Primer Premier
6.0

Manual

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Introduction to Primer Premier

Primer Premier is the most comprehensive tool for designing and analyzing PCR primers. Primer Premier 6 designs primers for standard PCR. It automatically interprets the BLAST search results and utilizes a proprietary algorithm to check for possible secondary structures. Homologies and structures are avoided for designing highly specific and efficient primers. Search algorithm finds optimal PCR, multiplex and SNP genotyping primers with the most accurate melting temperature using the nearest neighbor algorithm. Primers are screened for secondary structures, dimers, hairpins, homologies and physical properties before reporting the best ones for your sequence, in a ranked order

Primer Premier provides the following major functionalities:

Primer design

Optimal primers – designs primers free of dimers, repeats and runs.

Multiplexing – Pools of primers are checked for cross homologies to reduce primer dimer.
Allele Discrimination – designs primers for detection of both wild and mutant alleles.

SNP amplification – designs SNP flanking primers to amplify SNPs.

Multiplex primers – checks primers for cross reactivity preventing competition in multiplex reactions.

Evaluate pre-designed primers – allows the use of previously designed or published primers of standard PCR assays. Designs a compatible primer given an antisense or sense primer.

Avoid cross homology – ensures specificity by automatically avoiding homologies found using BLAST.

Primer and amplicon BLAST search – BLAST searches primers and amplicons to verify specificity of the design.

BLAST search – BLAST searches the entire sequence, designed primers and amplicon to visualize specificity.

BLAST database – searches local custom databases using StandAlone or Desktop BLAST or connects directly to the NCBI server for public databases.

Repeat and low complexity regions – optimizes BLAST search parameters to detect repeats and low complexity regions while searching the genomic databases available at NCBI.

BLAST result view – provides result view for BLAST searched on the sequence, primer-pair and amplicon.

Algorithm – calculates highly accurate Tm using nearest neighbor thermodynamic algorithm.
Rating – ranks primers as per their priming efficiency and screens them for their thermodynamic properties as well as secondary structures.

Database Management

Projects – creates multiple projects. Data of multiple experiments can be easily managed by creating separate projects for each experiment.

Application database – maintains a local database for sequence information and search results.

Web Integration

Web Integration – batches of sequence can be quickly retrieved from Entrez using accession numbers and from dbSNP using assay Ids.

BLAST Search – BLAST searches sequences against local custom databases using the StandAlone BLAST service and Desktop BLAST from NCBI.

Template Secondary structure search – uses an innovative proprietary algorithm to detect and display the possible template structures at folding temperatures. These structures are avoided during primer design.

Input/Output

Online sequence retrieval – automatically connects to Entrez or dbSNP and retrieves multiple sequences.

File format – supports GenBank, FASTA and dbSNP formats. GenBank and FASTA formated files can contain multiple sequences.

SNP Loading - easily adds SNP(s) at a time on a single sequence or load thousand of SNPs using standard GenBank variation files.

View output in spreadsheet – results can be viewed and manipulated in any spreadsheet program like MS Excel and lotus 123.

Flexible output format – tab delimited format of the output file can be easily loaded in to any database.

Working Environment

Menus and Toolbar

The Primer Premier toolbar is displayed at the top of the main window under the menu bar. It provides shortcut buttons for the following menu commands. The toolbar options can also be accessed from pop-up menu at right click (For Macintosh, CTRL+click) over the Sequence Information tab and Search Status tab.

Menus

The main menu bar is at the top of the main window

File Edit View Analyze Tools Online Help

Menu	Toolbar	Hot Keys (For windows)	Hot keys (For MAC)	Description
Primer Premier 6.0				Program version and PREMIER Biosoft contact information
About Primer Premier				
Quit Primer Premier				
File Menu		Alt+F		
New				
New Project		Ctrl+N	+N	Create a new project to manage the data of every new design project.
New Sequence		Ctrl+E	+E	Add a text sequence from any other application by cutting and pasting the sequence bases or by manually typing the sequence in the Sequence window.
Open				
Project		Ctrl+O	+O	Open to work on an existing project.
Sequence:				
From File		Ctrl+L	+L	Retrieve one Single or multiple

				more sequences from a local file. (must be in FASTA or GenBank format)
From Entrez		Ctrl+G	+G	Retrieve one or more sequences from Entrez. Program interprets the SNP information in the header and displays it
From dbSNP		Ctrl+S	+S	Retrieve one or more sequences from dbSNP
Save Project As				Save the existing project with different file name at specified location
Save Sequence As				Save the existing sequence with different name within the project
Close Project				Close and save the current project.
Export				Exports the primer and probe search results in a tab delimited spread sheet format file type(.xls,.csv).
Export Primer Results		Ctrl+Alt+E	Alt+E	Launch Export Results window for primer search result export.
Export Sequence		Ctrl+Shift+S	+Shift+S	Launch Export Sequence window for exporting sequence with/ without oligos.
Report				Export the design results in a report format that can be

				printed.
Generate Report				The user may select the data that needs to be included in the report by checking or un-checking the boxes of the corresponding fields.
Open report		Ctrl+Alt+N	+ Alt + N	Open the report from local file that can be printed.
Exit				Saves active working project and terminates program.
Edit Menu		Alt+E		
Copy				
Sense Primer		Ctrl+Shift+E	+Shift+E	Copy the Sense Primer of the selected sequence to the clipboard.
Antisense Primer		Ctrl+Shift+A	+Shift+A	Copy the Anti-sense Primer of the selected sequence to the clipboard.
Select All		Ctrl+A	+A	Select all sequences in the Sequence Information tab.
Find		Ctrl+F	+F	Search Sequences.
Delete Sequence		Delete	Delete	Delete selected sequences in the Sequence Information tab.
View Menu		Alt+V		
Sequence Details		Ctrl+Alt+Q	+Alt+Q	View selected sequence source file.
BLAST Details				
Complete Sequence		Ctrl+Shift+S	+Shift+S	View BLAST results information for the selected sequence.

Primer Pair		Ctrl+Shift+P	+Shift+P	View BLAST results information for the Primer Pair.
Amplicon		Ctrl+Shift+C	+Shift+C	View BLAST results information for the Amplicon.
Template Structure Details		Ctrl+Alt+T	+Alt+T	View Secondary structure information for the selected sequence.
Multiplex Primers		Ctrl+Shift+I	+Shift+I	Launch Multiplex Primers result window.(More than one sequences should be selected)
Cross Homology		Ctrl+Alt+H	+Alt+H	View Cross-Homology results data interpreted form the BLAST results for the selected sequence.
All				
Primer				View All primers for the selected sequence ordered by descending rating.
All Structures				
Primer				View graphical displays of primer secondary structures for the selected sequence.
Sequence View		Ctrl+Alt+Z	+Alt+Z	Show/Hide Sequence View.
Base Per Block				Shows either 3/10 bases per block in the sequence.
Sequence Strand				Shows either Single/Double strand in the sequence.
Tool bar				Show/Hide Tool bar.

Status bar				Show/Hide Status bar.
Analyze Menu		Alt+A		
Primer Search		Ctrl+L	+L	Launch Standard Primers selected, Primer Search parameter window.
Evaluate Predesigned Primers		Ctrl+Alt+P	+Alt+P	Launch Standard Primers selected in Evaluate Primers window for evaluating pre-designed primer pair.
Template Structure Search		Ctrl+T	+T	Launch Template Structure Search parameter window for Template secondary structures search.
BLAST Search				
Complete Sequence...		Ctrl+Alt+S	+Alt+S	Set parameters and launch BLAST Search for selected sequences.
Primer Pair		Ctrl+Alt+M	+Alt+M	Launch BLAST Search Primer Pair window to verify cross homology in the designed primer pair.
Amplicon		Ctrl+Alt+A	+Alt+A	Launch BLAST Search Amplicon window to verify cross homology in the designed Amplicon.
Tools Menu		Alt+T		
Reaction Conditions				Set reaction conditions for primer design.
SNP				Add or Delete SNP information
Add		Ctrl+Shift+N	+Shift+N	Launch window for adding SNP information to the selected

				sequence.
Delete		Ctrl+Shift+D	+Shift+D	Delete SNP information.
Online Menu		Alt+O		
Entrez Home				Launch the Entrez home page in default browser.
Internet Settings				Set up Internet proxy server.
Help Menu		Alt+H		
Contents		F1		View online help for Primer Premier.
Check Upgrades				Automatically detects for availability of a free program upgrade and installs it on your computer.
PREMIER Biosoft home page				Open PREMIER Biosoft home page in your browser.
Request Feature				Open an online form to request any new features to serve your research needs.
About Primer Premier				Program version and PREMIER Biosoft contact information.

Toolbar

Main Window

The main window displays the Sequence Information Tab, Search Status Tab, Sequence View and the Search Results Tab.

Sequence Information Tab

The table allows you to select sequences, primer design, BLAST search, template search. It displays the accession number/name, definition, and sequence length of all the sequences within a project. Sequences can be sorted by accession numbers, definition, or sequence length by clicking the respective column headers. You can also delete the selected sequence(s).

Search Status Tab

For convenience, an overall view of the designed primer is given in the Search Status Tab at the end of each search.
The search status is displayed in their respective modes. Primer search, BLAST search, template search and SNP status are available. Quality of the designed primer is displayed as Best, Good, Poor or Not Found. The primers with the rating greater than or equal to 75 are termed Best, those between 74 and 50 are termed Good, and those with the rating below 50 are termed Poor. If no primers are found that meet the selected set of parameters "Not Found" is displayed in the search status. By clicking the column header you can sort sequences by their quality. (Algorithm and Formulae) Similarly the BLAST search status is displayed as "Complete" or "Not Found" on the basis of the results returned from NCBI BLAST server. The Template structure search status is also displayed as "Complete" or "Not Found"on the basis of the results returned from Quikfold server. The SNP status for the sequence can be either "Selected" or "Not selected".

Sequence View

The Sequence View displays the nucleotide sequence. The selected sequence can be viewed as 3/10 bases per block. The sequence can be viewed in either as single stranded or double stranded. The designed primers are marked on the sequence .The sense primer is marked in blue and the antisense primer is marked in red. The selected SNP position is also marked in pink.

Result Display Tab

The detailed search results are displayed below the Sequence Information table. The result display tabs include primers Properties tab, BLAST Information tab and SNP Information tab.

Primer Properties Tab

You can view primer properties for a selected sequence under this tab. The primer results include both sense and antisense primer rating, primer sequence, position, length, melting temperature, GC%, maximum hairpin dG, maximum self dimer dG, repeat and run length, optimum annealing temperature, maximum cross-dimer dG, product length and product Tm. Apart from this you can also view primer secondary structures by clicking Secondary Structures option from pop-up menu available at right click (For Mac, CTRL+click) over the Primer Properties table or by selecting **View>All Structures > Primers**. You can view the alternate primer pairs for the selected sequence by clicking the All Primers button or by selecting **View >All > Primers**.

BLAST Information tab

The BLAST search information includes Genome and Database details against which BLAST search was performed and the Search status of the BLAST search for the sequence, primer-pair and amplicon. If there is any error in BLAST search or no significant cross homology is found a "Not Found" status is displayed.

You can view the information about sequences showing significant cross homology with the selected sequence by clicking the Cross Homology button or by selecting **View > Cross Homology**.
The BLAST results for a sequence against a database can be viewed in the Search Status tab or by selecting View > BLAST Details. This will open the BLAST result page in your default browser. The same information is available in the BLAST properties tab. The BLAST results for the primer pair, the amplicon can be viewed in the search status in the BLAST properties tab.

SNP Information tab

You can view SNP information for the selected sequence in this tab. The SNP information includes the SNP base position and the mutant nucleotide for all the SNPs on the sequence.

Primer Search Parameters

Primer Search Parameters for Standard PCR

The Primer Search Parameters window is launched when you select **Analyze>Primer Search** or click button from the toolbar.

Design Mutation Flanking Primers: Design a primer pair to amplify the template containing mutation.

You can set parameters for primer search in this window and launch a primer search.
Search parameters:

Avoid Template Structure: Default value: Checked. Template secondary structures will be avoided while designing primers.

Avoid Cross Homology: Default value: Checked. Interprets BLAST search results for the selected sequence to avoid designing primers in the region of cross homology.

Note: Avoid Cross Homology & Avoid Template Structure option is disabled when SNP is selected

Range Parameters

Search Location: Default value: Anywhere. The optimum primers are searched anywhere in the sequence or biased towards 5' end or biased towards 3' end of the selected sequence. Search Location selection is disabled, when the Avoid Template Structures checked.

Search Range: The optimum primers are searched within the user specified search range. The search range can be specified as From and To sequence base numbers. Search Range selection is restricted to the sequence bases analyzed for template secondary structures, when Avoid Template Structure is checked.

Note: When a SNP is selected, Search Location & Search Range options are not available.

Primer Parameters

Tm (oC): Default value = 50 +/- 5 ^{o}C Tm and the +/- tolerance. The search engine finds primers for all sequences as close as possible to the specified target value of Tm. For example: To specify a range of 60 ^{o}C to 70 ^{o}C, enter 65 ^{o}C for target Tmand 5 ^{o}C for the +/- tolerance. Tm values are calculated using the nearest neighbor thermodynamic algorithm.

TaOpt (oC): Default value = 50 +/- 5 ^{o}C. TaOpt and the +/- tolerance. The search engine finds primers for all sequences as close as possible to the specified target value of TaOpt. Formula for calculating TaOpt.

Primer length: Default value = 18 to 25 base pairs, the desired range for the primer length.

Primer Pair Parameters

Specify amplicon length: Default value = 100 - 200 base pairs. Target product length range in bp. The location of the amplicon dictates the location of the primers as the program tries to design primers within the specified location.

Include gap up and downstream of SNP: Default value = 30 this parameter excludes the region

upstream and downstream of a SNP for flanking primer design.

Alternate Primers: Default value = 5. The number of alternate primer pairs designed per sequence in a single search.

Advanced Search Parameters:

Hairpin maximum dG 3' End (kcal/mol): Default value = 3 -kcal/mol. The free energy of the most stable hairpin that is acceptable at 3' end of the primer.
Formula for calculating free energy . - dG = dH - T * dS,
Where dH = enthalpy, T = temperature, dS = entropy.

Hairpin maximum dG Internal (kcal/mol): Default value = 5 -kcal/mol. The free energy of the most stable hairpin that is acceptable in the primer.

3' End maximum stability dG (kcal/mol): Default = 12 -kcal/mol Primers with a 3' end stability greater than the specified value are not considered. Primers with stability greater than 4 and less than the specified value are rated lower.

Self-dimer maximum dG 3' End (kcal/mol): Default value = 6 -kcal/mol. The free energy of the most stable self-dimer that is acceptable at 3' end of the primer.

Self-dimer maximum dG Internal (kcal/mol): Default value = 8 -kcal/mol. The free energy of the most stable self-dimer that is acceptable for a primer.

