8	11.0	11.9	7.8	10.5	12.2	8.0	7.6
dG or G	5.6	11.1	11.0	6.5	13.3	14.2	12.2	10.2
dT ¹ or U	6.0	5.6	5.8	9.1	8.1	13.3	10.5	6.6

¹ Not listed nucleotides are treated as dT; $\Delta H_{ini} = 0$.

Table 5 Extinction coefficient values (ϵ , in A_{260} units/ μ mol; from Ref. 11).

Second Nucleotide →		dA	dC	dG	dT	dl	dN
First Nucleotide ↓							
dA		13.7	10.6	12.5	11.4	9.3	12.0
dC		10.6	7.3	9.0	7.6	7.2	8.6
dG		12.6	8.8	10.8	10.0	8.8	10.5
dT		11.7	8.1	9.5	8.4	8.1	9.4
dl		9.3	7.1	8.8	8.4	6.8	8.4
dN ¹		12.1	8.7	9.4	9.4	8.7	9.9
		A	C	G	U	I	N
A		13.7	10.5	12.5	12.0	9.3	12.2
C		10.5	7.1	8.9	8.1	7.1	8.6
G		12.6	8.7	10.8	10.6	8.8	10.5
U		12.3	8.6	10.0	9.8	8.6	10.2
I		9.3	7.1	8.8	8.3	6.8	8.4
N		12.1	8.7	9.4	9.4	8.7	9.9
dA	15.4	dT	8.7	A	15.4	U	9.9
dC	7.4	dl	7.2	C	7.2	I	7.2
dG	11.5	dN	10.7	G	11.5	N	11.0

¹ Nucleotides not listed here are treated as dN.

Nearest neighbor method:

$$\epsilon_{ABCDE} = [2 (\epsilon_{AB} + \epsilon_{BC} + \epsilon_{CD} + \epsilon_{DE}) - \epsilon_B - \epsilon_C - \epsilon_D]$$

Table 6 Molecular weight (M_r) values used by OLIGO.

A	329.21	dA	313.21
C	305.19	dC	289.19
G	345.21	dG	329.21
U	306.17	dT	304.20
I	330.20	dl	314.20
N	321.44	dN	308.95

$$M_r ABCDE = M_r A + M_r B + M_r C + M_r D + M_r E + 18.02 \text{ (for H}_2\text{O)}$$

Table 7 Ambiguous bases which are recognized by the OLIGO program (12). For calculations of thermodynamic parameters, all ambiguous bases and inosine are treated as deoxythymidine.

Symbol	Meaning
B	not A
D	not C
H	not G
I ¹	inosine
K	G or T
M	A or C
N	A C G T
R	A or G
S	C or G
V	not T
Y	C or T
W	A or T
X ¹	A C G T

¹ This symbol is not listed in Ref. 12.

Table 8 *Restriction Endonuclease Cleavage Efficiency (13).*
Linearized plasmids were incubated with the indicated enzymes (10 U/μg) for 1 h. More conservative values are presented.

Enzyme	Base Pairs from End	Cleavage Efficiency [%]
Aat II	1	95
Acc 65 I	2	99
Afl II	2	13
Age I	1	100
Apa I	2	100
Asc I	1	97
Avr II	1	100
BamH I	1	97
Bgl II	3	100
BsiW I	2	100
BspE I	2	100
BsrG I	1	88
BssH II	2	100
BstX I	1	>70
Cfr9 I	0	95
Cla I	1	>70
Dra II	3	>70
Eag I	2	100
Eco24 I	1	80
Eco88 I	0	100
EcoR I	1	100
EcoR V	1	50
Ecl136 II	1	100
Hinc II	1	33

Hind III	2	91
Kas I	1	93
Kpn I	1	99
Mlu I	2	99
Mun I	2	100
Nco I	2	100
NgoM IV	2	100
Nhe I	1	82
Not I	4	>70
Nsi I	2	95
Pae I	1	75
Pac I	1	76
Pme I	1	94
Ppu10 I	3	98
Pst I	3	98
Sac I	1	99
Sac II	3	>70
Sal I	3	89
Sma I	1	>70
Spe I	2	100
Sph I	1	92
Xba I	1	94
Xho I	1	97
Xma I	2	92

Appendix E: Amino Acid Symbols

Nondegenerate Amino Acid Symbols

Symbol	Name of Amino Acid
A	Alanine
C	Cysteine
D	Asparatic Acid
E	Glutamic Acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine
-	Termination Codon

Degenerate Amino Acid Symbols (Used in Search for Restriction Sites in Protein)

Symbol	Amino Acid Equivalent
a	CW-
b	DE
c	FL
d	HQ
e	IM
f	KN
g	RS
h	Y-
r	FLIV
s	GR-
t	LIV
u	LMV
v	KEQ-
w	PAST
x	SRGC
y	YNDH
z	WRG
1	AEGIKLPQRSTV-
2	AEGKLMPQRSTVW-
3	ACDFGHILNPRSTVY
4	SKRIMNT
5	VADEG
6	QRLPH
7	SYFLCW-

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Software User Comment Sheet

As hard as we try, we don't always get everything right. We're hoping you'll take a few minutes to let us know about any errors you found in this manual or any problems you had with the program. Please make a copy and complete this page and send it back to MBI (Fax: 1-719-684-7989 or use OLIGO.NET web page). We'll try to address your concerns in the next release of the program.

User Manual

If you found something we need to address in the manual, please describe it here:

_____Error _____Suggestion _____Comment

Chapter/Page Number: _____

Description:

Software Program

If you found something we need to address in the program, please describe it here:

_____Error _____Suggestion _____Comment

Menu Item: _____

Description:

About You

This section is optional. We'd like to be able to contact you for more information on some issues, but you can remain anonymous, if you prefer.

Name: _____

Institution: _____ Department: _____

Address: _____

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