PRISE 2.0

User Manual

UC Riverside, January 2012

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1. General Information

PRImer Selector 2 (PRISE2) is a software package developed at the University of California, Riverside that implements several features for improving and streamlining the design of sequence-selective PCR primers. It can also be used to produce primer-probe sets for qPCR assays such as TaqMan, and probes for hybridization-based assays such as FISH. It is available free of charge for non-commercial use at http://alglab1.cs.ucr.edu/OFRG/PRISE.php.

1.1 System requirements

PRISE2 requires a minimum of 512 MB of RAM (1 GB of RAM or more is recommended) and active Internet connectivity. It can be run on the following platforms:

- Mac OS X 10.5 or higher
- Windows 2000/NT/XP/2003 Server/Vista/7
- Ubuntu 10.04 or higher

Note: In order to install PRISE2 on Mac OS X Mountain Lion or higher, users may be required to change or bypass their Gatekeeper settings to allow the installation. Detailed information about this process can be found at http://www.imore.com/how-open-apps-unidentified-developer-os-x-mountain-lion.

1.2 Overview of the design process

Designing PCR primer pairs and primer-probe sets using PRISE2 involves two steps:

- Step 1, which is divided into two components (1.1 and 1.2), enables target and non-target DNA sequences to be identified and collected, and
- Step 2, which generates PCR primers/probes designed to amplify target but not non-target sequences.

Probes are designed along with primer pairs as a set, so primer pairs need to be generated first. After generating primer pairs, users can continue to generate probes corresponding to specific primer pairs from the menu option. In the current version of the program, designing probes for FISH analyses requires primers to be designed first, even though they will not be used.

A detailed step-by-step protocol (PRISE2 Tutorial), which demonstrates how the software was used to create sequence-selective PCR primers and probes for a specific fungal rRNA gene, can be accessed via the Instructions or Help links.

1.3 Starting the program

When the program is started, a window with four buttons appears. This window links to instructions and modules for performing steps in the primer/probe design process. Detailed information for each module will be described in following sections.

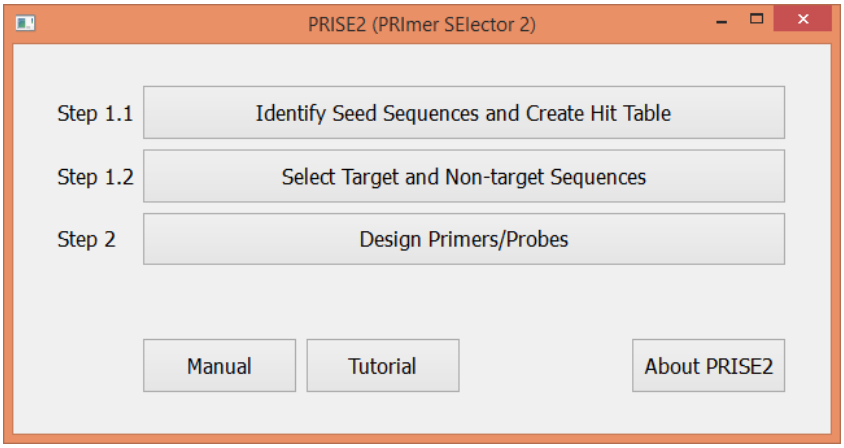


Figure 1: Opening window with links to instructions and modules of PRISE2

2. Step 1.1: Identify Seed Sequences and Create Hit Table

2.1 Overview

The first step in the design process is to identify the seed sequences and to create the hit table. The button **Identify Seed Sequences and Create Hit Table** opens a wizard page which guides users through this step. Seed sequences represent the DNA sequences that the primers are designed to amplify. The hit table is a list of DNA sequences with various degrees of similarity to the seed sequences, from which the target and non-target sequences can be derived. It is created by subjecting the seed sequences to an analysis using BLAST (blastn).

Note. Although steps 1.1 and 1.2 are designed to identify and collect target and non-target DNA sequences, there are certainly other strategies for accomplishing this task, which users may decide to use instead of or in combination with our steps. The only requirement for using the primer design module of PRISE2 (step 2) is that the target and non-target sequences be available in separate FASTA-formatted text files.

- *Identify Seed Sequences:* Identify the sequences that the primers are intended to amplify and save them in FASTA format as a text file. Use of large numbers of seed sequences requires longer processing times.

- *Create the Hit Table*: Subject the seed sequences to a nucleotide BLAST analysis. To create a hit table, BLAST analysis is required; this can be done by either using the program on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) or running BLAST command line application on local machine. It is essential for BLAST analysis to select the appropriate database and the maximum number of target sequences, which, in our experience, will typically be at least 500.

2.2 Create hit table using NCBI BLAST website

The hit table can be created by utilizing the NCBI BLAST website; users can adjust BLAST settings and get results through the web interface. For users' information, the ***Max target sequences*** option is located in the ***Algorithm parameters*** section. After clicking on the ***BLAST*** button, click on ***Formatting options***. Under the section ***Show,*** set ***Alignment*** as ***Plain text,*** and set ***Alignment View*** to ***Hit Table***. In addition, in the ***Limit results*** section set ***Alignments*** to the value that was used for the ***Max target sequences***. Click ***View report*** and save the output as a text file. This file is the Hit Table[1].

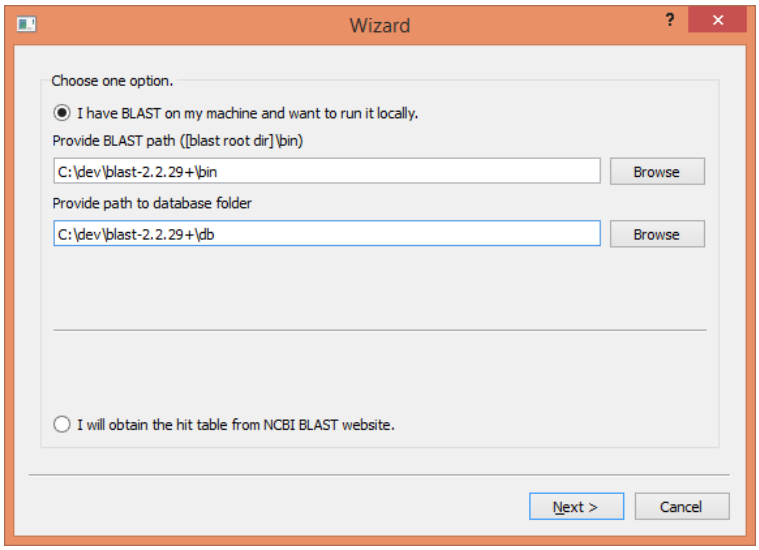
2.3 Create hit table using local BLAST application and database

[1] **Note:** There is an issue with BLAST that occurs if you do not select the alignment view to be a hit table and, after the blast analysis is completed, you attempt to re-format the BLAST run via the formatting options. We found that in this situation the hit table option is often not available. The following work-around has been provided by a BLAST technician: *(1) Click Download, then right click the "Hit Table(text)" link to copy it. (2) Open a new window/tab in the browser, paste in the link, and save the Hit Table as a text file.*

For users that have the BLAST command line application installed on their machine, PRISE2 provides an option to run BLAST locally using their own databases and settings, and get results through PRISE2's interface. A designated wizard page will help users through this process.

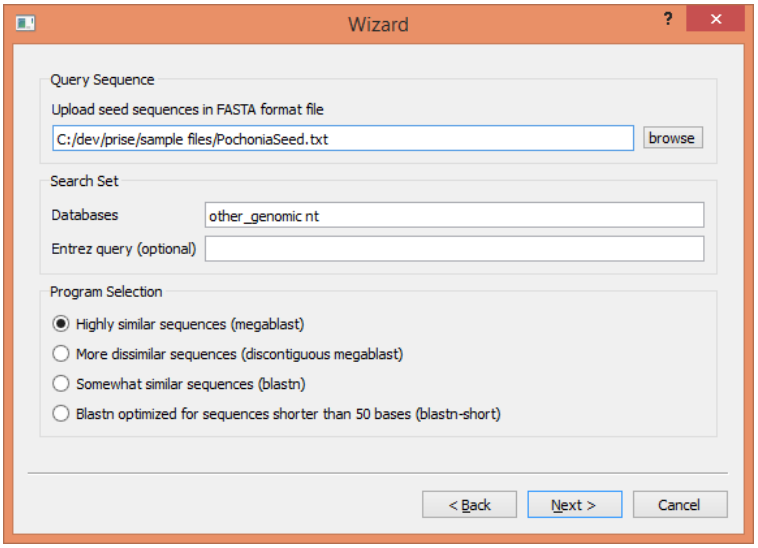
I. *Provide paths to BLAST and databases*

To run BLAST locally, users need to provide the path to BLAST folder and databases, as shown in the figure below. After selecting the "**I have BLAST on my machine and want to run it locally**" option, users can configure required paths for BLAST application, and then a similar interface as NCBI website will allow users to provide inputs and adjust parameters.