Run/repeat maximum length: Default value = 5 bp/dinuc. The maximum acceptable length of single nucleotide run (e.g. AAAA) or dinucleotide repeats (ATATATAT).

G/C Clamp- Target Consecutive G/Cs at 3' End- GC clamp is the presence of consecutive G or C bases within the last five bases from the 3' end of a primer. However, the first G or C base present within the last 5 bases and are not consecutive, are be interpreted as a GC clamp by the program.
eg. GCTTGCTGCGTTCACACT GC clamp is reported as 1.
It should be noted that presence of more than 3 G's or C's should be avoided. Primers with less than the specified number of G or C' s at the 3' end will be rated lower.
You can specify a GC clamp of 0 to 3 bp. If you specify a GC Clamp of 0, it intends that no GC clamp should be present at the 3' end of a primer. The program tries and designs such primers. If it fails, it reports primers with GC clamps. A demerit is assigned to these primers and are rated low.

An example of a primer pair in which the antisense primer does not have a GC clamp.
Sense Primer: AAACACAGAGAACACCACGAAA GC clamp-2
Antisense Primer: TGTCCCACAGCTTTAGCAAATT GC clamp-0

GC%: Default value = 40-60 %. Percentage of G and C bases tolerated in the designed primers.

Maximum ambiguous bases in the amplicon: Default value = 0. The number of ambiguous bases tolerated in the amplicon.

Maximum primer pair Tm mismatch: Default value = 4. The maximum acceptable difference between the Tm values of the two primers.

Cross-dimer maximum dG 3' End (kcal/mol): Default value =7 -kcal/mol. The free energy of the most stable cross-dimer that is acceptable at 3' end of a primer.

Cross-dimer maximum dG Internal (kcal/mol): Default value = 8 -kcal/mol. The free energy of the most stable cross-dimer that is acceptable in a primer.

Multiplexing maximum dG (kcal/mol): Default value = 6 -kcal/mol. The free energy of the most stable cross-dimer that is acceptable in a multiplex reaction.

BLAST Search Parameters

Primer Premier 6 supports BLAST search against all the genomic databases available at NCBI. Local customized databases can also be searched using the Standalone WWW BLAST service from NCBI.

In Primer Premier 6 BLAST is available for Complete Sequence, amplicon, primer pairs. The selected sequence is BLAST searched against any of the selected genomic database, the results are interpreted to designing primers avoiding cross homology. The cross homology in the thus designed primer pair/amplicon can be verified by BLASTing the primer pair/amplicon sequence against the same genomic database.

You can set parameters for BLAST search for selected sequences in the BLAST Search Parameter window. To launch the BLAST search parameters window select **Analyze>BLAST Search** or click from the toolbar.

To launch the BLAST Search parameters window for Primer pair/Amplicon select **Analyze>BLAST Search>Primer-pair/Amplicon**.
The following parameters are available for BLAST search:

Search Type

Human Genome BLAST: Search your query sequence against the genome (all assemblies), genome (reference only), Ref RNA (Default), Build RNA or Ab initio RNA databases at NCBI. http://www.ncbi.nlm.nih.gov/genome/seq/page.cgi?F=HsBlast.html&&ORG=Hs

Microbial Genome: Search your query sequence against the DNA databases (Default) of all the completed genomes at NCBI. Various organisms are listed in Search options >Genomes under two basic categories: Archaeal genome and Bacterial genome.
http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?

Eukaryotic BLAST: Search your query sequence against the genome databases (Default) available under Vertebrates, Plants, Fungi, Insects, Protozoa, Nematodes and other eukaryotes.

Vertebrates

Mouse Genome: Search your query sequence against the genome (all assemblies), genome (reference only), Ref RNA (Default), Build RNA or Ab initio RNA databases ofmouse available at NCBI. http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html

Cat Genome BLAST: Search your sequence against RefSeq RNA.
http://www.ncbi.nlm.nih.gov/genome/seq/FcaBlast.html

Chicken Genome: Search your query sequence against the genome (all assemblies), genome (reference only), Ref RNA (Default), Build RNA or Ab initio RNA databases ofcjicken available at NCBI. http://www.ncbi.nlm.nih.gov/genome/seq/GgaBlast.html

Chimpanzee Genome BLAST: Search your query sequence against the genome (all assemblies), Genome (reference only), RNA*(RefSeq, Ab Initio, build).
http://www.ncbi.nlm.nih.gov/genome/seq/PtrBlast.htm I

Cow Genome BLAST: Search your query sequence against the Genome (all assemblies), Genome (reference only), RNA*(RefSeq, Ab Initio, build).
http://www.ncbi.nlm.nih.gov/genome/seq/BtaBlast.html

Dog Genome BLAST: Search your query sequence against the Genome (reference

only),RNA*(RefSeq, Ab Initio, build). http://www.ncbi.nlm.nih.gov/genome/seq/CfaBlast.html

Pig Genome BLAST: Search your query sequence against the RefSeq (RNA). http://www.ncbi.nlm.nih.gov/genome/seq/SscBlast.html

Rat Genome: Search your query sequence against the genomic sequence, Ref RNA (Default), Build RNA or Ab initio RNA databases ofrat available at NCBI. http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html

Sheep Genome BLAST : Search your query sequence against the RefSeq RNA http://www.ncbi.nlm.nih.gov/genome/seq/OarBlast.html

Zebrafish Genome: Search your query sequence against the mRNAs or Reference mRNAs (Default) ofZebra fish available at NCBI. http://www.ncbi.nlm.nih.gov/genome/seq/DrBlast.html

Puffer fish: Search your query sequence against the Genomic Sequence (Default) of Puffer fish available at NCBI. http://www.ncbi.nlm.nih.gov/blast/Genome/fugu.htm l

Plants : Search your query sequence against DNA and mRNA (Default) database of all the completed genomes at NCBI . http://www.ncbi.nlm.nih.gov/BLAST/Genome/PlantBlast.shtml?10

Fungi: Search your query sequence against the DNA (Default) of databaseof all the completed genomes at NCBI: http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=fungi

Protozoa: Search your query sequence against the DNA (Default) of databaseof all the completed genomes at NCBI: http://www.ncbi.nlm.nih.gov/sutils/blast_table.cgi?taxid=Protozoa

Other Eukaryotes: Search your query sequence against the Genomic sequence (Default) of all the completed genomes at NCBI: http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi?organism=euk

nr BLAST: (Available only for complete sequence/Primer Pair / Amplicon BLAST Search) Search your query sequence against the chosen current nucleic acid database. Select from among organism-specific, inclusive, or specialized set databases:

nr: All GenBank+RefSeq Nucleotides+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences). No longer "non-redundant".
est: Database of GenBank+EMBL+DDBJ sequences from EST Divisions.
est_human: Human subset of GenBank+EMBL+DDBJ sequences from EST Divisions.
est_mouse: Mouse subset of GenBank+EMBL+DDBJ sequences from EST Divisions.
est_others: Non-Mouse, non-Human sequences of GenBank+EMBL+DDBJ sequences from EST divisions.
gss: Genome Survey Sequence, includes single-pass genomic data, exon-trapped sequences, and Alu PCR sequences.
htgs: Unfinished High Throughput Genomic Sequences: phases 0, 1 and 2 (finished, phase 3 HTG sequences are in nr)
pat: Nucleotides from the Patent division of GenBank.
pdb: Sequences derived from the 3-dimensional structure from Brookhaven Protein Data Bank.
month: All new or revised GenBank+EMBL+DDBJ+PDB sequences released in the last 30 days.
alu-repeats: Select Alu repeats from REPBASE, suitable for masking Alu repeats from query sequences. ftp://ftp.ncbi.nih.gov/pub/jmc/alu/
dbsts: Database of GenBank+EMBL+DDBJ sequences from STS Divisions .
chromosome: Searches Complete Genomes, Complete Chromosome, or contigs form the NCBI Reference Sequence project..
Local BLAST: Search any customized database set on your local server using the Standalone WWW BLAST service.

Server name / IP address: Host name of the server or IP address of the machine on which the Standalone WWW BLAST service is installed (Linux / Mac). This address will be used to connect the client (Primer Premier) to the server for local BLAST search.
Genomes: Select the local database category as of Human, Microbes or Others

Human: All the databases which belong to human.

Microbes: All the databases which belong to microbes.

Others: All the databases which belong to organisms other than human and microbes.

Desktop BLAST: Desktop BLAST can BLAST search sequences against local custom databases without the need of setting up a local BLAST server. You should have sequences in fasta format with either .txt or .fa file extension. Save them in a folder stored locally on your computer. Guide Primer Premier to this folder for performing Desktop BLAST. Database location: Specify the location to locate the directory having the sequence files agianst which you need to perform the desktop BLAST
Genomes : Select the local database category as of Human, Microbes or Others

Human : All the databases which belong to human. Microbes : All the databases which belong to microbes.

Others : All the databases which belong to organisms other than human and microbes.

Note: For Desktop BLAST and the local database there is an editable text field for Expect Value, for which the minimum value is 0.0001 and maximum value is 1000.

Search Options:

Program: Default = blastn. Standard Nucleotide-Nucleotide BLAST search.

Database: Displays the list of available database for each search type. The databases are same as mentioned in search type above.
To verify database to be used, follow this link :
http://www.ncbi.nlm.nih.gov/genome/seq/Database.html

Advanced options

Limit by Entrez Query:(Available only for theStandard BLAST type in the Primer Pair / Amplicon BLAST Search) BLAST searches can be limited to the results of an Entrez query against the database chosen. This can be used to limit searches to subsets of the BLAST databases. Any terms can be entered that would normally be allowed in an Entrez search session. To limit to a specific organism you can either select using the pulldown menu, form a list of the most common organism in the databases. Or enter the name of the organism in the Entrez Query field with the [Organism] qualifier. Default = (none)

Filter: Default = unchecked Filter mask off segments of your query sequence that have low compositional complexity, as determined by SEG programs. Filtering can eliminate statistically significant but biologically uninteresting reports from the BLAST output (e.g., hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences. Filtering is only applied to the query sequence (or its translation products) and not to database sequences. Default filtering is done by DUST for BLASTn and by SEG for other programs. It is not unusual for nothing at all to be masked by SEG, when applied to sequences in SWISS-PROT, so filtering should not be expected to always yield an effect. In some cases, sequences are masked in their entirety, indicating that the statistical significance of any matches reported against the unfiltered

query sequence should be suspect. This option masks Human repeats (LINE's and SINE's) and is especially useful for human sequences that may contain these repeats. Filtering for repeats can increase the speed of a search especially with very long sequences (>100 kb) and against databases which contain large number of repeats (htgs).
Note : The filter for Low-complexity and Human repeats can be checked individually in the Standard BLASTs Advanced options section of the advanced search parameters dialog box.

Expect: Default = 10 ,0.01 for Primer Pair /Amplicon BLAST.
The Expect value is the statistical significance threshold for reporting matches against database sequences; the default value is 10, meaning that 10 matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). If the statistical significance ascribed to a match is greater than the EXPECT threshold, the match will not be reported. The lower EXPECT thresholds are more stringent. Fractional values are acceptable. The Expect value can be used as a convenient way to create a significance threshold for reporting results. The E value describes the random background noise that exists for the matches between sequences. The Expect value (E) decreases exponentially with the Score (S) that is assigned to a match between two sequences. When the Expect value is set at 10, a larger list with more low-scoring hits can be reported. Similarly a lower Expect value can be set to avoid reporting of low scoring hits.

Program Advanced Options

-G Cost to open a gap [Integer]
default = 5

-E Cost to extend a gap [Integer]
default = 2

-q Penalty for a mismatch [Integer]
default = -3

-r reward for nucleotide match [Integer]
default = 1

|-e Expectation value [Real]
default = 10

-W wordsize [Integer]
default = 11

-v Number of one-line descriptions [Integer]
default = 100

|-b Number of alignments to show [Integer]
default = 100

Format Options

NCBI-gi: Default = Checked Results in NCBI gi identifiers to be shown in the output, in addition to the accession and/or locus name.

Descriptions: Default = 100, and 50 for Primer-pair / Amplicon BLAST Search. Restricts the number of short descriptions of matching sequences reported to the number specified.

Alignments: Default = 100,and 50 for Primer-pair / Amplicon BLAST Search. Restricts database sequences to the number specified for which high-scoring segment pairs (HSPs) are reported.

The default limits is100. If more database sequences than this happen to satisfy the statistical significance threshold for reporting (see EXPECT above), only the matches ascribed the greatest statistical significance are reported.

Cross Homology Threshold

Select Sequences with E-value >: Default= 0.0. Allows you to select homologous regions based on their E-values. Specify target value in? Select sequence with e-value >and click Update. The regions with E-value greater than the target value are selected and therefore avoided for primer design. The regions with E-value less than the target value remain unchecked.

Select Regions with Percent Identity <=: Default= 100. Allows you to select regions based on their percent identity. Specify the target value in

Select Regions with Percent Identity < and click **Update**. The regions with percent identity less than and equal to specified value will be selected and are avoided while primer design. The regions not satisfying percent identity criterion will remain unchecked.
AND & OR: Allows you to specify either one of the above two criteria or both for selecting the regions to be avoided. By selecting AND, the regions satisfying both the E-value and the percent identity criteria are checked and avoided. By selecting OR option, the regions satisfying either the E-value or the percent identity criterion are checked and avoided. Note: To overlook both the parameters for selecting homologous regions, uncheck both the options.

Template Structure Search

Significant template secondary structure may hinder the primers from annealing and prevent complete product extension by the polymerase. Template secondary structures search can be performed on the sequence to avoid designing primers in the regions which form stable secondary structures at the PCR extension temperature.

The Template Structures dialog is launched when you select **Analyze>Template Structures Search** or click button from the toolbar.

You can view the secondary structures for the selected sequence by selecting **View >Template Structure Details.**

Search Parameters

Sequence Range : Default value: 1- 1200 bp. The length of the sequence which is submitted for finding the secondary structures at the PCR extension temperatures.

Maximum 1200 bp can be submitted for folding.
Folding Temperature : Default value : 55o C . The temperature at which sequence is submitted for finding the secondary structures is settable.

E-mail address: Field for entering E-mail address. A valid E-mail address is essential for retrieving template structure search results.
The Sequence is folded at the PCR reaction conditions for most optimum folding results.

View Cross Homology

Primer Premier 6 avoids cross homologies, repeats and low complexity regions while designing primers. It interprets BLAST search results to determine relevant cross homologies for the selected sequence. You can view the relevant cross homologies in the Cross Homology window by selecting **View > Cross Homology** or clicking Button from the BLAST Information tab.
The window has following columns:

1. **Check box**: Checking or un-checking will select or unselect the sequences for avoiding cross homology while designing primers. By default all the checkboxes remain checked to ensure the relevant cross homologies for the sequence could be avoided.