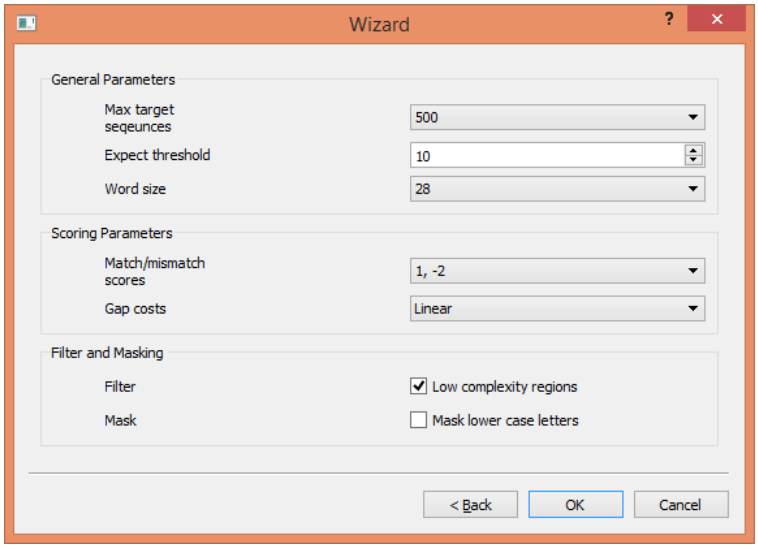
II. Specify query sequences, databases and applied algorithm

Next, users specify the query sequence, which is the same as the seed sequence. Also, to let BLAST program know where to search, the names of databases are required, which should be separated by spaces as show in the figure below. BLAST contains several different algorithms that are suitable for different similarity measures and the sequence lengths; by default the megablast algorithm is applied. Users can choose the desired algorithm according to the query sequence and usage.



III. *View and change parameters*

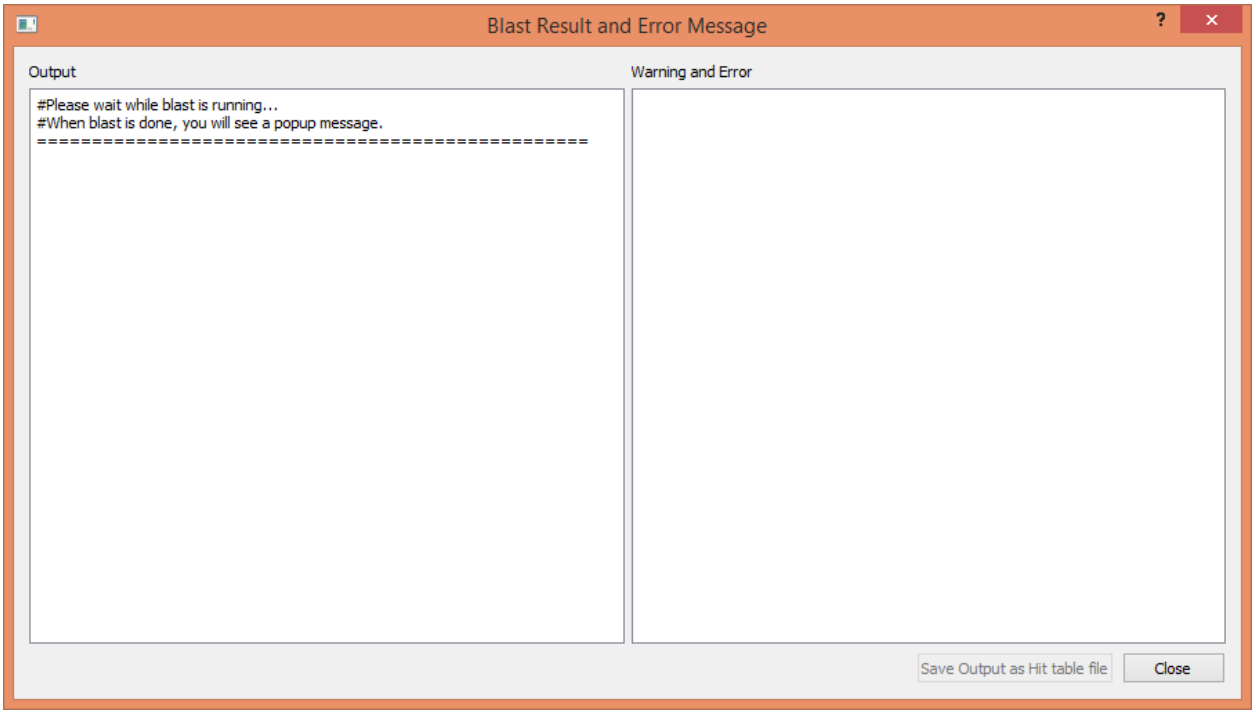
Each BLAST algorithm has a number of parameters. Before running BLAST analysis, PRISE2 allows users to view and adjust those parameters.



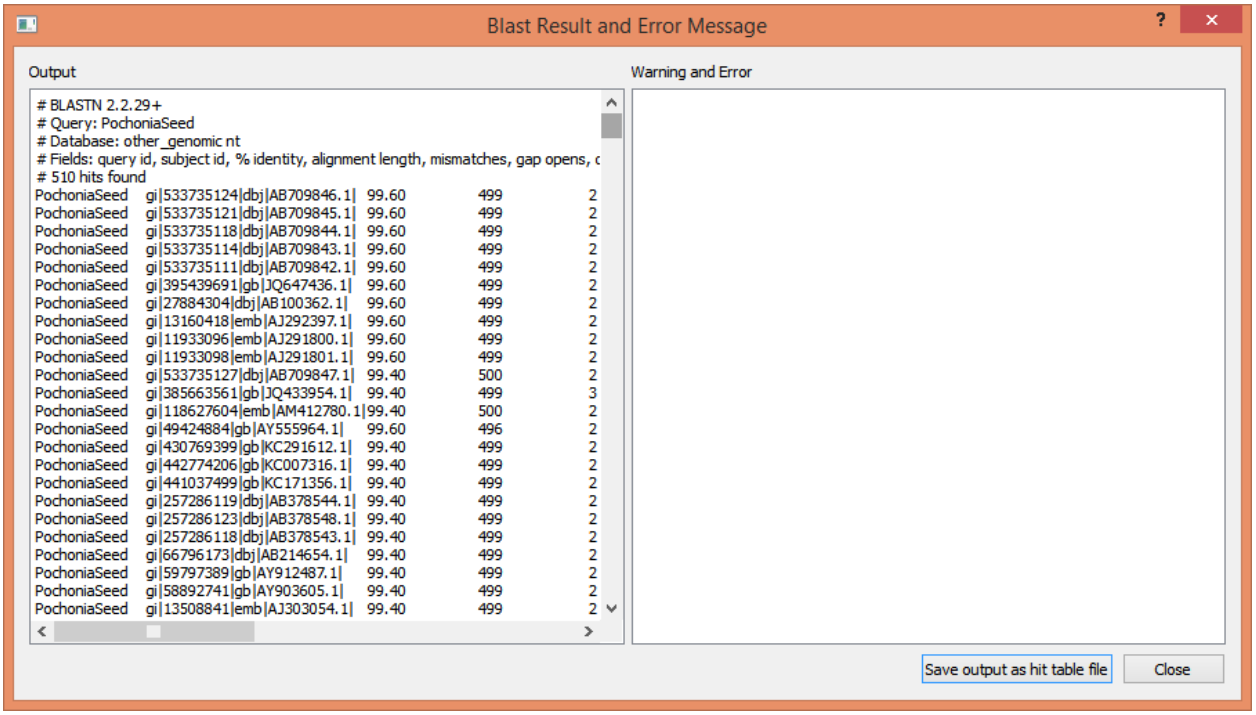
IV. *Run BLAST analysis and obtain hit table*

After providing above information and pressing the OK button, PRISE2 will try to run local BLAST. If BLAST cannot start successfully, a notification message will pop up. In most case this is because the BLAST path is not correct; please also check if the BLAST application is correctly configured and runnable.

If BLAST starts successfully, the result window appears. The BLAST process may take a few minutes to finish. When it finishes, PRISE2 will notify users with a pop-up message.



After the BLAST analysis finishes, the result or error/warning messages (if any) will show in the result dialog window as below. If there are any error/warning messages, users can check and change corresponding settings; otherwise they can save the result as a hit table file by clicking on the "**Save Output as Hit table file**".



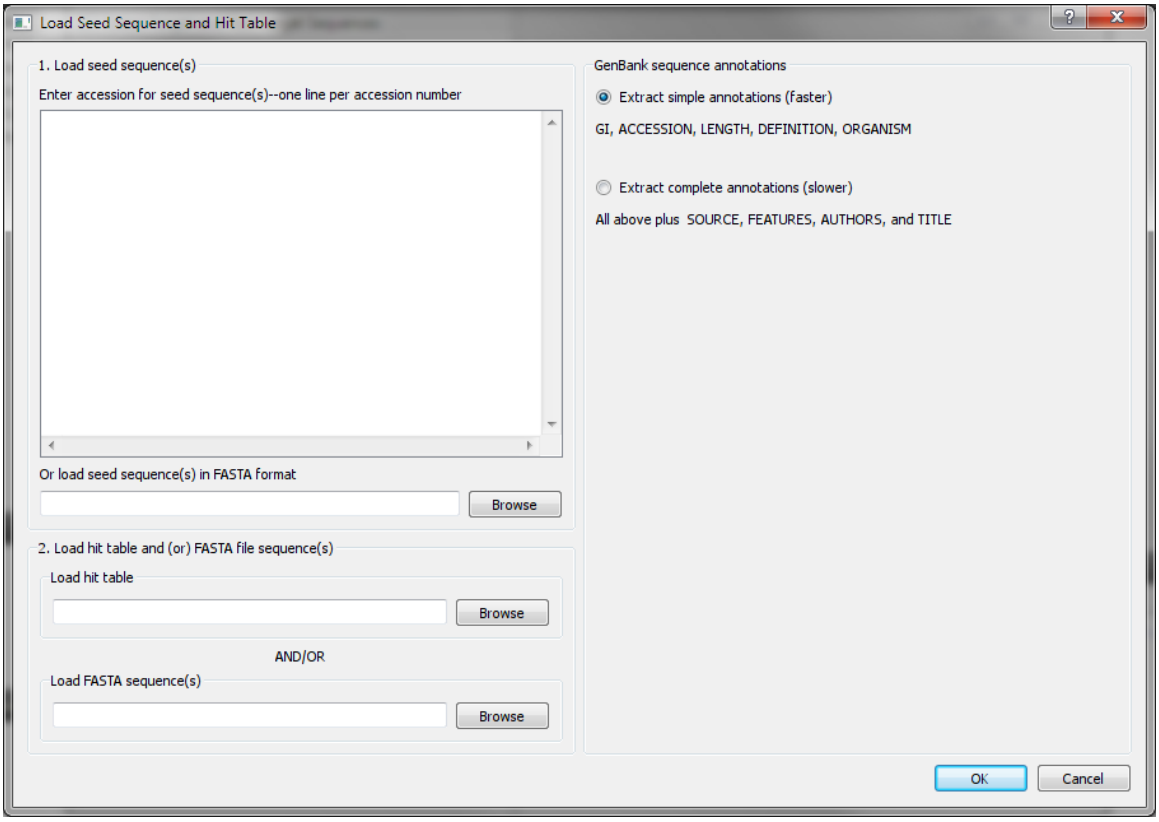
3. Step 1.2: Select Target and Non-target Sequences

Once the seed sequences and hit table are created, the next step is to identify and collect the target and non-target sequences in the ***Select Target and Non-Target Sequences*** module.

3.1 Using the module

I. *Load Sequences*

After opening the module, users can input the seed sequences and hit table files into the software by selecting the **Load Seed Sequence and Hit Table** option from the *File menu*. This option opens a window titled *Load Seed Sequence and Hit Table*, where the appropriate files can be input. Note that this window also allows FASTA files to be input instead of or along with the hit table, allowing sequences other than those generated by a BLAST analysis to be utilized. In the next window, titled *Sequence Alignment Settings for Pairwise Identity Analysis*, users can select settings for the pairwise identity analyses, which will be performed between the seed sequences and the hit table sequences (and/or FASTA sequences if there are any).



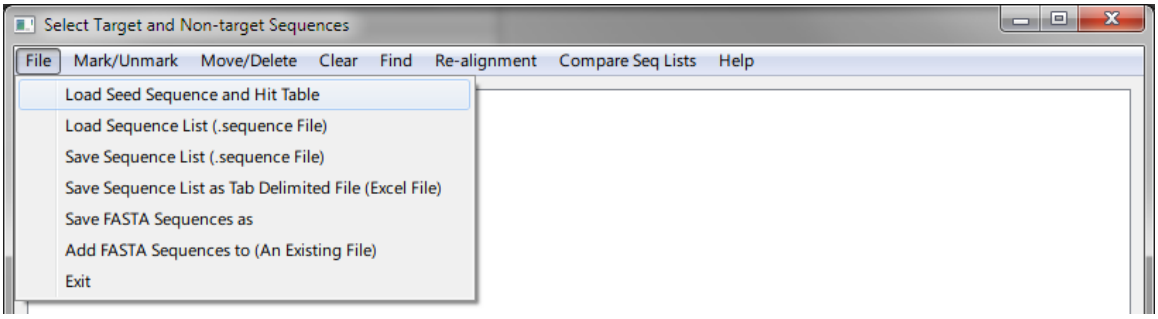
II. *Collecting and Parsing the Sequence Data*

After the sequences are uploaded, the software downloads all of the GenBank records associated with the seed sequences and hit table sequences, parses the data contained within them into separate components, performs pairwise identity analyses between the seed sequences and hit table sequences, and displays these data in tabular form in a report window. The title of this window will be the hit table file name followed *by "- Select Target and Non-Target Sequences."* After the program finishes processing the data, which could take minutes to hours, depending on the number of sequences in the seed sequence and hit table files, the speed of the internet connection and the capabilities of the computer, a sequence downloading report dialog appears. This report lists the accession number of sequences from the hit table that are too large to be analyzed. The information in the report can be saved as a text file for later.

III. *Sequence Selection*

Once these actions have been completed, users can identify and collect the target and non-target sequences by applying sorting tools to the sequences assembled in the table. This task is primarily done by using tools that allow the sequences to be selected by parameters including sequence length, sequence identity, or GenBank parameters such as Definition or Source. Sequences can also be sorted by clicking on the column headings. Below is a description of all of the functions in this module, organized by the pull down menu they reside in. See the PRISE2 Tutorial for a few examples of how they can be used.

3.2 File menu



- *Load Seed Sequence and Hit Table:* Allows Seed Sequences and hit tables to be loaded. This window also allows the user to load a FASTA file instead of or along with a hit table, allowing sequences from sources other than a BLAST analysis to be utilized.

- *Load Sequence List:* Allows previously created sequence lists (which are PRISE2 generated and formatted files) to be loaded into the software.

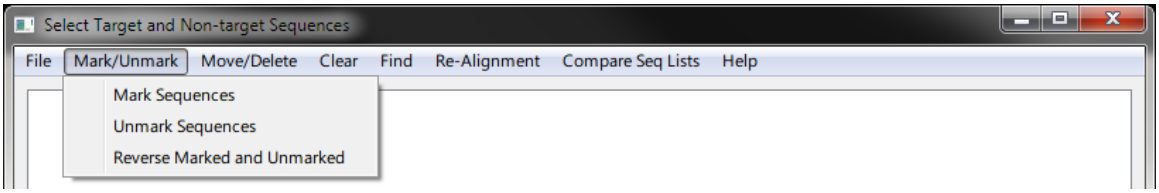
- *Save Sequence List:* Allows sequence lists to be saved in the format used by the PRISE2 software.

- *Save Sequence List as Tab Delimited File:* Allows sequence lists to be saved in a tab-delimited format, which can be used in standard spreadsheet software.

- *Save FASTA Sequences As:* Saves the sequences in the FASTA Sequence Box in FASTA format as a text file.

- *Add FASTA Sequences To:* Adds the sequences in the FASTA Sequence Box to another text file (typically one that contains other sequences in FASTA format).