2. **Reference:** Displayes the accession numbers of the sequences showing cross homology. This unique identifier can be used to access the sequence(s) and associated information from the database. Clicking the accession number opens the database records for the corresponding sequence in your default browser.

3. **Description:** Description of the sequences showing cross homology.

4. **E-Value:** Number of hits expected with similar or better score. It depicts the significance of match. E-value = 0 indicates complete homology.

5. **Percent identity:** Percentage of identical bases between query and subject sequence in an alignment. Percent identity = 100 indicates complete homology. 6. From - To: The region of the query sequence homologous with the database sequences is shown as the start and the end base number.

Cross Homology Threshold:

Unselect Regions with E-value <=: Default= 0.0. Allows you to select/unselect homologous regions based solely upon their E-values. Specify the target value in Unselect sequence with e-value <= and click Update . The regions with E-value less than or equal to the target value are unselected and are not avoided during primer design. The regions with E-value greater than the target value remain checked and are avoided during primer design.

Unselect Regions with Percent Identity >: Default= 98 for Human genome BLAST, 95 for eukaryotic genome BLAST, 93 for microbial genome BLAST and 98 for nr, local and desktop BLAST. Allows you to select/unselect regions based solely upon the percent identity exhibited. Specify the target value in Unselect Regions with Percent Identity > and click Update . The regions with percent identity greater than the target value are unselected and are not avoided during primer design. The regions with percent identity greater than the percent identity criterion remain checked and are avoided during primer design.

AND & OR : Allows you to specify either or both of the above two criteria for choosing the regions to be avoided. By selecting AND , the regions satisfying both the E-value and the percent identity criteria are unchecked and not avoided. By selecting OR option, the regions satisfying either the E-value or the percent identity criterion are unchecked and not avoided.

Note: To ignore both the parameters for selecting homologous regions, uncheck both the options. Unselect completely homologous sequences if E-Value > 0 : Default = checked. Allows you to select/unselect completely homologous sequences even if the E-value is greater than zero. The completely homologous regions with E-value greater than zero and percent identity =100% are unselected and therefore not avoided during primer design. Partially homologous regions with E-value greater than zero and percent identity less than 100% are checked and avoided during primer design.

The Cross Homology window displays:

1. **Description:** Description of the sequence showing cross homology.
2. **Alignment:** Portion of the query showing alignment with the subject sequence.
The Description table has four columns:
a. **Check box:** Selecting a description will allow you to select or unselect alignments ranges (from-to) in the Alignment table for avoiding cross homology. Unselecting a description will not avoid the description and it's alignments as relevant cross homology, while designing primers. Un-checking the sequence in the Description table, automatically un-checks all the sequences ranges (from-to) in the Alignment table.

b. **Reference:** Displays the accession numbers of sequences showing cross homology. This unique identifier can be used to access specific sequences and associated information from the database. Clicking the accession number opens the database records for the corresponding sequence in default browser.

c. **Description:** Description of the sequences showing cross homology. d. E-Value: Number of hits expected with similar or better score. It depicts the significance of match.

The Alignment table has four columns :
a. **Check box :** Selecting an alignment (from-to) will avoid only the selected alignment as relevant cross homology while designing primers. The sequence copy in database is unchecked by default. By default, all other check boxes remain checked to ensure the relevant cross homologies for the sequence are avoided.

b. **From - To:** The region of the query sequence homologous with the database sequence is represented in terms of the start and the end base number.

c. **E-Value :** It depicts the statistical significance of member of the set reported.

d. **Percent Identity:** It depicts the extent of similarity between two sequences.

View Secondary Structures

The graphical view of the primer structure is displayed in the Secondary Structures window. You can view the primer secondary structures by clicking **All Structures** option from pop-up menu available at right click (For Mac, CTRL+click) over the Primer Properties table or by selecting **View > All > Primers.** You can also view the primer secondary structures by clicking the 

button on the Primer Properties tab.

The Secondary Structures window displays the graphical view of all the possible structures for dimers, hairpins, cross-dimers, repeats and runs for the selected primer. The dG values for each structure and its position as internal or 3' end is also shown. The sense structures are displayed in blue while the antisense structures are displayed in red.

View Template Secondary Structures

As soon as you run a template structure search, the regions involved in template structures are underlined in orange in the sequence view.

Note: To turn off the display, select **View> Template Structure Regions** and uncheck the option. Alternatively, right click in the sequence view and uncheck **Display Secondary Structure Regions**.

The functionality described above is available for all the modes.

Note: Regions involved in template structures are displayed in the sequence view irrespective of whether the "**Avoid Template Structure**" option in the Primer Design dialog is checked. This will enable you to check if the designed primer falls within a template structure.

Multiplex Results

With Primer Premier 6 you can multiplex primers for each of the selected sequences. The multiplex results are displayed in the Multiplex Results window.
The window has four columns:

1. **Accession number**: Displays the accession numbers of multiplexed sequences.

2. **Type:** Displays the type of oligonucleotide being multiplexed.

3. **Graphical view:** Displays the graphical view of the most stable cross dimer formed between the multiplexed oligonucleotides. The sense sequence is displayed in blue and the antisense in red.

4. **Most stable cross-dimer dG (kcal/mol):** Displays the free energy of the most stable cross-dimer formed between two primers of the selected sequence

Tutorial

Create and Manage a Project

Primer Premier 6 enables you to organize your work by creating separate Projects.
You can create any number of projects using Primer Premier 6. But only one project can be opened at a time. You can delete an existing project from Finder by deleting the project folder and the .prp file.

Create New Project

You can start working with Primer Premier 6 by creating new project.

To create new project:

1. Choose **File> New>Project** or click  button from the toolbar. Enter the project name.
2. By default a project is created in the PP Projects folder. You can browse in the Save File dialog and create a project at any other location on your local drive.

Open Project

To open an existing project:

1. Choose **File > Open > Project or click**  from the toolbar. Browse and select the .prp file of the project you want to open and click Open.
2. You can also open the project by selecting a project displayed in the history list at the bottom of the file menu. Primer Premier 6 displays four most recent projects in the history list.
3. An open project is automatically closed when you open another project.

Note: To transfer a previously created project from original location to a new folder or to another drive or even to another machine, copy both the project.prp file and the project folder and paste at the new destination.

Close Project

To close the project
1. Choose **File>Close Project**.
2. Choose **File>Open>Sequence>From Entrez**. This will close the project and exit the program.

Save Project As

You can save number of projects but only one can be opened at a time.
To save a project
1) Select **File>Save Project As**.
2) Enter the project name.
3) Select the location where you want to save the project.

4) Primer Premier 6 will save the project at specified location.

Save Sequence As

You can save existing sequence as a new sequence in a project:
To save the sequence
1) Open the project.
2) Select the sequence to be saved.
3) Select **File> Save Sequence As.**
4) Save Sequence As dialog will be launched.
5) Specify the name.
6) Selected sequence will be saved with specified name within the project.

Note: Sequence will be saved with specified name, only feature information of the sequence will be copied.

Open Sequence

Sequences can be opened directly from Entrez, dbSNP or from files saved on your local drive.

Open Sequence from Entrez

Primer Premier provides online access to Entrez. Sequences can be directly retrieved from Entrez using this web integration feature.

1. Choose **File>Open>Sequence>From Entrez** or click  button from the toolbar. Enter the accession/ GI number.
2. You can open multiple sequences at a time. To open multiple sequences, type or paste the list of accession/ GI numbers separated by comma, tab or new line.
For example you can type the following accession numbers: AY634314, EF428566, NM_002018 in the open sequence window and click **OK**. The accession or GI numbers can be separated by commas or tabs, or entered one number per line. The downloaded sequences will appear in the Sequence Information table.

Open Sequence From dbSNP

Primer Premier provides online access to dbSNP. Sequences can be directly retrieved from dbSNP using this web integration feature and opened in the current project.

1. Choose **File>Open>Sequence>From dbSNP** or click  button from the toolbar. Enter the Assay ID.

2. You can open multiple sequences at a time. To open multiple sequences, type or paste the list of Assay ID separated by comma, tab or new line.

Open Sequence From File

To open a sequence from a file saved on the local drive:

1. Choose **File>Open>Sequence>From File** or click  button from the toolbar. In the file open dialog, browse and select the sequence file you want to open and click **Open**. You can open multiple sequences by selecting multiple files using Shift / Ctrl and click **Open**.
Open a different format sequence:
Primer Premier supports sequences in GenBank and FASTA formats. To open a sequence of another format first convert it to FASTA format as follows:
Copy the sequence in a text file and type a greater than sign (>) followed by a single line comment before the first base.
For example:

```
>A0001,Sample sequence in FASTA format
ATCCTAGCACGACGTGTTTACGTGCACCCGTTTCACCGATCGGCGCACCGTTTAAGCTGC
ACGAGTGGGACTCGAGCTGGACTGACGGTTTTCGACTAGCGAAAGTGGACCTGGACGTTG
TCCCAGGTCGAGGCATCGCGACGGCATGGGTTTAAATCGATCGACTACGATCGAGCTCAG
CTAGCAATCGACGATCGAGGCATTATCGCGATTATCGAGCAATCGATGAGACGAGAAGCA
TTACCGCGCCGATATAGCTATATTCGAGAGTTCGACCTTTACAAATCGCATCGGACTCAC
```

Once a sequence is opened in the program it is automatically saved in the application database and it will remain in the program until it is explicitly deleted. The sequence in the program is displayed in the Sequence Information table in the main window. You can view the complete sequence in your default browser by selecting **View>Sequence Details**.

Add Text Sequence

You can add a text sequence or a sequence from any other application not in supported format by using this option.

1. Choose **File>New>Sequence** or click  button from the toolbar.
2. In the New Sequence window type a single line definition for the sequence in the Sequence Definition text field.
3. Now Type or paste the sequence bases in Sequence window and click **Add**.

You can import sequence from any other application by cutting and pasting the sequence bases in the Sequence window. You can also edit the sequence manually by typing the sequence in the editor.

Note: In demo mode, the program does not allow you to open a sequence from your local drive or Entrez.

Load Multiple SNP

In Primer Premier 6 you can load hundreds of SNPs for designing probes for multiplex detection. SNP's can be defined as Variation feature in GenBank file format.
You can open multiple sequences from Entrez containing SNP information in the following format:

```
Variation                1256
                         /replace="t"
```

Where Variation feature represent SNP information, 1256 is SNP position as base number, and "t" represents the mutant base.
Primer Premier will automatically identify the variation feature in this format as SNP information and display it under the SNP Information Tab.
You can edit the GenBank sequences to add or modify the variation feature to match the supported format.

To edit a sequence:

1. Download the sequence from Entrez. Select **Online > Entrez Home** to launch the Entrez home page in the default browser.
2. Save the searched sequence on the local drive.
3. Open the sequence in a text editor such as Notepad and edit it to add SNP information as variation feature in the supported format explained above.
4. Save the modified file and open it in the project using Open Sequence from File option . You can open multiple sequences by selecting multiple files using Shift / Ctrl and clicking **Open** or by selecting the sequence file containing the multiple sequences.

Load Unpublished SNP information

You can even load non-GenBank SNP locations by first converting the sequence information into a GenBank like format with SNP information as Variation features. To convert the format, copy the sequence in a text file and add mandatory fields to it, as shown in the template.
Sample SNP information file

```
LOCUS       Z2354     400 bp     SNP
DEFINITION
ACCESSION   123456
KEYWORD
FEATURES             Location/Qualifiers
     variation       278
                     /replace="t"
     variation       281
                     /replace="A"

ORIGIN
ggattcccttcgatcatgggctcttctcaactctccactcttctcttttt
tcaccgtcgtctccagcggccgtgatctccccggagactatctccgattg
ccttctgaaacctccagattcttccgtgaacctaaaaacgatgacgactt
aggtgggcgattttactagctggttctaatggttactggaattatagaca
gtttgtcatgcgtatcaattctgaggaaaggtggttcgaaggaagaaaat
ttcatgtatgatgatattgcttccaatgaagagaatccaaggcctggtgt
tgatggggatgatgtttatgcaggagttccaaaggattatactggtgcag
gacaatttctatgctgctttacttggaaataaatcagctcttacggtgat
//
```

Please refer the template to incorporate the SNP information in the non-GenBank sequences.

Add SNP

You can also add SNP information to the sequences already present in the project using the Add SNP option.

1. Select the sequences in Sequence Information table.

2. Select menu **Tools>SNP>Add** or click button from the toolbar.

3. The accession number of the sequence to which SNP will be added is displayed in the text field.

4. Add Mutant base information in the Mutant Base field and SNP position as base number in SNP Position field and click Add SNP button. More than one SNP can be added for the sequence.

Select/Unselect SNP

To overlook the SNP for a sequence the **Select/Unselect SNP** option is used. This is useful when the SNP information is automatically interpreted from the sequence annotations while sequence loading.

You can select multiple sequences and use this option to overlook SNP information.

1. Select the sequences in the Sequence Information table.

2. Click button from the toolbar.

3. The available SNP will be unselected in the SNP information tab.

Delete SNP

You can delete the SNP information for a sequence using Delete SNP option.

1. Select the sequence in the Sequence Information table.

2. Select **Tools> SNP>Delete** or click button from the toolbar.

3. The selected SNP for the sequence is deleted.

Search Sequence

Sequences can be searched by their Accession number or Sequence Definition.

1) To search a sequence, choose **Edit > Find** or click the  button from toolbar.

2) Select the Search Criteria and type in either an accession number or Sequence Definition keyword in the corresponding field.

3) The search can be made case-sensitive by checking the **Match Case** option.

Note: When searching by accession number complete accession number or the initial character of the accession number can be used.

For example:

1. Select Accession Number under Search Criteria, Type **L13736** in the corresponding field and click **Find**.

2. The corresponding sequence gets highlighted. In case only the partial accession number is known (L13), type the first few characters or numbers.

3. The **Find** function displays all the accession numbers starting with L13, choose the appropriate and click **Find**.

4. Similarly, a search can be conducted using a keyword that appears in the sequence definition.

5. In case of **L13736**, the definition contains word "Adenosine".

6. Select Definition under Search Criteria, key-in "Adenosine" the corresponding field and click **Find**.

7. Sequence definition in the Search window, will display search results for all the sequences that include the word "Adenosine" in their sequence definition.

Avoid Cross Homology

Primers can be designed avoiding regions of cross homology. Primer Premier 6 will BLAST your sequences, automatically interpret the results and will then design highly specific primers. Both primers and amplicons can be BLAST searched using genomic database available at NCBI or local customized database using the Standalone WWW BLAST server. Primers will already have been designed to avoid cross homology. This will allow visualizing primer specificity.

Set Parameters for BLAST Search

To BLAST search a Sequence:

1. Select a sequence in the Sequence Information table.
2. Select menu **Analyze > BLAST Search > Sequence** or click  button from the toolbar.

3. In the BLAST Search Sequence window, set parameters for BLAST Search and click Search.

4. As soon as the search is complete, the BLAST results are interpreted to identify the sequences showing cross homology.