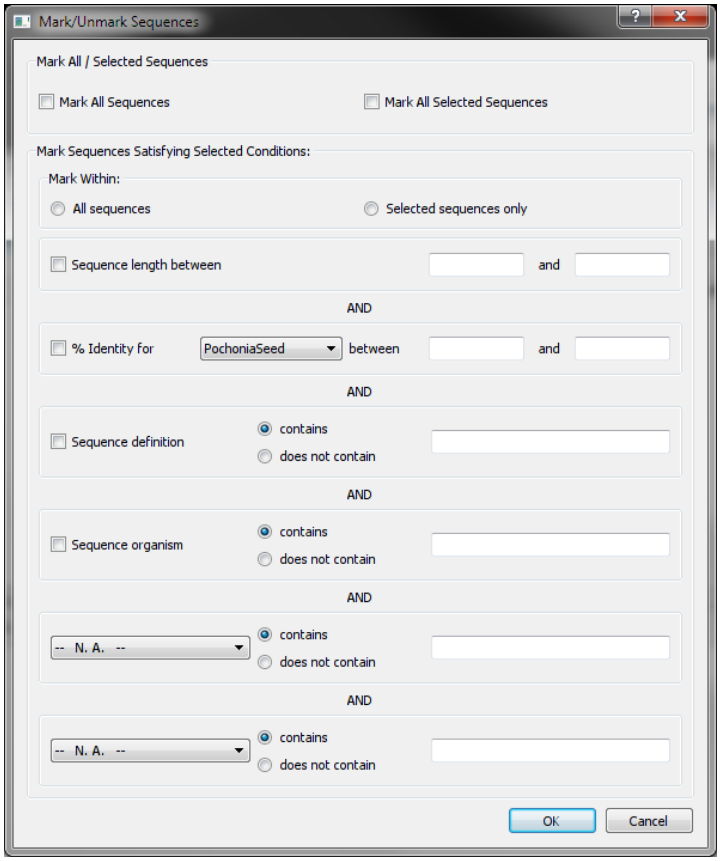
3.3 Mark/Unmark menu



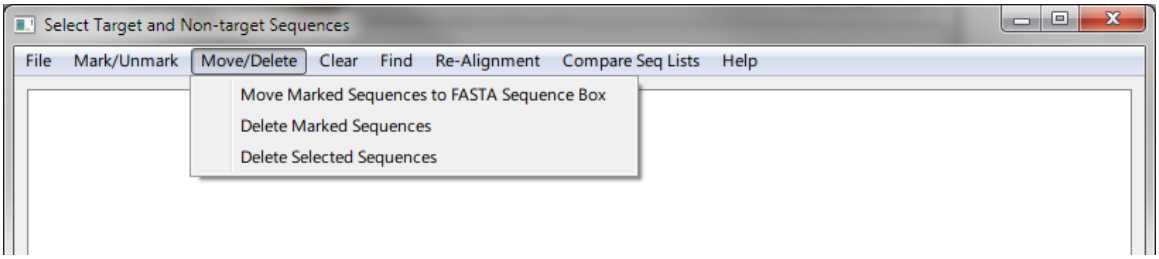
- *Mark Sequences:* Allows sequences to be marked if they possess user-defined criteria. Marked sequences are designated by a check mark in the box in the second column (and a yellow-highlighted row). Sequences that are marked can be moved to the FASTA Sequence Box, and then saved or merged with other FASTA files to make target and non-target sequence files.

- *Unmark Sequences:* Allows sequences to be unmarked if they possess user-defined criteria.

- *Reverse Marked and Unmarked Sequences:* Reverses the marked and unmarked designations.



3.4 Move/Delete menu



- *Move Marked Sequences to FASTA Sequence Box:* Moves marked sequences to the FASTA Sequence Box. Marked sequences are designated by a check mark in the box in the second column. Sequences that are marked can be moved to the FASTA Sequence Box, and then saved or merged with other FASTA files to make target and non-target sequence files.

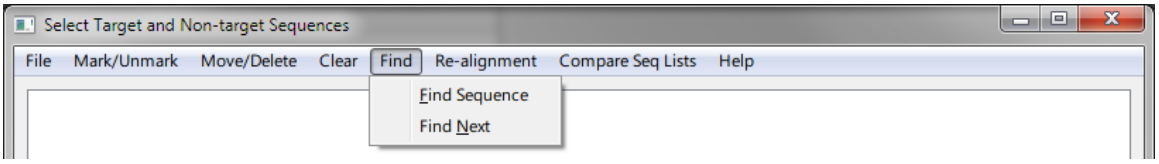
- *Delete Marked Sequences:* Deletes marked sequences from the sequence list. Marked sequences are designated by a check mark in the box in the second column (and a yellow-highlighted row).

- *Delete Selected Sequences:* Deletes selected sequences from the sequence list. Selected sequences are designated by their rows being highlighted in blue. Sequences can be selected by clicking on any part of the row except the boxes in the second column. Standard key commands such as shift and control can be used with this function, allowing groups of sequences to be selected. Once sequences are selected, they can be marked or unmarked using the functions in the Mark/Unmark menu.

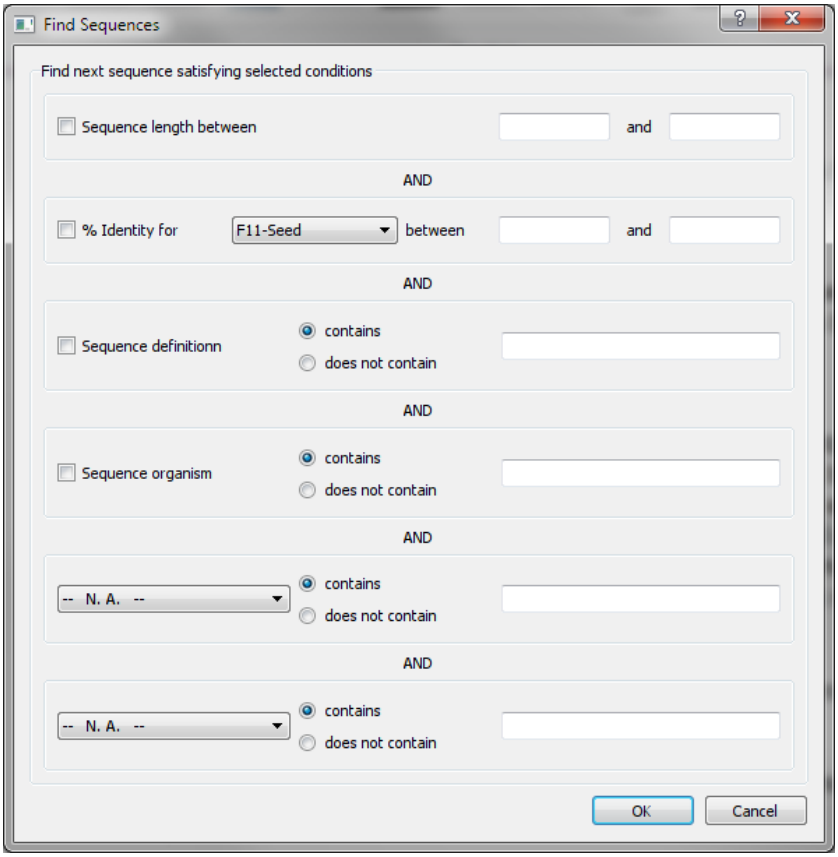
3.5 Clear menu

- *Clear FASTA Sequence Box:* Deletes the sequences from the FASTA Sequence Box.

3.6 Find menu

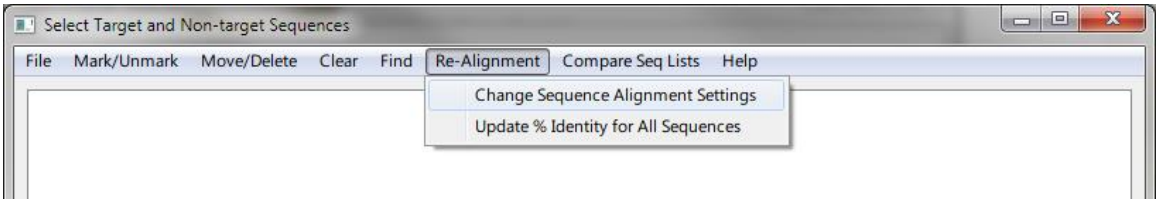


- *Find Sequence:* Allows the user to search for sequences by user-defined criteria.



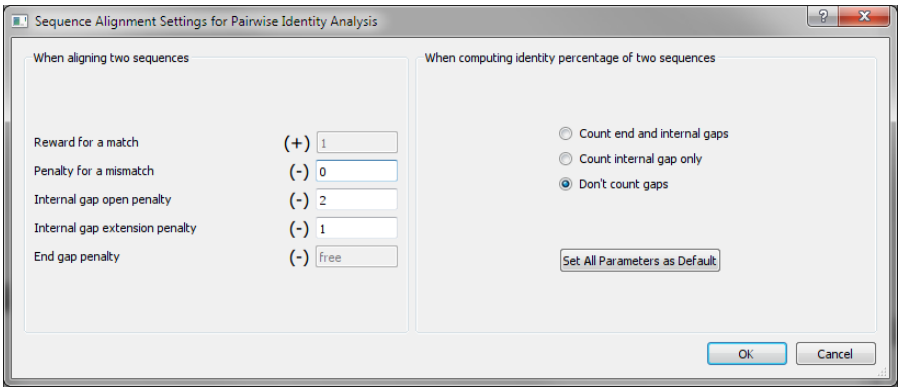
- *Find Next:* Allows the user to search for sequences using the criteria that were input in the last *Find Sequence* search.

3.7 Re-alignment menu

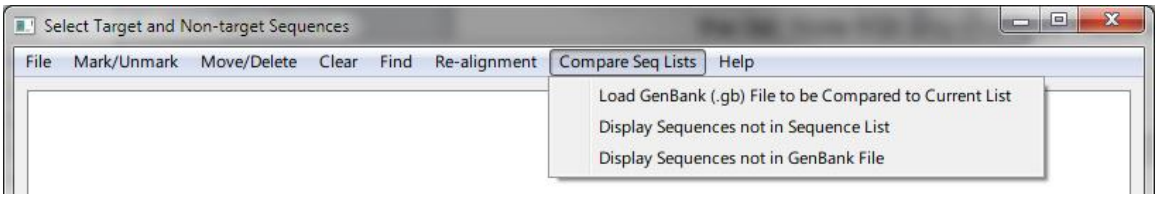


- *Change Sequence Alignment Settings:* Allows the user to change the settings used for the pairwise identity analyses. The resulting changes in the alignment values for individual sequences can be viewed by using the **Display Pairwise Alignment** option, which is accessed via a right click. Note that these settings will not be saved unless the **Update % Identity for All Sequences** option is used (see immediately below).