5. You can view the cross homology results by clicking the **Cross Homology** button in the BLAST Information tab.

6. You can view BLAST result details in your default browser by selecting View>BLAST Details. The time required to return the results for a sequence varies dramatically, depending on the load on NCBI servers and the speed of your internet connection, from just a few seconds to several minutes per search. If a sequence is BLASTed for a second time, the new results replace the previous results. Thus Primer Premier always displays the most recent BLAST results.

Example:
To BLAST search the sequence for avoiding cross homology:

1. Select sequences NM_001101 in the Sequence Information table.
2. Select **Analyze> BLAST Search> Sequence** from the menu or click  from the toolbar.

3. In the BLAST Search Sequence window, all the parameters are set to default. i.e. Search Type: Human genome BLAST, Genomes: Human Database: Ref (all assemblies), Expect value is 10, Filter checked.

4. Launch search by clicking the **Search** button.

5. As soon as the search is complete, the BLAST search results are interpreted to identify the sequences exhibiting cross homology.

6. You can view the cross homology results by clicking the Cross Homology button in the BLAST Information tab.

Set Parameters for Local BLAST Search

1. Select menu **Analyze> BLAST Search> Sequence** or click  button from the toolbar.

2. In the BLAST search window, select Search Type as Local Database BLAST.

3. Enter Server Name/ IP Address.

4. Click **Genomes**.

5. Select the local database category as of Human, Microbes or Other Genome and click **OK**.

6. Select the Database for BLAST search from the dropdown menu.

7. Click Search. As soon as the search is complete, the BLAST results are interpreted to identify the sequences showing cross homology.

8. You can view the cross homology results by clicking the Cross Homology button in the BLAST properties tab.

9. You can view the BLAST result details in your default browser by selecting menu **View>BLAST Details**.

Note: The default parameters for BLAST search are optimized to report all the relevant cross homologies for the query sequence. We recommend not changing these default values.

Set Parameters for Desktop BLAST Search

Desktop BLAST can BLAST search sequences against local custom databases without the need of setting up a local BLAST server. For Desktop BLAST search, you must have sequence files either .txt or .fa stored locally on your computer. You need to guide Primer Premier 6 to the directory which contains .fa or .txt sequences and then BLAST search sequence against the sequences.

The following instructions show how Primer Premier 6 can perform Desktop BLAST.

1) Select **Analyze > BLAST Search > Sequence** or click the  button on the toolbar.

2) In the BLAST search window, select Desktop BLAST search.

3) Click Advance button. You can specify the word size. The default Word size for nucleotide sequences is 11 bp. Click **OK**.

You can specify a Word Size of upto 18 or 22 for a file size of 500 MB or more to facilitate rapid searches.

Note: Smaller the Word size, slower the BLAST search will be.

System Recommendations

The memory of a machine is normally more important than the CPU speed for "Desktop BLAST" searches.If the file size is say, 500 MB or 1 GB, then it is recommended to use a 2GB RAM.
Other suggestions to decrease system resource requirements are:
a. BLAST searching one template at a time
b. Changing some of the parameters on Advanced BLAST Search Parameters window:
- Increase the word size.
- Decrease the Expect value.
- Check the Filter option.
- Decrease the Alignment and Description values.

4) Click on the Browse button to locate the directory having the sequence files.

5) Click **Genomes**.

6) Select the database category as of Human, Microbes or Others Genome and click OK.

7) Select the Database for BLAST search from the drop down menu.

8) Click Search. As soon as the search is complete, the BLAST results are interpreted to identify the sequences showing cross homology.

9) You can view the homology results by clicking the  button on the toolbar.

10) You can view the BLAST result details in your default browser by selecting menu **View > BLAST Details**.

Note: For Desktop BLAST and the local database there is an editable text field for **Expect Value**, for which the minimum value is 0.0001 and maximum value is 1000.
For BLAST, the search parameters are defaulted as:
Filter: Unchecked. The program automatically avoids low complexity and sequence repeats. If the filter named 'Default' is checked, the query sequence will be masked and the cross homology for the regions will not be reported, leading to a design in low complexity or repeat regions.

Note: Filter option is checked by default when you select Human Genome BLAST or Eukaryotic Genome BLAST as the Search type.
Expect = 10. It is the statistical significance threshold for reporting matches against database sequences. If the statistical significance ascribed to a match is greater than the EXPECT threshold, the match will not be reported. Lower EXPECT thresholds are more stringent, leading to fewer chance matches being reported. Increasing the threshold shows less stringent matches. In order to avoid cross homology, it is essential to select less stringent EXPECT thresholds for which all relevant matches are reported.

Cross Homology Threshold

Select Sequences with E-value>: Allows you to select homologous regions based on their E-values. Specify target value in "**Select sequence with e-value>**" and click **Update**. The regions with E-value greater than the target value are selected and therefore avoided for primer design. The regions with E-value less than the target value remain unchecked.

Select Regions with Percent Identity<=: Allows you to select regions based on their percent identity. Specify the target value in "**Select Regions with Percent Identity<=**" and click **Update**. The regions with percent identity less than and equal to specified value will be selected and are avoided while primer design. The regions not satisfying percent identity criterion will remain unchecked.
AND & OR: Allows you to specify either one of the above two criteria or both for selecting the regions to be avoided.
By selecting **AND**, the regions satisfying both the E-value and the percent identity criteria are checked and avoided.

By selecting **OR** option, the regions satisfying either the E-value or the percent identity criterion are checked and avoided.

Note: To overlook both the parameters for selecting homologous regions, you uncheck both the options.

To BLAST Search the Primer-pair / Amplicon
1. Select sequence in the Sequence Information table.
2. Select menu **Analyze > BLAST Search > Primer-pair / Amplicon**.
3. The Genome and database fields for BLAST search are consistent with the genome and database fields chosen while BLAST searching the sequence. Set other parameters for BLAST

search and launch search.
4. The search results are displayed in BLAST Information tab in the main window.
5. You can view BLAST Primer-pair/Amplicon result details in your default browser by clicking on "Completed" in the Search Status column in the BLAST Information tab in the Main Window or by selecting menu **View>BLAST Details > Primer-pair / Amplicon**.

Interpreting Cross Homology Results

The database sequences showing cross homology with the query sequence are displayed in the **Avoid Cross Homology** window. In this window, all the relevant cross homologies are checked. The program considers the checked sequences for avoiding cross homology. You can uncheck a sequence if it is not a relevant cross homology.

1. Select the sequence AY634314 in the Sequence Information table.

2. Select **View >Cross Homology** from the menu or click the **Cross-Homology** button in the BLAST Information Tab.

3. In the cross homology results for AY634314, the first sequence shows complete homology with the query sequence. The hit has an E-Value of 0.0. This encodes for Soleropsis invictra Virus 1.

4. This sequence remains unchecked by default. The program will not consider this sequence as a relevant cross homology as it is the database copy of the query sequence.

5. For example: For sequence AY634314, the first 9 sequences shows 100% homology.

6. If you select this sequence for avoiding homology with, the program will fail to design any primers because the entire sequence will be deemed as cross homologous.

7. The sequences showing relevant cross homology are EU436455.1, EF219380.1, EU436456.1, EU436423.1, AY275710.1 and others.

8. By default these sequences are checked. The program will not design primers in the regions where these sequences show homology with the query sequence.

9. You can save the selection by clicking **OK** and proceed with primer design.

Interpreting Human BLAST Results

Human Ref RNA database is available for BLAST search at NCBI. It consists of ref-sequence records. The query sequences submitted for BLAST search will show complete homology with either the database copy of the mRNA sequence or its modeled transcript or with both, depending on the differences in two sequences.
The database sequences showing cross homology with the query sequence are displayed in the Avoid Cross Homology window. In this window, all the relevant cross homologies are checked. The program considers the checked sequences for avoiding cross homology. You can uncheck a sequence if you think it is not a relevant cross homology.
1. Select sequence EF428566 in the Sequence Information table. BLAST the sequence against Virus database of nr Blast.

2. Select **View>Cross Homology** from the menu or click the Cross-Homology button in the BLAST Information Tab.

3. In the cross homology results for the sequence EF428566, the first sequence shows complete homology with the query sequence. These hits have E-Value of 0.0.

4. The sequences having E-Value of 0.0 remain unchecked by default. The program will not consider these sequences as relevant cross homologies as these are the splice variants or mRNA of the query sequence itself, present in the database .

5. For example: for sequence EF428566, the first sequence having E-value 0.0 is EF428566.1.

6. The sequence EF428566.1 is the complete genome of Solenopsis invicta virus 2, which is the same as the query sequence.

7. If you select this sequence for avoiding cross homology by checking the checkboxes opposite it, the primer search will fail to design any primers because the entire sequence will be deemed cross homologous.

8. The sequences showing relevant cross homology are AL844505.1 and AF486073.2 which are sequences of Plasomodium falciparum and Acute bee paralysis vitus respectively.

9. These sequences are checked by default. The program will not design primers in the region where these sequences show homology with the query.

10. You can save the selection by clicking the **OK** button and proceed with primer design.

Interpreting Microbial BLAST Results

When the complete genome of an organism is represented in a single accession number, then BLASTing a sequence against such a database will result in a single description The copy of the query sequence in the database and all other relevant cross homologies are reported in that single hit. To avoid relevant cross homologies, which are reported as different alignments, microbial BLAST results are interpreted differently.
The database sequences showing cross homology with the query sequence are displayed in the Avoid Cross Homology window. In this window, all the relevant cross homologies are checked. The program considers the checked sequences for avoiding cross homology. You can uncheck a sequence if you think it is not a relevant cross homology.
1. Retrieve the sequence M95491 from GenBank in the Sequence Information table.

2. Select **View>Cross Homology** from the menu or click the **Cross-Homology** button in the BLAST Information Tab.

3. In the cross homology results for the sequence M95491, the alignment which shows 100% homology with the query sequence, remains unchecked by default. The program will consider this alignment as the database copy of the query sequence. For example: for the sequence M95491, the alignment showing complete homology is from 39 to 56 bases.

4 If you select this alignment for avoiding homology, the program will fail to design any primers because the entire sequence will be deemed as cross homologous.

5. Rest of the alignments from 2179-2212, 2364-2379, 2643-2661, 2921-2936, 5648-5663, 5842-5860, 6151-6168, 7253-7271 bases show cross homology with the query sequence.

6. By default these alignments are checked. The program will not design primers in the region where these alignments show homology with the query sequence.

7. You can save the selection by clicking the **OK** button and proceed with primer design.

Avoid Template Structures

A successful real-time PCR reaction requires efficient amplification of the product. Both primers and target sequence affect this efficiency. Significant template secondary structure may prevent the primers from annealing and therefore prevents complete product extension by the polymerase.

While designing primers please note the following points:
1. Avoid designing primers in the region of template secondary structures, as at annealing temperature these regions will not be available for primer- template hybridization. (The kinetics always favors the intramolecular template secondary structure over the desired template-primer annealing).

2. Avoid designing primers in the internal hairpin loop regions.

3. Avoid designing primers flanking template secondary structures.

4. Avoid designing primers immediately upstream of a secondary structure, the farther the primer is from a secondary structure, the better.

Set Parameters for Template Secondary Structure Search

To search a sequence for Template Secondary Structures:

1. Select a sequence in the Sequence Information table.

2. Select menu **Analyze> Template Structure Search** or click  button from the toolbar.

3. In the Template Structure Search window, set the parameters for Template Search and click **Search**.

4. As soon as the search is complete, the Template Search results are interpreted to identify regions of stable secondary structures.

5. You can view the Template Search results in your default browser by selecting **View >Template Structure Details**.

6. The regions involved in template structures are underlined in orange in the sequence view.

To turn off the display, select **View> Template Structure Regions** and uncheck the option. Alternatively, right click in the sequence view and uncheck **Display Secondary Structure Regions**.

If a sequence is Template Searched for a second time, the new results replace the previous results. Thus, Primer Premier always displays the most recent Template Structure Search results. Up to 1200 bp at a time can be submitted for folding. Also, a valid e-mail address is essential for processing the request.
Example:

To search a sequence for Template Secondary Structures:

1. Select sequences M33019 in the Sequence Information table.

2. Select **Analyze> Template Structure Search** from the menu or click  from the toolbar.

3. In the Template Structure Search window, set Sequence Range as 1000 to 2100. Rests of the parameters are set at their default values.

4. Enter a valid e-mail Address.

5. Launch search by clicking the **Search** button.

6. As soon as the search is complete, the Template Structure search results are interpreted to identify regions of stable secondary structures.

7. You can view Template Search results in your default browser by selecting **View >Template Structure Details**.

PCR Primers

Primer Search Parameters for Standard PCR

The Primer Search Parameters window is launched when you select Analyze>Primer Search or click button from the toolbar.

Design Mutation Flanking Primers: Design a primer pair to amplify the template containing mutation.

You can set parameters for primer search in this window and launch a primer search.
Search parameters:
Avoid Template Structure: Default value: Checked. Template secondary structures will be avoided while designing primers.

Avoid Cross Homology: Default value: Checked. Interprets BLAST search results for the selected sequence to avoid designing primers in the region of cross homology.
Note: Avoid Cross Homology & Avoid Template Structure option is disabled when SNP is selected

Range Parameters

Search Location: Default value: Anywhere. The optimum primers are searched anywhere in the sequence or biased towards 5' end or biased towards 3' end of the selected sequence. Search Location selection is disabled, when the Avoid Template Structureis checked.

Search Range: The optimum primers are searched within the user specified search range. The search range can be specified as From and To sequence base numbers. Search Range selection is restricted to the sequence bases analyzed for template secondary structures, when Avoid Template Structure is checked.

Note: When a SNP is selected, Search Location & Search Range options are not available.
Primer Parameters

Tm (oC): Default value = 50 +/- 5 ^{o}C Tm and the +/- tolerance. The search engine finds primers for all sequences as close as possible to the specified target value of Tm. For example: To specify a range of 60 ^{o}C to 70 ^{o}C, enter 65 ^{o}C for target Tmand 5 ^{o}C for the +/- tolerance. Tm values are calculated using the nearest neighbor thermodynamic algorithm.

TaOpt (oC): Default value = 50 +/- 5 ^{o}C. TaOpt and the +/- tolerance. The search engine finds primers for all sequences as close as possible to the specified target value of TaOpt. Formula for calculating TaOpt.

Primer length: Default value = 18 to 25 base pairs, the desired range for the primer length.

Primer Pair Parameters

Specify amplicon length: Default value = 100 - 200 base pairs. Target product length range in bp. The location of the amplicon dictates the location of the primers as the program tries to design primers within the specified location.
Include gap up and downstream of SNP: Default value = 30 This parameter excludes the region upstream and downstream of a SNP for flanking primer design.

Alternate Primers: Default value = 5 . The number of alternate primer pairs designed per sequence in a single search.