- *Update % Identity for All Sequences:* Allows the user to change the settings used for the pairwise identity analyses and then perform a new pairwise analysis on all sequences in the list. Note that any changes made with this option will be automatically saved in the Sequence List file.



3.8 Compare Seq Lists menu



- These functions allow the user to compare sequences in the Sequence List, which is currently loaded in the PRISE2 software, to sequences in a GenBank file. Note that these sequences will be compared by their GenBank Accession number, not their nucleotide sequences.

- *Load GenBank (.gb) File to be Compared to Current Sequence List:* Allows the GenBank file to be loaded into the software.

- *Display Sequences Not in Sequence List:* Displays the sequences that are in the GenBank file but not in the Sequence List.

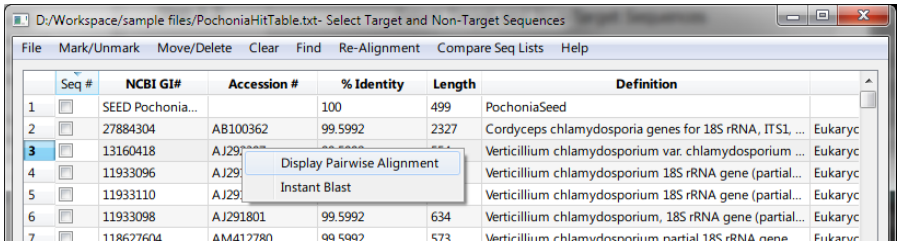
- *Display Sequences Not in GenBank File:* Displays the sequences that are in the Sequence List but not in the GenBank file.

3.9 Help menu

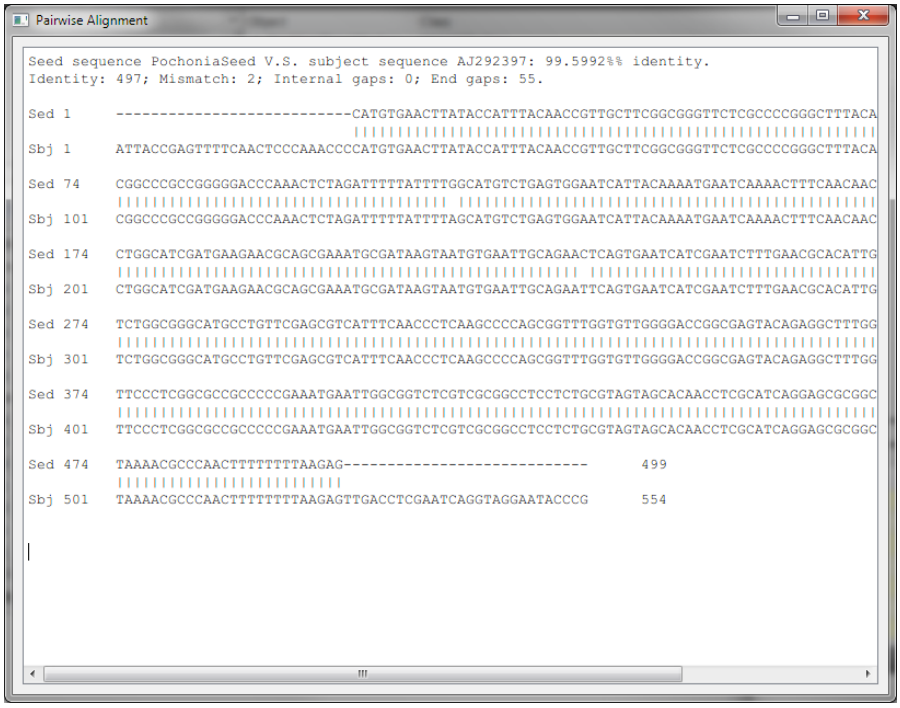
- *PRISE2 Manual:* Opens the PRISE2 Manual.

- *PRISE2 Tutorial:* Opens the PRISE2 Tutorial, which provides a step-by-step protocol showing how the software was used to create sequence-selective PCR primers and probes for a specific fungal rRNA gene.

3.10 Right-click options



- *Display Pairwise Alignment:* Opens a window showing the alignment of the selected sequence and the Seed Sequence. Note that this function only works when one sequence is selected and the seed sequence contains one sequence.



- *Instant Blast:* Allows the sequence to be subjected to a BLAST analysis, by opening the BLAST page at NCBI and loading the sequence. Note that this function only works when one sequence is selected.

4. Step 2: Design Primers/Probes (Choosing Primers)

PRISE2 allows selection of both standard PCR primer parameters, such as GC content, primer length, inter- and intra-complementarity, as well as criteria for sequence-selectivity. Selectivity is accomplished by identifying primers that should amplify target sequences but not non-target sequences. The prediction as to whether a PCR product will be made is based on a number of criteria that can be customized by the user to suit the application at hand.

One of the criteria used in this process is a scoring scheme that is used to define the likelihood that specific primer-template combinations will produce a PCR product. This scheme allows the user to set the design criteria for each position in the primer. Here, we describe only a simple version of this scheme that focuses on last three 3' positions. (For more detailed information on Primer Selectivity Settings, please refer to Appendix I.)

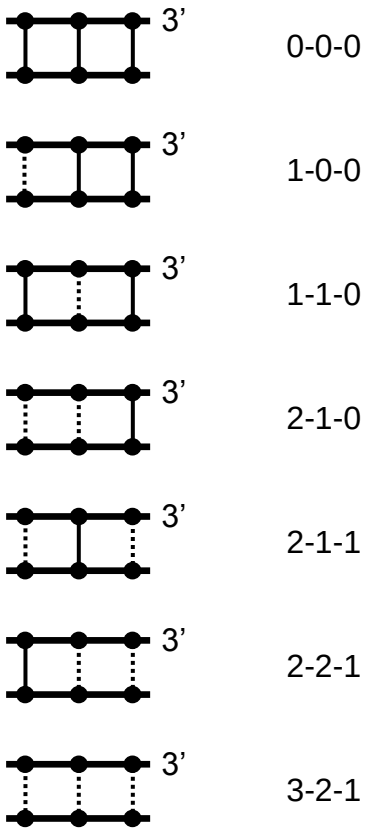


Figure 2: Scoring scheme for the sequence-selectivity component of the Design Primers module. On the left side are depictions of the last three 3' nucleotides of a primer and its corresponding template. The primer is the top strand. Base-paired nucleotides are designated by solid lines. Non-based paired nucleotides are designated by dashed lines. The score (3 digits) assigned to each type of template-primer pair is shown to the right.

Figure 2 shows various match-mismatch configurations and corresponding parameter settings. If the setting is set to xyz, then only primer-template pairs that satisfy this xyz match-mismatch configuration and those above will be considered as producing a PCR product. For example, for

0-0-0 setting, only exact matches at all three positions will be scored as creating a PCR product. If the setting is 2-1-0, then any primer-template pair with match-mismatch configurations of 0-0-0, 1-0-0, 1-1-0, and 2-1-0 will be counted as producing a PCR product. (One match-mismatch setting does not appear in the figure for technical reasons – see Appendix I for details.)

This scoring scheme can be set separately for target and non-target sequences. This useful feature gives a user the flexibility to define different stringency requirements for primer annealing within these two classes of sequences.

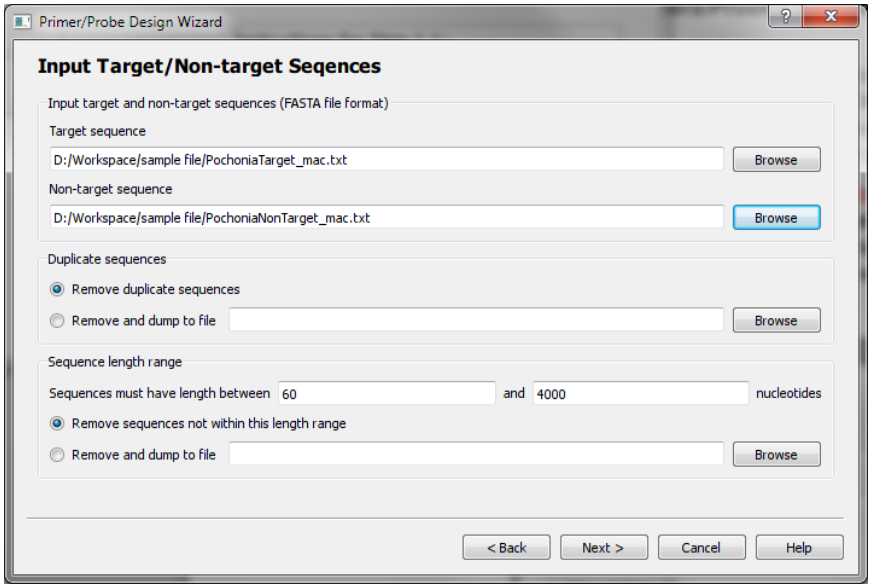
4.1 Using the module

I. Loading the Sequences

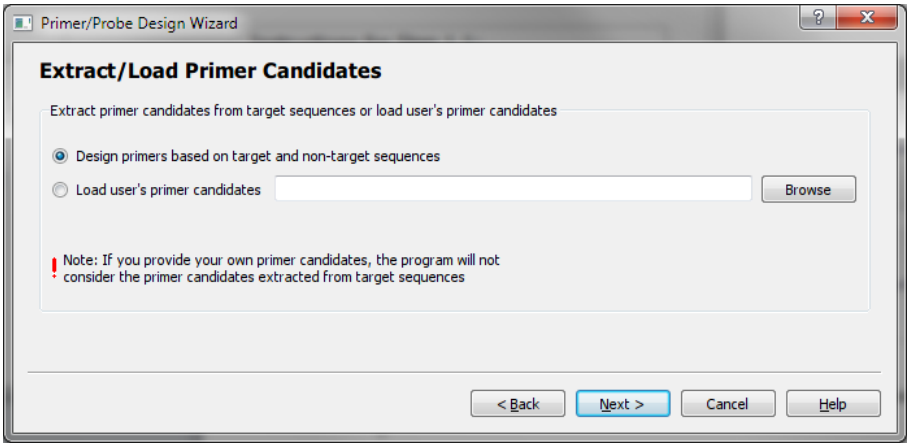
After opening the ***Design Primers/Probes*** module, the Primer/Probe Design Wizard will help users to go through this step.

First is the *Load or Design New List* page, where users can load a previously created primer list file or initiate a new primer design project. Next is the *Input Target/Non-Target Sequences* page. On this page, users can load the target and non-target sequence files and select options to remove duplicate sequences and those that do not meet user-selected size criteria.

Note that size selection could have a dramatic impact on the quality of the primers produced**.** For example, if one included sequences with a large size range, a primer could be scored as not being present in a given sequence, only because that sequence was relatively short, and therefore did not contain the region that the primer was targeting.



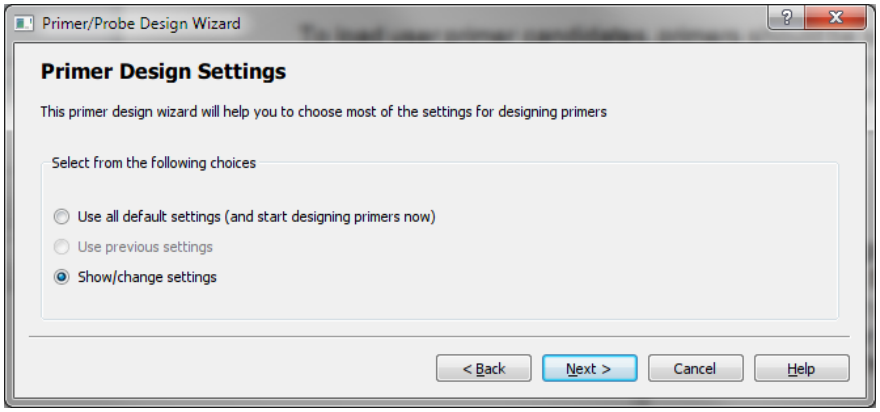
The next page is the *Extract/Load Primer Candidates* page, where users can choose from (i) **Design primers based on the target and non-target sequences** (and user defined primer criteria) or (ii) **Load user primer candidates** to assess their properties in relation to the target and non-target sequences and user-defined primer criteria.



To load user primer candidates, primers should be saved as text files in the following format. The sequences of the primers are written 5’ to 3’ (left to right), with the forward primer placed before the reverse primer, and the primer sequences separated by two periods (not spaces). When multiple primer pairs are analyzed, they need to be written on separate lines.

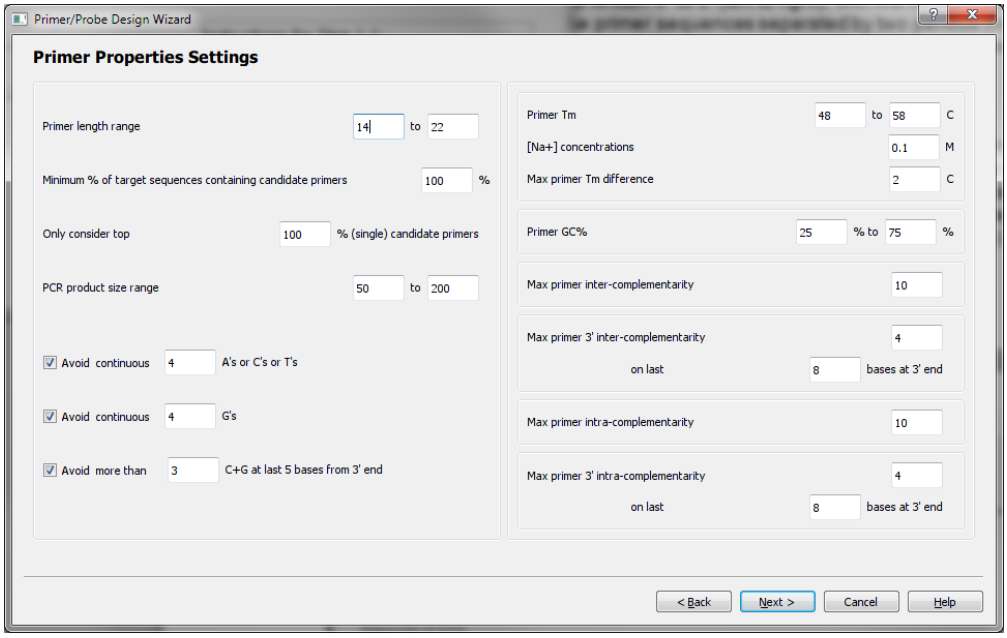
II. Primer Property Settings

In the next page, titled *Primer/Probe Design Settings*, the user can select (i) **Use all default settings**, (ii) **Use previous settings**, or (iii) **Show/change settings**.



The last option allows users to review and change the current used primer settings; it opens the Primer Properties Settings window, showing primer properties such as primer length, PCR product size, GC content and melting temperature. The melting temperature (Tm) is calculated with the following formula:

$T_m = 81.5 + 16.6 \log [Na^+] + 41(G + C)/length - 500/length$



III. *Primer Selectivity Settings*

The primer selectivity settings are located in the next two windows. These two successive windows are ordered by increasing user complexity and control.

The purpose of the selectivity settings is to identify highly selective primers, those that will bind to most target sequences but to as few as possible non-target sequences. In these settings the user defines what constitutes a match between a primer and a sequence. These settings can be defined separately for target and for non-target sequences. Roughly, stringent (high) settings correspond to nearly perfect matches, while more flexible settings (low) represent inexact matches. The more stringent the settings, the more likely the primer is to bind at a position where a match occurs. At the same time, more stringent settings result in fewer sequences matching the primer. Thus an ideal primer would be such that it

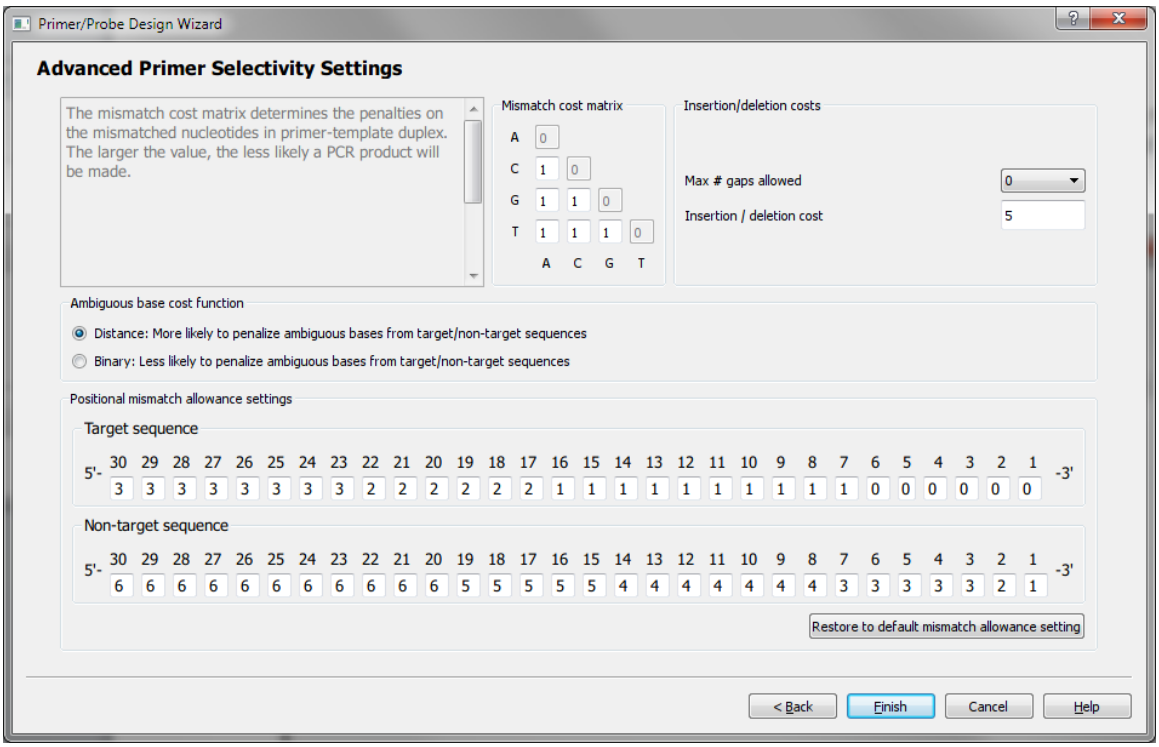
- Matches most of target sequences with respect to very stringent settings,
- Matches very few non-target sequences with respect to very flexible settings.