Advanced Search Parameters:

Hairpin maximum dG 3' End (kcal/mol): Default value = 3 -kcal/mol. The free energy of the most stable hairpin that is acceptable at 3' end of the primer.
Formula for calculating free energy . - dG = dH - T * dS,
Where dH = enthalpy, T = temperature, dS = entropy.

Hairpin maximum dG Internal (kcal/mol): Default value = 5 -kcal/mol. The free energy of the most stable hairpin that is acceptable in the primer.

3' End maximum stability dG (kcal/mol): Default = 12 -kcal/mol Primers with a 3' end stability greater than the specified value are not considered. Primers with stability greater than 4 and less than the specified value are rated lower.

Self-dimer maximum dG 3' End (kcal/mol): Default value = 6 -kcal/mol. The free energy of the most stable self-dimer that is acceptable at 3' end of the primer.

Self-dimer maximum dG Internal (kcal/mol): Default value = 8 -kcal/mol. The free energy of the most stable self-dimer that is acceptable for a primer.

Run/repeat maximum length: Default value = 5 bp/dinuc. The maximum acceptable length of single nucleotide run (e.g. AAAA) or dinucleotide repeats (ATATATAT).

G/C Clamp: Target Consecutive G/Cs at 3' End- GC clamp is the presence of consecutive G or C bases within the last five bases from the 3' end of a primer. However, the first G or C base present within the last 5 bases and are not consecutive, are be interpreted as a GC clamp by the program.
eg. GCTTGCTGCGTTCACACT GC clamp is reported as 1.

It should be noted that presence of more than 3 G's or C's should be avoided. Primers with less than the specified number of G or C' s at the 3' end will be rated lower.
You can specify a GC clamp of 0 to 3 bp. If you specify a GC Clamp of 0, it intends that no GC clamp should be present at the 3' end of a primer. The program tries and designs such primers. If it fails, it reports primers with GC clamps. A demerit is assigned to these primers and are rated low.

An example of a primer pair in which the antisense primer does not have a GC clamp.
Sense Primer: AAACACAGAGAACACCACGAAA GC clamp-2
Antisense Primer:TGTCCCACAGCTTTAGCAAATT GC clamp-0

GC%: Default value = 40-60 %. Percentage of G and C bases tolerated in the designed primers.

Maximum ambiguous bases in the amplicon: Default value = 0. The number of ambiguous bases tolerated in the amplicon.

Maximum primer pair Tm mismatch: Default value = 4. The maximum acceptable difference between the Tm values of the two primers.

Cross-dimer maximum dG 3' End (kcal/mol): Default value =7 -kcal/mol. The free energy of the most stable cross-dimer that is acceptable at 3' end of a primer.

Cross-dimer maximum dG Internal (kcal/mol): Default value = 8 -kcal/mol. The free energy of the most stable cross-dimer that is acceptable in a primer.

Multiplexing maximum dG (kcal/mol): Default value = 6 -kcal/mol. The free energy of the most stable cross-dimer that is acceptable in a multiplex reaction.

Primer Search Results

As soon as the primer search is complete the search quality status is displayed in the Search Status Tab and the detailed result is displayed in the Primer properties table under Primer Properties tab.

1. The displayed result include both the sense and antisense primer rating, primer sequence, position, length, melting temperature, GC%, maximum hairpin dG, maximum self dimer dG, repeat and run length, optimum annealing temperature, maximum cross dimer dG, product length, product T_m.

2. You can view all possible primer secondary structures in View Secondary Structures window.

3. In the Sequence View, both sense and antisense primer positions are marked on the sequence.

4. You can copy the primer sequence to the clipboard. Select **Edit > Copy > Primer Sequence** or select **Copy Primer Sequence** option from pop-up menu available at right click (For Mac, CTRL+click) over the primer sequence.

Example:
1. The primer search result for sequence M35019 displays sense and antisense primers along with the free energy values for secondary structures and product properties. The designed primer pair amplifies the H. influenzae 16s ribosomal RNA.

2. Similarly, the primer search result for sequence NM_001101 displays sense and antisense primers along with the free energy values for secondary structures and product properties. The designed primer pair amplifies Homo sapiens actin, beta(ACTB), mRNA

3. You can view the graphical view of all the primer secondary structures in Secondary Structures window by clicking **Primer All Structures** option from pop-up menu available at right click (For Mac, CTRL+click) over the Primer Properties table or by selecting **View > Alternate > Primers**.

You can replace the default primer pair with an alternate primer pair to meet your research needs.

1. Properties of the alternate primers are displayed in the All Primers window. Select any primer pair.

2. Click the **Replace** button.

3. The selected primer pair will replace the default primer pair in the Primer Properties table.

Select alternate primer:

After designing primer you can replace the default primers by selecting any other primer pair from the All Primers window.

1. To view alternate primers click 

button.

2. The window displays the alternate primers. The window displays both the default primer displayed under Primer Properties Tab and alternate primers. The primer which is displayed under Primer Properties tab will be highlighted in All Primer window.

2. Select any primer pair in this window, it will be highlighted.
3. Click the **Replace** button.

4. The selected primer replaces the default primer pair in the Primer Properties tab and now will

be highlighted with grey color in All Primers window. The primer sequence and properties can be printed and saved outside the program by using Export Primer Results.

Sort Primers:

In All Primers window there will be facility to sort sense primer, antisense primer and product on the basis of rating, position, length, Tm , GC%, Hairpin Delta G value and Self dimer delta G values. Sorting is not available on sequence and S.No.

Note: Whenever All Primers window will be launched primers are always sorted on the basis of Product rating.

Export Results

Export Primer Search Results

Primer Premier 6 can save the primer search results outside the program by selecting the Export Results option. The results are exported in a Tab delimited file that can be loaded into any spreadsheet such as MS Excel and Lotus 123 for printing or further processing.

To Export the Primer Search Results:

1. Select one or more sequences in the Sequence Information table.

2. Choose **File > Export Primer Results** or click  button from the toolbar. Primer Premier saves the output file with a .xls extension registered to Microsoft Excel and with .csv extension for opening in any other spreadsheet program.

3. Primer Premier has two option for exporting
a. **Best Primer**- At default this will be selected. Selecting this option will let you export primer pair displayed under primers Properties tab.
b. **All Primers** - Selecting this option will let you export All primers pairs designed on sequence.

4. At default, Primer Premier creates the output file in the Primer Premier installation folder. You can select the output file name and location in the Export Result window.

5. At default, the primer search results include pair quality, pair rating, product length, product Tm, TaOpt and primer location, primer sequence and free energy values of the most stable secondary structures for both the sense and the antisense primers. Sequence definition and length can also be exported. The Export Result window allows you to select any or all of the available data using the appropriate check boxes.

Primer Premier allows exporting of sequences along with the primer. **Select File > Export Sequence** or click  the button from the toolbar. You can choose between a single or double stranded display and specify the base grouping (3 or 10). Browse to the location for exporting the file and click **Export**.

Evaluate predesigned primer

Evaluate Predesigned primers with Template
In Primer Premier 6, you can add predesigned primers instead of designing primers using the program. This is useful when you are working with published primer sequences or already have a predesigned preused set of primers. You can use a predesigned primer pair or a sense primer or an anti-sense primer. Primer Premier would then design a compatible primer for a standard PCR assay.

To add and evaluate a primer pair for a sequence:
1. Select the sequence in the Sequence Information table.
2. Select **Analyze > Evaluate Predesigned Primers** or click In the evaluate predesigned primer click button from the toolbar, to launch the predesigned Primers window.
3. Type or paste the primer sequences in the given text field. The primers should always be added in the 5' to 3' direction.
4. Primer Premier analyzes and rates the added primers.

The predesigned primers are analyzed using existing primer search parameters from the Primer Search Parameters window.
Note: If the added predesigned primer pair is not targeted on the template or if targeted on template but not evaluated then, Primer Premier simply calculate following properties - length, Tm, GC%, hairpin, self dimer, cross dimer, run length, GC clamp for your predesigned primer pair and will display them in primer properties tab.
Note: If BLAST search and template structure search is performed for a sequence, then the program will evaluate the primer pair considering the cross homologies and secondary structure. If the evaluated primer pair falls in homology region or in secondary structures, a warning message is displayed in status line.

For example1:
1. Select the sequence NM_170745.
2. Add the following primer sequences: Sense Primer: 5' ATGTCTGGACGAGGGAAG 3'. Antisense Primer: 5' ACTTGCTTTGGGCTTTATGG 3'.
3. Primer Premier analyzes and rates these primers using existing primer search parameters, which are currently set as default. The primer properties are displayed in Primer properties table.
4. To verify the specificity of the evaluated primer pair, you can BLAST search the primer pair against the relevant database using **Analyze>BLAST Search>Primer Pair/Amplicon**.
5. Detailed BLAST search results are displayed in the default browser.

Evaluate Predesigned primers without Template
In Primer Premier 6, you can analyze single primer or primer pair without any template sequence. Primers are checked for amplification related properties including melting temperatures (Tm) and secondary structures.

To analyze a primer, just type in the primer sequence and click the Analyze button. All of the properties of the primer are displayed.

To analyze a primer pair, select the primer pair option from the drop down menu and then type in both sense and antisense primer sequence in 5'-3' direction.

Cross dimers are now calculated in addition to the properties for each primer.
A well organized primer report can be exported directly from the properties pane. It consist of detailed information on the primer pair. Primer name and description areas are provided to aid in identification of the primer from the printout. The report can be exported in .html and .csv format.

To evaluate a primer/primer pair:

1. Select **Analyze > Evaluate Predesigned Primers** or click In the evaluate predesigned primer

click button from the toolbar, to launch the predesigned Primers window.
2. Select the "No template" radio button.
3. Type or paste the primer sequences in the given text field. The primers should always be added in the 5' to 3' direction.
4. Primer Premier analyzes the added primers and displays the properties on a new properties pane.

For example:

1. Add the following primer sequences: Sense Primer: 5' TGTCTGCATGTACCTCTACC 3'. Antisense Primer: 5' AGTTCTTCTATTCGCTTGTAGG 3'.
2. Primer Premier analyzes these primers using existing primer search parameters, which are currently set as default. The primer properties are displayed in a launched Primer properties table.
3. Export the primer properties result by clicking on Export button on the properties pane. You can either export it in a *.html or *.CSV format.

Multiplex Primers

In Primer Premier 6.0 you can design primers for multiplex reactions. By avoiding homologies, competition can be avoided to maximize signal strength.

In order to multiplex the primers:

1. Select the sequences in Sequence Information table. You can select up to five sequences for multiplexing.

2. Select **View > Multiplex Primers** or click  from the toolbar.

3. Primer Premier instantly analyzes all the selected primers and displays the most stable cross dimers that are formed between the selected primers.

4. The multiplex results are displayed in Multiplex Results Window**.**

For example:

1. Select sequences AY34314, EF428566, FJ528584 and NM_007393 in Sequence Information table. Each of these sequences has a primer pair, which you have designed or added in previous steps.

2. Multiplex these by selecting **View > Multiplex Primers**.

3. Multiplex results show the most stable cross-dimer formed between two primers.

4. The cross dimers are displayed graphically along with the cross dimer dG.

5. By interpreting these results you can easily choose the most appropriate primers for a multiplex reaction.

Generate Report

Primer Premier 6 can export the design results in a report format that can be printed. The report includes the results, the design parameters used and a graphical view of sequence with positions of all the primers marked apart from the amplicon, sequence and primer properties. The user may select the data that needs to be included in the report by checking or un-checking the boxes of the corresponding fields.
The Print Preferences window is launched when you select **File>Generate Report** or click icon  on tool bar
Primer Properties: This include the Quality, Rating, Position, Length, Tm, Self Dimer dG, Hairpin dG, Run/Repeat Length, Cross Dimer multiplex dG. A user may select the fields to be included in the report with the help of check boxes of the corresponding fields.
Primer Pair Properties: There are two. In the Primer tab a user can specify the primer pair properties and in the Product tab the amplicon properties to be included in the report.

Search Parameters:

Select Corresponding parameters: Default: unselected. When selected, only the parameters corresponding to the selected properties used for designing the assay will be included in the report.

Select all Parameters: Default: Unselected. When selected, all the parameters used for designing the assay, will be included in the report.

None: Default: Selected. None of the parameters used for designing the assay, will be included in the report.

Include BLAST Parameters: Default: Unchecked. If the sequence is BLAST searched the search parameter details will be included in the report.

Sequence Information: Information pertaining to the sequence such as Accession no., Definition, Length and SNP.

Advanced Project Information:

Project Information:

Author: You may specify a name to be included in the report.

Comments: To add notes, observation or comments about the experiment.

Note: You can generate the report for multiplex Set

Here is how you can generate the report for multiplex set
1) Select Multiplexed group for which report is to be generated.
2) Select **File> Generate report**
3) Generate report dialog will be launched.
4) Select the parameters which needs to be included in the report.
5) Click **Ok**.

Formatted report will be launched.

Print Preview

To preview the appearance of the generated report

You can click **Print Preview** to see how a printed Web page will look.

1. Select **File>Generate Report** or click  icon from tool bar

2. Report Preference dialog is launched.

3. Click **Print Preview** Button.

4. Choose from the following commands:

Click This	Tool Bar	To Do This
Save		Saves the generated report. Report can be saved as either.prr or .html file format.
Print		Set printing options and print the page.
Page Setup		Change paper, headers and footers, orientation, and margins for this page.
First page		Display the first page to be printed.
Previous page		Display the previous page to be printed.
Next		Display the next page to be printed.
Last		Display the last page to be printed.
Zoom in		Increase the magnification.
Zoom out		Decrease the magnification.
Default Size		Display the default size of the page.
Help		Display the help for print preview.
Close		Close the Print Preview window.

Preferences

Reaction Condition

Primer Premier designs optimal primers based on standard reaction conditions. You can view and change the reaction conditions by selecting **Tools>Reaction Conditions.**
The following are the default reaction conditions:

Nucleic Acid Concentration: Default value = 0.25 nM. Nucleic Acid concentration refers to the concentration of annealing oligonucleotides in the reaction mixture according to Rychlik et al. (Nucleic Acids Research, vol 18, num 21). Thus it refers to the concentration of primers.

Monovalent Ion Concentration: Default value = 50 mM this value is the concentration of all the monovalent ions present in the reaction mixture.

Free Mg++ Ion Concentration: Default value = 1.5 mM this is the concentration of Mg++ ions, used as binders, in a reaction mixture.
The program computes the total Na [+] equivalent concentration and displays it. The value with default settings is 204.92 mM.

Temperature for Free Energy Calculation: Default value = 25 ^{o}C. This is the value of temperature that will be used to calculate the dG values. It is calculated using the formula dG = dH - T * dS.

Application Preferences

Default Project location:

Projects are, by default, saved in the PRProjects folder located in the product installation directory (C:\Program Files\Primer Premier\PRProjects for Windows and /Primer Premier/PRProjects for Mac). You can change the default location of this folder by specifying a new one.