However, good judgment needs to be exercised when choosing the settings, as using too high settings for target sequences and too low settings for non-target sequences can actually result in filtering out highly selective primers. This can happen, for example, if there is a primer that binds to all target sequences in spite of a single-base mismatch at the 5' end of the primer, but the settings for target sequences require a perfect match.

In the *Basic Primer Selectivity Settings* page, the user can select to either use the default settings or adjust the scoring scheme (described above and in Appendix I) for both target and non-target sequences. This window allows users to set the selectivity settings for two separate regions of the primers: the last three 3' nucleotides and the other nucleotides.

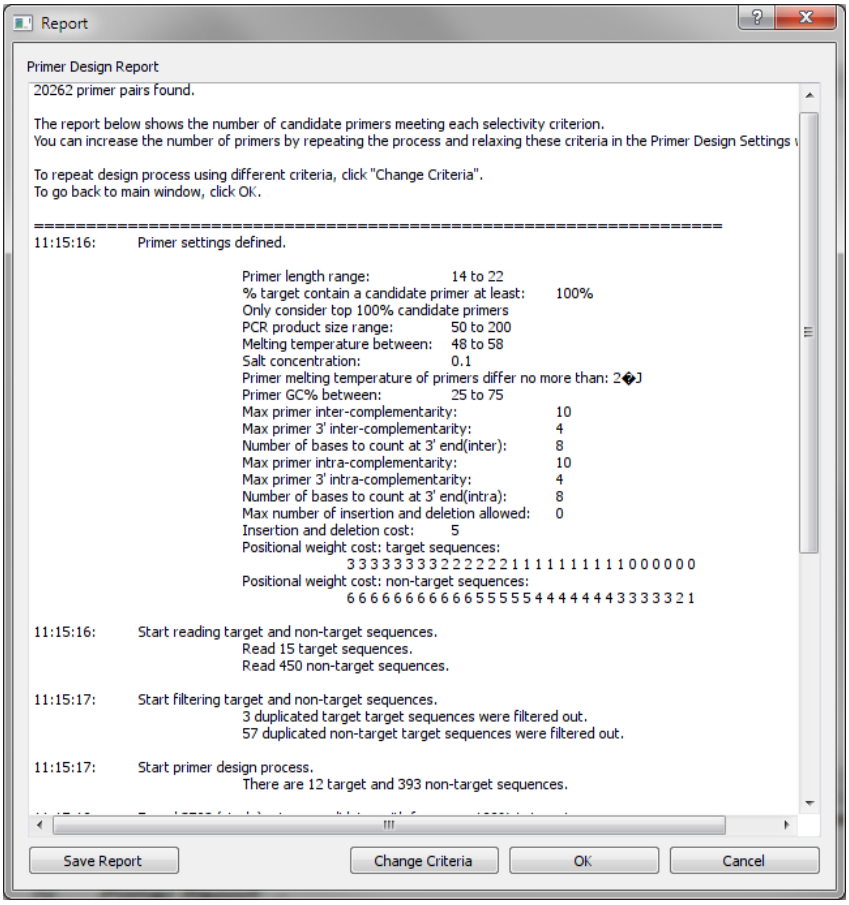
As explained earlier, theoretically, highly selective primers should be obtained when both target settings are set to *high* and both non-Target settings are set to *low*. However, when making primers from conserved sequences, such as rRNA genes, such settings may not produce PCR primers that meet these criteria. Therefore, for such analyses, we recommend using the middle (2-1-0) or the third from the bottom setting (2-1-1) for the “Base 1-3 on 3’ end” option for non-target sequences.

In the *Advanced Primer Selectivity Settings* page, the user can adjust the scoring function for ambiguous bases, mismatch cost matrix and Insertion/Deletion costs. More detailed information about the selectivity settings is listed above and in Appendix I.



The designing process could take minutes to hours, depending on the size and complexity of the sequences in the target and non-target files. After the designing process is finished, a report dialog will pop up, showing detailed information of this designing process such as how many candidates were left after each single step. This information is useful for finding which selection criteria may be too stringent, causing many primer candidates to be filtered out.

If no primer pair is found, or users are not satisfied with the found primer pairs, clicking on “Change criteria” button will allow users to change criteria and restart the designing process again. Otherwise users can continue to see the current result by clicking “OK” or go back to the main menu by clicking “Cancel”.

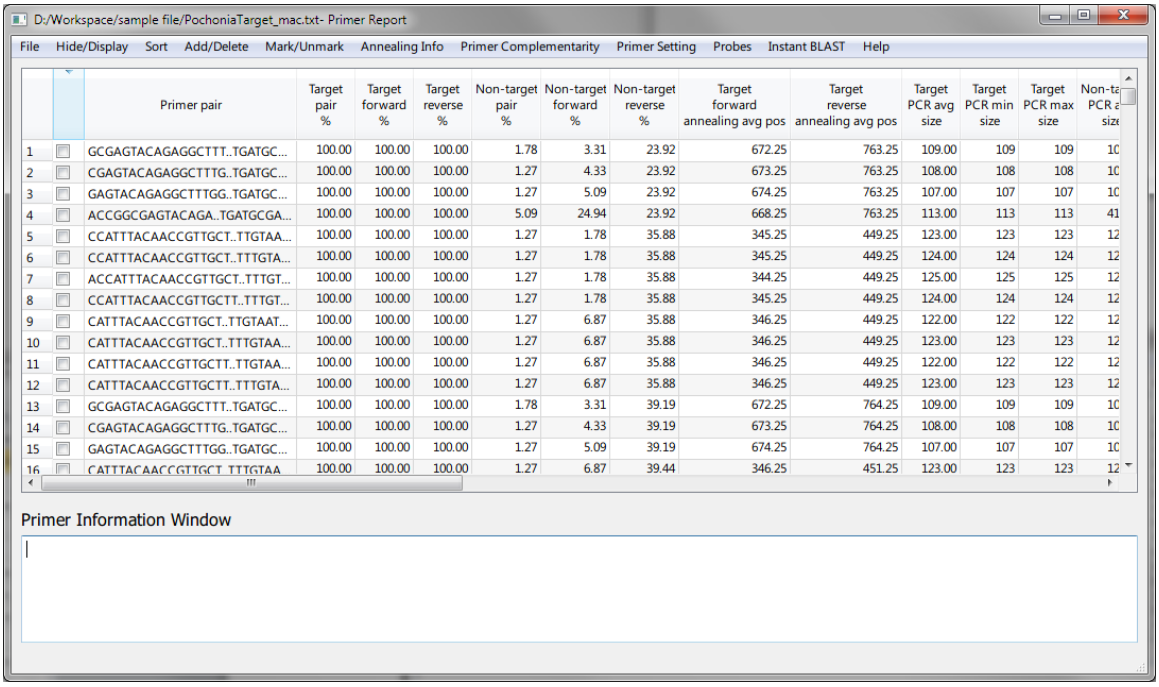

Report
Primer Design Report
20262 primer pairs found.
The report below shows the number of candidate primers meeting each selectivity criterion.
You can increase the number of primers by repeating the process and relaxing these criteria in the Primer Design Settings
To repeat design process using different criteria, click "Change Criteria".
To go back to main window, click OK.
11:15:16: Primer settings defined.
Primer length range: 14 to 22
% target contain a candidate primer at least: 100%
Only consider top 100% candidate primers
PCR product size range: 50 to 200
Melting temperature between: 48 to 58
Salt concentration: 0.1
Primer melting temperature of primers differ no more than: 2
Primer GC% between: 25 to 75
Max primer inter-complementarity: 10
Max primer 3' inter-complementarity: 4
Number of bases to count at 3' end(inter): 8
Max primer intra-complementarity: 10
Max primer 3' intra-complementarity: 4
Number of bases to count at 3' end(intra): 8
Max number of insertion and deletion allowed: 0
Insertion and deletion cost: 5
Positional weight cost: target sequences:
3 3 3 3 3 3 3 3 2 2 2 2 2 2 2 1 1 1 1 1 1 1 1 1 1 0 0 0 0 0 0
Positional weight cost: non-target sequences:
6 6 6 6 6 6 6 6 6 6 6 5 5 5 5 5 4 4 4 4 4 4 4 3 3 3 3 3 2 1
11:15:16: Start reading target and non-target sequences.
Read 15 target sequences.
Read 450 non-target sequences.
11:15:17: Start filtering target and non-target sequences.
3 duplicated target target sequences were filtered out.
57 duplicated non-target target sequences were filtered out.
11:15:17: Start primer design process.
There are 12 target and 393 non-target sequences.
Save Report
Change Criteria
OK
Cancel

IV. *Primer Report*

After the design process is finished, a dialog titled *Display Primer List* pops up. Here users have the options of **Display all primer pair**, **Display top # primer pairs** or **Display partial primer pair list according to** user-defined conditional constraints.