File Chooser:

For Mac: You could use a File Chooser dialog of your choice. The options available are
(1) OS Specific and (2) Customized.

When you select OS Specific, the native Mac file chooser dialog is displayed. The dialog includes the following options:
New project
Open project
Save project
Open sequence
Export

When you select Customized, a author-defined custom file chooser dialog is displayed. Click Demo to view each of these
.

Technical Information

Internet Setting

Primer Premier 6 has strong web integration. The program connects to NCBI Entrez and dbSNP for retrieving sequences online. It uses Quikfoldserver for calculating primer T_m and determining the template secondary structures and BLAST search the selected sequences by connecting to NCBI BLAST server.

Primer Premier 6 supports both direct Internet connection and Internet connection through a proxy server. If your organization has a firewall, Primer Premier may need to go through a proxy server before connecting you to the Internet. The proxy server acts as an intermediary between your internal network (Intranet) and the Internet for retrieving files from remote web servers.

To set Primer Premier 6 to work with a proxy Internet connection:

1. From the **Online** menu, choose **Internet Settings**.

2. In the **Proxy Server** section check the **'Use a Proxy Server'** option.

3. Add the proxy server's host name or IP address under "**Address**".

4. Add the proxy server's port number under "**Port**".

5. Click **OK** to save the settings.

Verify Proxy Internet Settings

Primer Premier 6 allows you to verify your Internet connection and proxy settings.

To verify proxy Internet settings:

1. Set Primer Premier to work with a proxy Internet connection (as discussed above).

2. Click **"OK"**. The program will prompt you to verify proxy Internet settings.

3. Click **"Yes"** to verify connection. For Internet connection setting verification, program will attempt to connect to NCBI server.

4. If the proxy server is password protected, the program will automatically detect the proxy server setting and ask you to authenticate the connection by specifying a **User Name** and **Password**.

Note: If the proxy server uses a domain name for authentication as well, the domain name must be entered in the **user name** text field in domain-name\user-name or user-name@domain-name format.

5. In the Authenticate Proxy window, specify **User Name** and **Password**. The fields are case sensitive.

6. If Primer Premier fails to establish a connection with the NCBI server, a warning message is displayed and the program prompts you to correct proxy Internet setting information.
If you proceed without verifying the proxy Internet setting the Internet settings will be verified the first time you try to load sequence from Entrez.

Note: The Internet setting is similar to the Proxy server setting used in Internet Explorer.

Primer Premier Automatic Upgrade

We frequently publish new versions of our products which contain fixes for reported problems and contain improvements over previous versions. These are available free of charge to our customers and may be downloaded from our web site.

Primer Premier's intelligent updater automatically detects the availability of a free upgrade and installs it on your computer.

Whenever you launch the program, an upgrade message will pop-up if a free upgrade is available on our web site. This message includes a brief description of the upgrade, a link for its detailed description, and its size in MB.

You may choose to upgrade according to your convenience. Click **Now** to start updating the version currently installed or click **Later** to upgrade at some convenient time. Check option **Do not remind me again** to stop checking for automatic upgrades.

Once your program is upgraded please relaunch the program.

If you have stopped the automatic checking of free upgrades, select **Help>Check Upgrade** menu option to check for a free upgrade manually.

To confirm the build number of the program installed on your own computer:

Select **Help > about Primer Premier** menu option if you use Windows.

Select **Primer Premier > about Primer Premier** menu option if you use Mac.

Standalone WWW BLAST for Linux

Standalone WWW BLAST Server is available for UNIX/Mac OS X web servers. The following steps are necessary to set up a Standalone WWW BLAST server on Linux:

Install RedHat Linux Server

Setup Firewall

Setup Apache webserver

Download BLAST Binaries

Install Standalone WWW BLAST Server

Setup Standalone WWW BLAST Server

Format databases

Update Standalone WWW BLAST Server configuration files

System Requirements

Troubleshooting

Install RedHat Linux Server

You can skip this step, if you have a RedHat Linux Server already installed.

Primer Premier and Standalone WWW BLAST server are tested with RedHat Linux webserver successfully.

Primer Premier and Standalone WWW BLAST server may work with other UNIX Apache webservers also.

RedHat Linux server is downloadable from the RedHat Linux Homepage.

Please download RedHat Linux 7.1 from
ftp://ftp.redhat.com/pub/redhat/linux/7.1/en/

For Firewall setup for RedHat Linux 7.1, please refer the Setup Firewall section.

Please download RedHat Linux 7.2 from
ftp://ftp.redhat.com/pub/redhat/linux/7.2/en/

For Firewall setup for RedHat Linux 7.2, please consult your System administrator.

For further information, please refer http://www.redhat.com/software/linux/

Setup Firewall

Firewall set up for RedHat Linux ver 6.2, 7.0 or 7.1

1. Check Firewall setup
Run the following command (at the command prompt)
ipchains -L
If you find
input Policy REJECT
output Policy REJECT
forward Policy REJECT
The result shows that the Firewall is working and your server will not listen to any client. You should set the Firewall rules so that the server will respond to clients.

2. Disable Firewall
If you want to give access to your Linux server and do not have any security risks then you can disable the ipchains service using the following command at command prompt:
ntsysv
Look for the ipchains service in the services scroll box and remove " * " against it by pressing the "spacebar".

3. Set up restricted Firewall
If you want to give restricted access of your Linux server then update the ipchains file with the help of your system administrator.
ipchains file is available at "/etc/sysconfig/ipchains"

After the Firewall setup, set up the Apache server. Apache is basically a webserver, which is required for accessing any web page like Standalone WWW BLAST.

Setup Apache webserver
Apache is open source HTTP server for UNIX, OS/2, Windows and other platforms . It is available as a built-in server in all the versions of the RedHat Linux server. Apache is a well known internet

webserver. It listens and forwards the client request to the Server. For e.g., Apache enables client applications (such as Primer Premier) to execute Local BLAST at the server. The inbuilt Apache version for RedHat Linux 7.1 is 1.3.19. To set up the Apache webserver, it requires a change in the httpd.conf file. The default location of httpd.conf file is "/etc/httpd/conf/httpd.conf".

Following are the directives to be setup in the httpd.conf file
1. ServerName
2. Port information
3. Listen ports
4. DocumentRoot
5. Start and stop commands for Apache

# indicates comment lines. These lines are provided for easy location of the command lines.

1. ServerName
#
ServerName: It allows you to set a host name which is sent back to clients for your
server if it's different than the one the program would get (i.e., use "www" instead of
the host's real name).
#
You will have to access it by its address (e.g., http://192.168.30.3) anyway.
#
ServerName <your machine IP address>
Example:
Servername 192.168.30.3
Server / Client machine IP address troubleshooting
To find your machine IP address please type 'ifconfig' at the command prompt. Please refer to the troubleshooting section for complete details.

2. Port information
#
Port: The port to which the standalone server listens. For ports < 1023, you will need
httpd to be run as root initially.
Change this port, if this port is being used by other application.
Port 80

3. Listen port
#
Listen: Allows you to bind Apache to specific IP addresses and/or ports, in addition
to the default. See also the <VirtualHost> directive. If you have changed port number
in step no. 2 then add this port to Listen directive also.
Listen 3000
Listen <IP address:Port>
Example:
Listen 192.168.30.3:80

4.DocumentRoot
#
DocumentRoot: The directory out of which you will serve your documents. By default,
all requests are taken from this directory, but symbolic links and aliases may be
used to point to other locations.
#
DocumentRoot <"Path of your document root directory">
Example:
DocumentRoot "/home/administrator/document"
Find directory directive for DocumentRoot. This starts as

#
This should be changed to whatever you set DocumentRoot to.
#
<Directory "Path of your document root directory end with/">
Example
<Directory "/home/administrator/document/">

5. Start and stop command for Apache webserver
Save the httpd.conf file with all those changes in above steps.
Use following commands for start, stop and restart the Apache webserver
Start Apache webserver : /etc/rc.d/init.d/httpd start
Stop Apache webserver : /etc/rc.d/init.d/httpd stop
Restart Apache webserver : /etc/rc.d/init.d/httpd restart
Note : If httpd.conf is modified, then only use restart command instead of start.

Test Apache webserver setup
To check Apache webserver,create a testapache.html file in your DocumentRoot directory (e.g. "/home/administrator/document") and paste or enter following html in it :
<html>
<title> Apache setup testing </title>
<body>
Hello from Apache Webserver
</body>
</html>
Now access this file with your default web browser by writing following address in browser
http://Your Linux Machine Server IP Address/testapache.html
Example
http://192.168.30.3/testapache.html
The page should show the following content "Hello from Apache Server". If you face difficulties in setting up the Apache webserver please refer to Apache Webserver set up troubleshooting.

Download BLAST Binaries

Connect to the NCBI server as an anonymous user via ftp link ftp://ftp.ncbi.nlm.nih.gov/blast/executables/ Download the wwwblast-xxx-xxx-Your_platform.tar.gz from "LATEST_WWWBLAST" folder. For example for Linux platform, download **"wwwblast-xxx-xxx-Linux.tar.gz"** file.

Install Standalone WWW BLAST Server for Linux,

After downloading the file "wwwblast-xxx-xxx-Your_platform.tar.gz", place it in the document directory of HTTPD server. (Document root directory set up at the time of Apache config file httpd.conf) and uncompress it by :
gzip -d wwwblast-xxx-xxx-Your_platform.tar.gz
tar -xvpf wwwblast-xxx-xxx-Your_platform.tar

Note : Access options stored in the distribution that parameter "p" in tar options is significant. It will preserve file access options stored in the distribution.

For Linux platform, the commands are:
gzip -d wwwblast-xxx-xxx-Linux.tar.gz
tar -xvpf wwwblast-xxx-xxx-Linux.tar

If the distribution file is uncompressed successfully, a 'blast' directory will be created in the DocumentRoot directory (e.g. "/home/administrator/document" directory).

Setup Standalone WWW BLAST Server for Linux

To set up Standalone WWW BLAST, make some more changes in httpd.conf file. The default location of httpd.conf file is **"/etc/httpd/conf/httpd.conf".**

Following directives are to be setup in the httpd.conf file
1. ScriptAlias
2. Add Handler

1. **ScriptAlias**

```
#
# ScriptAlias: This controls which directories contain server scripts. ScriptAliases are
# essentially the same as Aliases, except that documents in the realname directory
# are treated as applications and run by the server when requested rather than as
# documents sent to the client. The same rules about trailing "/" apply to ScriptAlias
# directives as to Alias.
#
```

ScriptAlias /cgi-bin/ "your DocumentRoot Directory/blast/"

Example

```
ScriptAlias /cgi-bin/ "/home/administrator/document/blast/"
#
# "/home/httpd/cgi-bin" should be changed to your ScriptAliased CGI
# directory exists, if you have configured.
#
<Directory "your DocumentRoot directory/blast/"
    AllowOverride None
    Options ExecCGI
    Order allow,deny
    Allow from all
</Directory
```

Example

```
<Directory "/home/administrator/document/blast/"
    AllowOverride None
    Options ExecCGI
    Order allow,deny
    Allow from all
</Directory
```

2. **Add Handler**

```
#
# AddHandler: It allows you to map certain file extensions to "handlers", actions
# unrelated to file type. These can be either built into other server or added with the
# Action command (see below)
#
# If you want to use server side includes, or CGI outside ScriptAliased directories,
# uncomment the following lines, by removing # from its start.
```

AddHandler cgi-script .cgi

Test Standalone WWW BLAST Server

Now access the main BLAST page by your default web browser by
http://your Linux machine server IP address/blast/blast.html
Example
http://192.168.30.3/blast/blast.html
It launches the main BLAST search page.
Refer to the Main BLAST page troubleshooting if you can not access it.

Enter the sequence of interest and BLAST search against test_na_db. If the search results are

displayed it means that local BLAST is set up correctly. If you are not able to generate the results, refer to BLAST search troubleshooting

Download Databases

1. Connect to the NCBI server as anonymous user via ftp link ftp://ftp.ncbi.nlm.nih.gov/blast/db/ Databases can be downloaded from any standard source.
2. Download the databases.
3. Extract the database in /blast/db directory.
4. Format the database.

Format Databases

Database Formatting Tool

1. For BLAST Server released before Jan 2002, formatdb program exists in /blast/db directory.

2. For BLAST Server released in Jan 2002, formatdb, makemat and copymat binaries are removed from the archive - those should be taken from the ftp site ftp://ftp.ncbi.nlm.nih.gov/blast/executables/release/.

This connects to NCBI server as anonymous user. For Linux, download the archive blast-x.x.x-ia32-linux.tar.gz Copy the blast-x.x.x-ia32-linux.tar.gz file to the blast/db directory. Uncompress it using the following commands
gzip -d blast-x.x.x-ia32-linux.tar.gz
tar -xvpf blast-x.x.x-ia32-linux.tar
It will extract the formatdb file.

Format Databases

Formatdb must be used in order to format nucleotide source databases before these databases can be searched by blastall, blastpgp or MegaBLAST.

Requirement:

The source database may be in FASTA format. Copy the db file to your db directory of installed blast, also place formatdb in this directory, if it not present here.
Unzip/uncompress compressed databases
Default command line:
formatdb -i filename -p F -o T
Example : alu.n is input db file
formatdb -i alu.n -p F -o T
This will generate some supporting file that is used by BLAST algorithm for BLAST search.

Additional command line options for formatdb:
-t Title for database file [String]
Optional
-i Input file for formatting (this parameter must be set)
[File In]
-l Logfile name: [File Out]
Optional
default = formatdb.log
-p Type of file
T - protein
F - nucleotide [T/F] Optional
default = T
-o Parse options
T - True: Parse SeqId and create indexes.
F - False: Do not parse SeqId. Do not create indexes.
[T/F] Optional default = F
If the "-o" option is TRUE (and the source database is in FASTA format), then the database identifiers in the FASTA definition line must follow the convention of the FASTA Definition line Format.
-a Input file is database in ASN.1 format (otherwise FASTA is expected)
T - True
F - False
[T/F] Optional default = F
-b ASN.1 database in binary mode

T - binary
F - text mode
[T/F] Optional default = F
A source ASN.1 database may be represented in two formats - ascii text and binary. The "-b" option, if TRUE, specifies that input ASN.1 database is in binary format. The option is ignored in case of FASTA input database.
-e Input is a Seq-entry [T/F]
Optional
default = F
-n Base name for BLAST files [String]
Optional
-v Number of sequence bases to be created in the volume [Integer]
Optional
default = 0
-s Create indexes limited only to accessions - sparse [T/F]
Optional
default = F
-A Create ASN.1 structured deflines [T/F]
Optional
default = F
-L Create an alias file with this name use the gifile arg (below) if set to calculate db size use the BLAST db specified with -i (above) [File Out] Optional
-F Gifile (file containing list of gi's) [File In] Optional
-B Binary Gifile produced from the Gifile specified above [File Out] Optional
Test the added database through Primer Premier
1. Launch BLAST search dialog.
2. Select the Local BLAST search option.
3. Select the "added database" in the Database dropdown menu.
4. If "added database" is not visible in Database dropdown menu, refer to Server configuration file syntax.

Configuration Files

Server configuration file and log file

Default configuration file is "blast.rc" and logfile "wwwblast.log" located at installed blast directory. Setting tag WWW_BLAST_TYPE to specific value may change these names. Here is a sample configuration file comes with this distribution:

```
# Number of CPUs to use for a single request
NumCpuToUse 4
#
# Here is list of combination program db1 db2 db3..., that are allowed by BLAST
# service. Format: ...
#
blastn test_na_db
blastp test_aa_db
blastx test_aa_db
tblastn test_na_db
tblastx test_na_db
```

To add your newly formatted db in local BLAST server, append your db input file name at the end of its corresponding program separated by a space.

For example, if you format file alu.n then append "alu.n" at the end of line
blastn test_na_db. Now the database alu.n is associated with blastn program.
Changed program/database (s) combination looks as follows:

```
blastn test_na_db alu.n
blastp test_aa_db
blastx test_aa_db
tblastn test_na_db
tblastx test_na_db
```

System Requirements

RedHat Linux 7.2 Server requirements:

For RedHat Linux 7.2	Required	Recommended
CPU	486DX processor	Pentium
RAM	32 MB	64 MB
Hard Disk Drive Space	1.0 GB	2.0 GB
Local BLAST Server Space	50 MB	50 MB
Space for Local Databases	-	Depends on number of databases for Local BLAST

Note: If the input FASTA file is about 500 MB then it needs around 170-200 MB additional hard disk space for translated BLAST database. At least another 100-200 MB should be allowed for memory consumption by the actual BLAST program.

Troubleshooting

1. Network connection

Make sure the client and server computers are connected through a network.
Use ping command at the command prompt
ping IP address/Server name
If sever and clients aren't connected, the following messages are generated
Request timed out (on Windows)
Destination Host Unreachable (on UNIX)
Please consult System administrator.

2. Apache Webserver

a) Check if the Apache webserver is running
Use the following command to verify that Apache webserver is running
ps -A | grep "httpd"
Output line(s) with "httpd" string indicate Apache webserver is running.
If not, please Restart Apache webserver
b) Check "testapache.html" is in the DocumentRoot directory.
c) Check directory directive corresponding to DocumentRoot. It gives access to pages in the DocumentRoot directory. Please refer to DocumentRoot
If server does not accepts requests from your system, access to testapache.html file is denied. It may be due to Firewall being implemented by RedHat Linux Server. Please consult System administrator.

3. Extract BLAST server

During untar, if a message appears "UnExpected End of file", it indicates that the downloaded file is either corrupted or incompletely downloaded. Please download it again.

4.Main BLAST page

Check the URL used.
a) Proper Server IP address.
b) Make sure "DocumentRoot" directory is the parent directory of blast directory or BLAST server has been extracted in the directory which is being used as DocumentRoot.
c) Address should consists of 3 parts ("http://Server name/blast/blast.html")
http protocol
Server name or Server IP address
main page location in the blast directory

5.BLAST Search

Request method POST is not allowed for the URL
While BLASTing, if the message "Request method POST is not allowed for the URL blast/blast.cgi" is generated,
a) "AddHandler cgi-script .cgi" is not uncommented. Find the command and uncomment it in the httpd.conf file. Please refer AddHandler
b) CGI executables scripts of BLAST server does not have executable permissions. Please refer to ScriptAlias
6. Server / Client machine IP address

Use the following commands at the command prompt to get the IP address of your server / client machine:
1. ipconfig (on Windows 98/ME/2000/XP)
2. ifconfig (on RedHat Linux server)

1. IP address on Windows machine
ipconfig [press Enter] on Windows gives
IP Address. . . . . : 192.168.30.18
Subnet Mask . . . . . : 255.255.255.0
Default Gateway . . . . : 192.168.30.1
192.168.30.18 is client machine's IP address.

2. IP address on RedHat Linux machine
ifconfig [press Enter] on RedHat Linux gives
eth0 Link encap:Ethernet HWaddr 00:10:B5:F8:DC:0A
inet addr:192.168.30.4 Bcast:192.168.30.255 Mask:255.255.255.0
UP BROADCAST RUNNING MULTICAST MTU:1500 Metric:1
RX packets:992240 errors:0 dropped:0 overruns:0 frame:0
TX packets:1464662 errors:0 dropped:0 overruns:0 carrier:0
collisions:0 txqueuelen:100
Interrupt:11 Base address:0xc000
lo Link encap:Local Loopback
inet addr:127.0.0.1 Mask:255.0.0.0
UP LOOPBACK RUNNING MTU:3924 Metric:1
RX packets:130 errors:0 dropped:0 overruns:0 frame:0
TX packets:130 errors:0 dropped:0 overruns:0 carrier:0
collisions:0 txqueuelen:0
192.168.30.4 is server machine's IP address.

7.Uncompress the compressed database

If database archive ends with ".Z" then uncompress "blast.linux.tar.Z" with gunzip command.
For example : gunzip -d blast.linux.tar.Z
If database archive ends with ".gz" then uncompress "blast.linux.tar.gz" with following commands.
For example :
gzip -d blast.linux.tar.gz
tar -xvpf blast.linux.tar

8. Specify valid Server name/IP address

Please specify valid Server name/IP address.
1. If main BLAST page is not accessible through browser. Refer to Main BLAST page troubleshooting
2. If main BLAST page is accessible through browser and Primer Premier generates an error, It may be due to one of the following reasons:
a) When you specify the IP address, please exclude "http://" from it.
b) The BLAST page name is included in the IP address. Please refer to BLAST server path and IP address troubleshooting.
c) Given IP address/server name does not exist. Please refer to Server / Client machine IP address troubleshooting.
d) Given server IP address or server name is incorrect. Please refer to Server / Client machine IP address troubleshooting.
e) Client and server are not connected through local network. Please refer to Network connection troubleshooting.
f) BLAST server may not exist in the server's DocumentRoot directory. Please refer to BLAST server path and IP address troubleshooting.

9. BLAST server path and IP address

Suppose your server IP address or host name is "MyServer".

1. Default BLAST server path
If BLAST server is installed in the DocumentRoot directory, then main BLAST page should be accessible by the following URL
http://MyServer/blast/blast.html
Primer Premier expects "MyServer" as Server IP address.
2. User defined server path
If BLAST server is not installed in the DocumentRoot directory and main BLAST page is accessible by the following URL
http://MyServer/../blastparent/blast/blast.html
Primer Premier expects "MyServer/../blastparent" as Server IP address.
For example
If main BLAST page is accessible by the following URL
http://MyServer/blastparent/blast/blast.html
Primer Premier expects "MyServer/blastparent" as the Server IP address.

Standalone WWW BLAST for Mac

Download Blast Binaries for Mac

Connect to the NCBI server as an anonymous user via ftp link
ftp://ftp.ncbi.nlm.nih.gov/blast/executables/

Download the wwwblast-xxx-xxx-Your_platform.tar.gz from "LATEST_WWWBLAST" folder. For example for Mac platform, download **"wwwblast-xxx-powerpc-macosx.tar.gz"** file.

Install Standalone WWW BLAST Server for Mac

After downloading the file "wwwblast-xxx-xxx-Your_platform.tar.gz", place it in the document directory of HTTPD server. This is the DocumentRoot directory (e.g. “/Library/WebServer/Documents”) specified in the Apache config file httpd.conf and uncompress it with the commands :
gzip -d wwwblast-xxx-xxx -Your_platform.tar.gz
tar -xvpf wwwblast-xxx-xxx -Your_platform.tar

Note : The parameter "p" in tar options is significant. It will preserve file access options stored in the distribution.

For Mac platform, the commands are:

gzip -d wwwblast-xxx-powerpc-macosx.tar.gz
tar -xvpf wwwblast-xxx-powerpc-macosx.tar

If the distribution file is uncompressed successfully, a 'blast' directory will be created in the DocumentRoot directory (e.g. "/Library/WebServer/Documents" directory).

Download Format db

Extract BLAST server troubleshooting

Setup Standalone WWW BLAST Server for Mac

To set up Standalone WWW BLAST, we need to make some more changes in httpd.conf file. The default location of httpd.conf file is **"/etc/httpd/httpd.conf"**.

Note: Only **root** or **super** user can modify this file.

Following directives need to be setup in the httpd.conf file
1. ScriptAlias
2. Add Handler

ScriptAlias

#
ScriptAlias: This controls which directories contain server scripts. ScriptAliases are
essentially the same as Aliases, except that documents in the realname directory
are treated as applications and run by the server when requested rather than as
documents sent to the client. The same rules about trailing "/" apply to ScriptAlias
directives as to Alias.
#
add following lines after </Directory> and before </ifModule> existed in ScriptAlias
ScriptAlias /cgi-bin/ "your DocumentRoot Directory/blast/"
<Directory "your DocumentRoot directory/blast/">
Options +ExecCGI
</Directory>
Example :
ScriptAlias /cgi-bin/ "/Library/WebServer/Documents/blast/"
<Directory "/Library/WebServer/Documents/blast/">
Options +ExecCGI
</Directory>

2. Add Handler

#
AddHandler: It allows you to map certain file extensions to "handlers", actions
unrelated to file type. These can be either built into other server or added with the
Action command (see below)
#
If you want to use server side includes, or CGI outside ScriptAliased directories,
uncomment the following lines, by removing # from its start.
AddHandler cgi-script .cgi

Test Standalone WWW BLAST Server

Now access the main BLAST page using your web browser by entering the address:
http://your Mac machine server IP address/blast/blast.html

Example :
http://192.168.30.3/blast/blast.html
It launches the main BLAST search page.
Refer to the Main BLAST page troubleshooting if you can not access it.

Enter a sequence and BLAST search against the test database test_na_db. If search results are displayed, the local BLAST is set up correctly. If not, refer to BLAST search troubleshooting

Download Databases

The following steps are required to obtain databases for local BLAST search:

1. Connect to the NCBI server as anonymous user via ftp link ftp://ftp.ncbi.nlm.nih.gov/blast/db/. Databases can be downloaded from any standard source.

2. Download the databases.

3. Extract the database in /blast/db directory.

4. Format the database.

Format Databases for Mac

Database Formatting Tool

1. For BLAST Server released before Jan 2002, the formatdb program exists in the /blast/db directory.

2. For BLAST Server released in Jan 2002, formatdb, makemat and copymat binaries have been removed from the archive - they should be downloaded from the ftp site ftp://ftp.ncbi.nlm.nih.gov/blast/executables/release/ . This connects to the NCBI server as an anonymous user. For Mac, download the archive blast-x.x.x-powerpc-macosx.tar.gz. Copy the blast-x.x.x-powerpc-macosx.tar.gz file to the blast/db directory. Uncompress it using the following commands

gzip -d blast-x.x.x-powerpc-macosx.tar.gz
tar -xvpf blast-x.x.x-powerpc-macosx.tar

It will extract the formatdb file.

Format Databases

Formatdb must be used to format nucleotide source databases before they can be searched by blastall, blastpgp or MegaBLAST.
The source database may be in FASTA format. Copy the db file to your db directory of installed blast, also place formatdb in this directory,
Unzip/uncompress compressed databases
with the command :
./formatdb -i filename -p F -o T
Example : alu.n is input db file
./formatdb -i alu.n -p F -o T
This will generate some supporting files that are used by the BLAST algorithm for BLAST search.

Additional command line options for formatdb:
-t Title for database file [String]
Optional
-i Input file for formatting (this parameter must be set)
[File In]
-l Logfile name: [File Out]
Optional
default = formatdb.log
-p Type of file
T - protein
F - nucleotide [T/F] Optional
default = T
-o Parse options
T - True: Parse SeqId and create indexes.
F - False: Do not parse SeqId. Do not create indexes.
[T/F] Optional default = F
If the "-o" option is TRUE (and the source database is in FASTA format), then the database identifiers in the FASTA definition line must follow the convention of the FASTA Definition line Format.
-a Input file is database in ASN.1 format (otherwise FASTA is expected)
T - True
F - False
[T/F] Optional default = F
-b ASN.1 database in binary mode
T - binary

F - text mode
[T/F] Optional default = F
A source ASN.1 database may be represented in two formats - ascii text and binary. The "-b" option, if TRUE, specifies that input ASN.1 database is in binary format. The option is ignored in case of FASTA input database.
-e Input is a Seq-entry [T/F]
Optional
default = F
-n Base name for BLAST files [String]
Optional
-v Number of sequence bases to be created in the volume [Integer]
Optional
default = 0
-s Create indexes limited only to accessions - sparse [T/F]
Optional
default = F
-A Create ASN.1 structured deflines [T/F]
Optional
default = F
-L Create an alias file with this name use the gifile arg (below) if set to calculate db size use the BLAST db specified with -i (above) [File Out] Optional
-F Gifile (file containing list of gi's) [File In] Optional
-B Binary Gifile produced from the Gifile specified above [File Out] Optional
Test the added database through Primer Premier.
1. Launch BLAST search dialog.
2. Select the Local BLAST search option.
3. Select the "added database" in the Database dropdown menu.
4. If "added database" is not visible in Database dropdown menu, refer to Server configuration file syntax.

Configuration Files for Mac

Server configuration file and log file

The default configuration file is "blast.rc" and the logfile is "wwwblast.log" are located in the installed blast directory. Setting tag WWW_BLAST_TYPE to a specific value may change these names. Here is a sample configuration file that comes with this distribution:

```
# Number of CPUs to use for a single request
NumCpuToUse 4
#
# Here is list of combination program db1 db2 db3..., that are allowed by BLAST
# service. Format: ...
#
blastn test_na_db
blastp test_aa_db
blastx test_aa_db
tblastn test_na_db
tblastx test_na_db
```

To add your newly formatted db to the local BLAST server, append your db input file name at the end of its corresponding program separated by a space.

For example, if you format file alu.n then append "alu.n" at the end of line
blastn test_na_db. Now the database alu.n is associated with blastn program.
The new program/database (s) combination looks like :

```
blastn test_na_db alu.n
blastp test_aa_db
blastx test_aa_db
tblastn test_na_db
tblastx test_na_db
```

System Requirements for Mac

Mac OS X 10.6 Server requirements:

For Mac OS X 10.6	Required	Recommended
CPU	IMac Power PC G3 350	IMac Power PC G3 350
RAM	256 MB	384 MB
Local BLAST Server Space	50 MB	50 MB
Space for Local Databases	-	Depends on number of databases for Local BLAST

Note: If the input FASTA file is about 500 MB then it needs around 170-200 MB additional hard disk space for the translated BLAST database. At least another 100-200 MB should be allowed for memory consumption by the actual BLAST program.

Troubleshooting for Mac

1. Extract BLAST server
During untar, the message "UnExpected End of file", indicates that the downloaded file is either corrupted or incompletely downloaded. Please download it again.

2. Main BLAST page
Check the URL used.
a) Proper Server IP address.
b) Make sure "DocumentRoot" directory is the parent directory of blast directory or BLAST server has been extracted in the directory which is being used as DocumentRoot.
c) Address should consists of 3 parts ("http://Server name/blast/blast.html")
http protocol
Server name or Server IP address, and
main page location in the blast directory
Note: We can use localhost host in place of IP Address if we are using it as a client and server both.

3. BLAST Search
Request method POST is not allowed for the URL
While BLASTing, if the message "Request method POST is not allowed for the URL blast/blast.cgi" is generated,
a) "AddHandler cgi-script .cgi" is not uncommented. Find the command and uncomment it in the httpd.conf file. Please refer Add Handler
b) CGI executables scripts of BLAST server does not have executable permissions. Please refer to ScriptAlias

4. Uncompress the compressed database
If database archive ends with ".Z " then uncompress "blast.Your_Platform.tar.Z" with gunzip command.
For example : gunzip -d blast.Your_Platform.tar.Z
If database archive ends with ".gz " then uncompress "blast.Your_Platform.tar.gz" with gzip commands.
For example :
gzip -d blast.Your_Platform.tar.gz
tar -xvpf blast.Your_Platform.tar

5. Specify valid Server name/IP address

Please specify valid Server name/IP address.
1. If main BLAST page is not accessible through browser. Refer to Main BLAST page troubleshooting.
2. If main BLAST page is accessible through browser and Primer Premier generates an error, It may be due to one of the following reasons:
a) When you specify the IP address, please exclude "http://" from it.
b) The BLAST page name is included in the IP address. Please refer to BLAST server path and IP address troubleshooting.
c) BLAST server may not exist in the server's DocumentRoot directory. Please refer to BLAST server path and IP address troubleshooting.

6. BLAST server path and IP address

Suppose your server IP address or host name is "MyServer".
1. Default BLAST server path
If BLAST server is installed in the DocumentRoot directory, then the main BLAST page should be accessible by the following URL

http://MyServer/blast/blast.html
or
http://localhost/blast/blast.html(if using as client server both)

Primer Premier expects "MyServer" as Server IP address.

2. User defined server path
If BLAST server is not installed in the DocumentRoot directory and main BLAST page is accessible by the following URL
http://MyServer/../blastparent/blast/blast.html

Primer Premier expects "MyServer/../blastparent" as Server IP address.

For example
If main BLAST page is accessible by the following URL
http://MyServer/blastparent/blast/blast.html

Primer Premier expects "MyServer/blastparent" as the Server IP address.

Algorithm and Formulae

Formula for Melting Temperature Calculation

The T_m for the primer is calculated using the following:

C - Primer concentration: 0.25 nM (acceptable range 0.1 - 100 nM)

Mg++: 1.50 mM (acceptable range 0 - 100 mM)

Monovalent ion concentration: 50 mM (acceptable range 0 - 1000 mM)

[Na+] Equivalent Algorithm: The sodium equivalent is calculated by:
[Na+] = Monovalent ion concentration + 4 X (Free Mg2+)$^{1/2}$ (all in molar concentration)

dH Calculation:
The dH is found by adding up all the di-nucleotide pairs values from the table below. If the primer begins with a G or a C, then the init G-C correction is added, otherwise the init A-T correction is added. The same is done for the base at the end of primer.

dS Calculation:
The same method as the dH is used. An addition salt correction term is added where N is the number of nucleotide pairs in the primer (primer length - 1):
dS (salt corrected) = dS (1 M NaCl) + 0.368 * N * ln ([Na+])

T_m Algorithm:
The T_m for a primer is calculated from its H, S, R the gas constant (1.987 cal/kmol), and C the primer concentration:
T_m = dH / (dS + R * ln C)

Example:
Here is an example of a T_m calculation using: Salt equiv: 204.92 mM

Primer Sequence: CAGAAGTCCACTCATTCTCG
CA AG GA AA AG GT TC CC CA AC
dH = - (8.5 + 7.8 + 8.2 + 7.9 + 7.8 + 8.4 + 8.2 + 8.0 + 8.5 + 8.4
CT TC CA AT TT TC CT TC CG
7.8 + 8.2 + 8.5 + 7.2 + 7.9 + 8.2 + 7.8 + 8.2 + 10.6) = -156.1 kcal/mol
Now we add in init G-C+ init G-C = -156.1kcal/mol + 0.1 kcal/mol + 0.1 kcal/mol = -155.9 kcals/mol
CA AG GA AA AG GT TC CC CA AC
dS = - (22.7+ 21.0 + 22.2 + 22.2 + 21.0 + 22.4 + 22.2 + 19.9 + 22.7 + 22.4
CT TC CA AT TT TC CT TC CG
21.0 + 22.2 + 22.7 + 20.4 + 22.2 + 22.2 + 21.0 + 22.2 + 27.2) = -419.8 cal/K-mol
Now we add the init G-C + init G-C =-419.4 cal/kmol + -2.8 + -2.8 = -425.4 cal/K-mol
Now we add salt correction,
NaEqiv = Na+ + 4 x sqrt(Mg++) (all concentrations molar)
= 0.05 + 4 x sqrt (0.0015)= 0.20492M
dS= -425.4 + .368 x N x ln [Na]
= -425.4 + .368 x 19 x ln .20492
= -425.4 + .368 x 19 x (-1.585)
= -436.48 cal/K-mol
T_m= H/(S + R ln C)
= -155.9 kcal/mol / (-436.48 cal/K-mol + 1.987 cal/kmol * ln(0.25e-9))

= -155.9 kcal/mol / (-436.48 cal/ K-mol + 1.987 cal/kmol * -22.109)
= -155.9 kcal/mol /(-480.41 cal/K-mol)
= 324.51 ^{o}K
= 324.51 - 273.15 = 51.36 ^{o}C

T_m for primer as predicted by Primer Premier using Breslaur values: 52.5 ^{o}C

Dinucleotide Table Values:

Dinucleotide Sequence	**H kcal/mol**	**S cal/k mol**
AA OR TT	-7.9	-22.2
AT	-7.2	-20.4
TA	-7.2	-21.3
CA OR TG	-8.5	-22.7
GT OR AC	-8.4	-22.4
CT OR AG	-7.8	-21.0
GA OR TC	-8.2	-22.2
CG	-10.6	-27.2
GC	-9.8	-24.4
GG OR CC	-8.0	-19.9
Init w G-C	0.1	-2.8
Init w / term	2.3	4.1

Formula for Calculating TaOpt

Primer Premier 6 displays calculated optimal annealing temperature Ta Opt for current primer pair in the Primer Properties tab. It is the recommended annealing temperature in the PCR reaction and is expected to yield the best PCR product yield with minimal false product production.

We use the following formula:

Ta Opt = 0.3 * Primer Tm + 0.7 * Product Tm – 14.9 ^{o}C

where Primer Tm is the melting temperature of the less stable primer and Product Tm is the melting temperature of the PCR product in oC. The less stable primer is the primer with lower Tm of the two primer Tm's.

The Ta Opt is generally lower than the Tm of the less stable primer by around 5 oC. For templates with high GC content, the calculated Ta Opt may be too high, even higher the Tm of the less stable primer. Running the reaction at this high temperature may result in reduced or unspecific annealing, and smaller or unspecific product yield may result. **The best method to avoid these deleterious effects is to increase the concentration of the less stable primer with respect to the other in the reaction.** We highly recommend following this suggestion.

1 Rychlik, W., Reference (13) in Appendix D.

Formula for Free Energy

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

dG = dH - T * dS

dH is the enthalpy.

T is the temperature.

dS is the entropy.

Formula for Rating

In Primer Premier 6 the sophisticated search algorithm calculates all properties of every possible primer within the allowed length and positional boundaries and rates them. Tm is calculated based on nearest neighbor thermodynamic theory using SantaLucia values. Primer Premier 6 then calculates the self-dimer and hairpin stability of each primer. To quantify the merit of each primer, a rating number is calculated based on a number of parameters; Tm , the stability of possible secondary structures and the location of each primer compared to the specified site and all of their individual tolerances. The rating determines how well the designed primer meets the search parameters relative to the tolerance limits specified for each parameter. The parameters included in arriving at the rating are maximum hairpin dG, maximum self-dimer dG, maximum run/repeat length, maximum Cross-dimer dG and Tm mismatch.

$$\text{Rating} = 100\left[1 - \sqrt{\frac{1}{N}\sum_{n=1}^{N}\left(\frac{\text{Parameter}_n - \text{Target}_n}{\text{Tolerance}_n}\right)^2}\right]$$

From the formula it is evident that if all parameters were exactly on target or summation (S) of all the variables is 0, the rating would be 100. On the other hand, if summation of all the variables (parameters) is greater than zero then the rating can vary between 100 to 0. It would be 0 when the summation of all the variables is equal to 1 or when all parameters were out at their tolerance limits.

Tolerance is a permissible value of variation acceptable by the program for designing primers from a preset standard (Default) value. For instance variation of +/- 5°C is the tolerance value specified for Target Tm parameter of 55°C.

The tolerance plays two related but distinct roles. First, it is used to reject primers with parameters outside of the specified range. Then, for acceptable primers, the inverse tolerance is the rating weight given to each parameter. There are no negative rating values because all primers with one or more parameters out of limits are rejected.

The rating of a given primer, depends on two factors:
1. How close the primer is to the target value of each parameter.
2. How tightly the tolerances are specified.

The default tolerances provided in the program are chosen to represent the requirements of typical experiments. Highly rated primers have most or all parameters near their ideal values and are very likely to work well, but it is important to note that all designed primers meet all the specifications, no matter what their rating.

Target is a specified value for a particular given parameter. For instance if we specify a value for Target Tm parameter as 55°C then this value is the Target value.

For instance one can specify the Tm tolerance, which corresponds to the acceptable range of temperature over and below the optimal Tm for which primers are acceptable. Default for the temperature tolerance is +/- 5 degrees.

Thus at a temperature of 55°C, the temperature will range from 50 to 60°C, allowing design of more primers that fit into the five degree range. The primers qualifying as per criteria set will be designed by the program. In other words, if tolerance increases then chances of primers qualifying at majority of parameters increases and so rating also increases. Other parameters are

also considered by the program which determines the quality of any oligo for an experiment to be a success.

NOTE: If you change the tolerance to 0, the program will display an error as: “Please enter a number between 0.1 and 99.9”. For example, for a temperature selection of 55°C, if you give a Tolerance of 0, the above error message is displayed.

For primer search, the quality of the designed primer is displayed as Best, Good, Poor or Not Found in the Search Status tab. The primers having rating greater than or equal to 75 are termed Best, rating between 74 and 50 are termed as Good and those having rating below 50 are termed Poor. If no primer meeting the specified criteria is found, a "Not Found" status is displayed. The search status appears in RED under Primer Properties tab.

For instance: Status: No primer pairs found : 294 rejected (ProdLen: 188 TmMatch: 106) indicates the total number of primer rejected and the parameters they failed on.

To find a suitable primer, one may change the search parameters based on the search status message. For the above example, one can increase both the product length and Tm to check if its possible to design primers.

The rating assigned is just a facilitator for you to take an informed decision and indicates how closely a primer meets all the target values. When setting the tolerance limits, the sole criteria should be acceptability. Though counter intuitive, specifying tolerances tightly does not assure better results. A low rated primer would not necessarily fail. It may be worthwhile to consider its properties relative to your experimental needs and decide whether the primer is acceptable or whether the low rating is an artifact of narrow tolerances.

System Specifications

Supported Operating Systems	Microsoft's Windows 98/ME/2000/NT/XP/Vista/Win 7 Mac OS X 10.4.x, 10.5.4, 10.6 (Snow Leopard)

System Requirements:

For Windows OS	Required	Recommended
CPU	Pentium-IV 1.80 GHz	Pentium-IV 3.00 Ghz or higher
RAM	256 MB	512 MB
Hard Disk Drive Space	500 MB	1 GB
Screen Resolution	800 X 600	> 800 X 600

For Macintosh OS X	Required	Recommended
CPU	IMac Power PC G5 1.6 GHz	IMac Power PC G5 1.6 GHz
RAM	256 MB	512 MB
Hard Disk Drive Space	500 MB	1 GB
Screen Resolution	800 X 600	> 800 X 600

For Intel Based Mac OS X	Required	Recommended
CPU	Intel 1.8 GHz Intel Core Duo	Intel 1.8 GHz Intel Core Duo
RAM	256 MB	512 MB
Hard Disk Drive Space	500 MB	1 GB
Screen Resolution	800 X 600	> 800 X 600

Suggestion

1.Switch to a computer with larger RAM.

For further information, please see:
http://www.premierbiosoft.com/products/system_requirements/system_requirements.html

2.Try and decrease system resource requirements by:
a. BLAST searching one template at a time.
b. Changing some of the parameters on Advanced Search Parameters window:
- Increase the word size.
- Decrease the Expect value.
- Check the Filter option.
- Decrease the Alignment and Description values.

Software Limits

Sequence Supported	15 MB
Sequence format Supported	GenBank, FASTA, GenBank Like, dbSNP, Text sequence

Primer Search limit:

Search Range Supported	50,000 bp
Primer Search Range	25 bp
Minimum Primer length	10 bp
Minimum Amplicon length	50 bp
Maximum Amplicon length	5000 bp
Maximum Primer length	99 bp
Maximum Alternate Primer pairs	50

BLAST limit:

RID Polling	30 sec for first time and every 60 sec after that
In case of ISA/NTLM proxy	7000 bps
Other proxy and direct internet connection	NO LIMIT

Template structure search limit:

Number of sequence searched at a time	5
Maximum length of bases	1200BP
Minimum length of bases	50BP

Additional Information:

Multiplex Primer -Primer	10 Sequences
Number of SNP selected per sequence	1
Number of SNP per sequence	Unlimited
SNP format supported	/replace="t"
Number of SNP deleted per sequence	All
Number of Primers/primer pair evaluated at once	1
SNP Position	No limit

Terms of Use

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http://www.ncbi.nlm.nih.gov/About/disclaimer.html

BLAST Server - Terms of Use
Primer Premier connects to the BLAST server at:
http://www.ncbi.nlm.nih.gov/blast/Blast.cgi
This server is run by the U.S. Government. The user will be responsible for following the guidelines and policies set up by the U.S. Government for using the BLAST server.
PREMIER Biosoft International assumes no liability for unauthorized or illegal use of this server.
For your convenience, we urge you to strictly follow these guidelines:-
- Submit only a few thousand individual requests per day (not more than 2000 per day),
- Limit all large searches to the off peak hours of 9 PM to 5 AM Eastern Standard Time (USA).
For further information or queries please visit:
http://www.ncbi.nlm.nih.gov/BLAST/

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