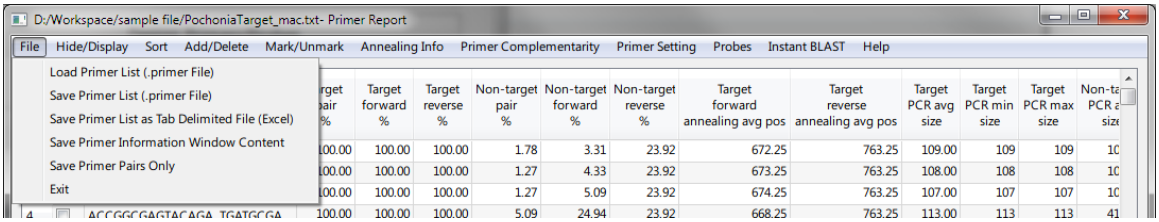
The next window shows the primer pairs. The title of this window will be the Target sequence file name followed by "*- Primer Report.*" The primer report window is a table that displays the primer pairs and their properties, including the percentage of target and non-target sequences predicted to be amplified, PCR product size, etc.

To assist the process of selecting optimal primers, the primer pairs in the table can be sorted by their parameters and by a formula that identifies primers that are most likely to amplify target but not non-target sequences (the "Selectivity Formula"). In addition, primers can be sorted by clicking on the column headings. This module also provides tools enabling the user to obtain detailed information about the selectivity of the primer pairs. These data include the percent of each nucleotide, at each position, in the target and non-target sequences in relation to the nucleotides in each position of the primers. In addition, the user can identify the target and non-target sequences that should or should not be amplified by each primer pair. He/she can also load additional primer pairs, not necessarily created by PRISE2, enabling the properties of these primers to be examined in relation to the target and non-target sequences and compared to the PRISE2-generated primers. The primers and their properties can be saved in a tab-delimited format, so that the user can import the data into other programs such as spreadsheet software.



Below is a description of all of the functions in this module, organized by the pull down menu they reside in. Note that some of the functions are also available by right clicking on a row. See the PRISE2 Tutorial for a few examples of how they can be used.

4.2 File menu



- *Load Primer List:* Allows previously created primer lists (which are PRISE2 generated and formatted files) to be uploaded into the software.

- *Save Primer List:* Allows primer lists to be saved in the format used by the PRISE2 software.

- *Save Primer List as Tab Delimited File:* Allows primer lists to be saved in a tab-delimited format, which can be used in standard spread sheet software.

- *Save Primer Information Window Content:* Saves information in the Primer Information Window as a text file.

- *Save Primer Pairs Only:* Saves primer pairs as a text file. Such files can be used for a variety of purposes, including being loaded in the Extract / Load Primer Candidates window (see above) in future experiments.

- *Exit:* Closes the Design Primer module.

4.3 Hide/Display menu

- *Display All Columns:* Allows all data columns to be viewed. This function is only needed if the user had previously hidden columns.

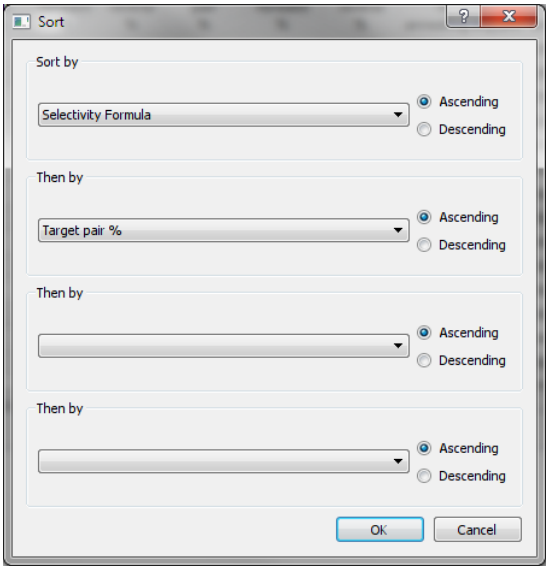
- *Hide/Display Columns:* Allows selected data columns to be hidden or displayed.

- *Hide/Display Primer Pairs:* Allows selected primers to be hidden or displayed.

4.4 Sort menu

- *Sort Primer List:* Allows the primers in the list to be sorted by a variety of user-selected criteria. One parameter that we find particularly useful is the Selectivity Formula, which is
  (100 - % of target sequences estimated to be amplified)^2
  + ½ (% of non-target sequences estimated to anneal with forward primer)^2
  + ½ (% of non-target sequences estimated to anneal with reverse primer)^2.

  The smaller the value generated by the Selectivity Formula, the more likely the primers will amplify target sequences and not amplify non-target sequences.

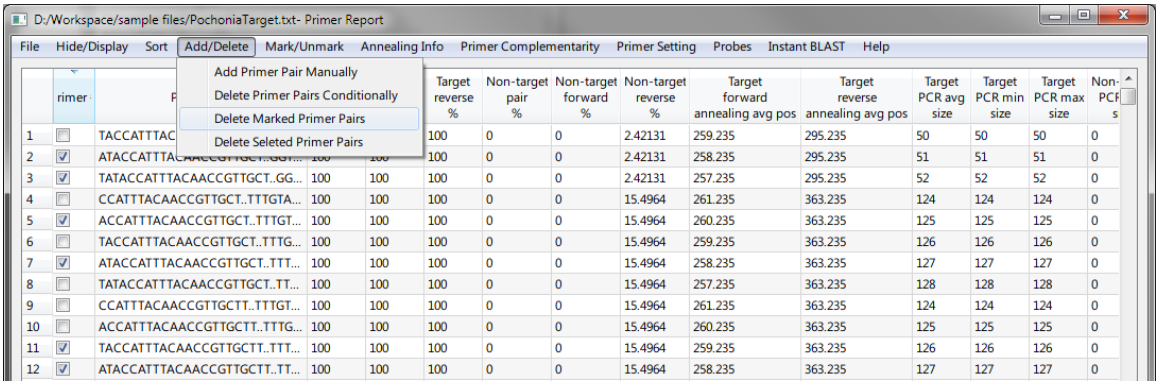


4.5 Add/Delete menu

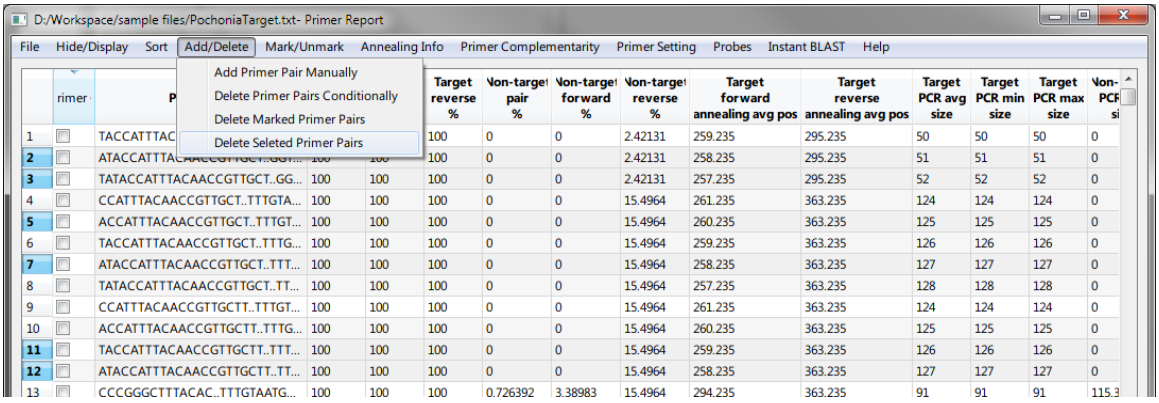
- *Add Primer Pair Manually:* Allows an individual primer pair to be added to the primer list, and its properties determined in relationship to the target and non-target sequence files and user-defined primer design settings. The primer pair must be entered in the format given earlier.

- *Delete Primer Pairs Conditionally:* Allows primer pairs to be deleted from the primer list by user-specified criteria.

- *Delete Marked Primer Pairs:* Allows marked primers to be deleted. Marked primers are designated by a check mark in the second column (and a highlighted row). Primers can be marked by clicking on the boxes in the second column or by using the Mark/Unmark functions below.



- *Delete Selected Primer Pairs:* Allows selected primers to be deleted. Selected primers are designated by their rows being highlighted in blue. Primers can be selected by clicking on any part of the row except the boxes in the second column. Standard key commands such as shift and control can be used with this function, allowing groups of primer pairs to be selected.



4.6 Mark/Unmark menu

- *Mark Selected Primer Pairs:* Allows selected primer pairs to be marked. Marked primer pairs are designated by a check mark in the box in the second column (and a yellow-highlighted row). Marked primers can be saved in the PRISE2 program format or tab-delimited format using options in the File menu.

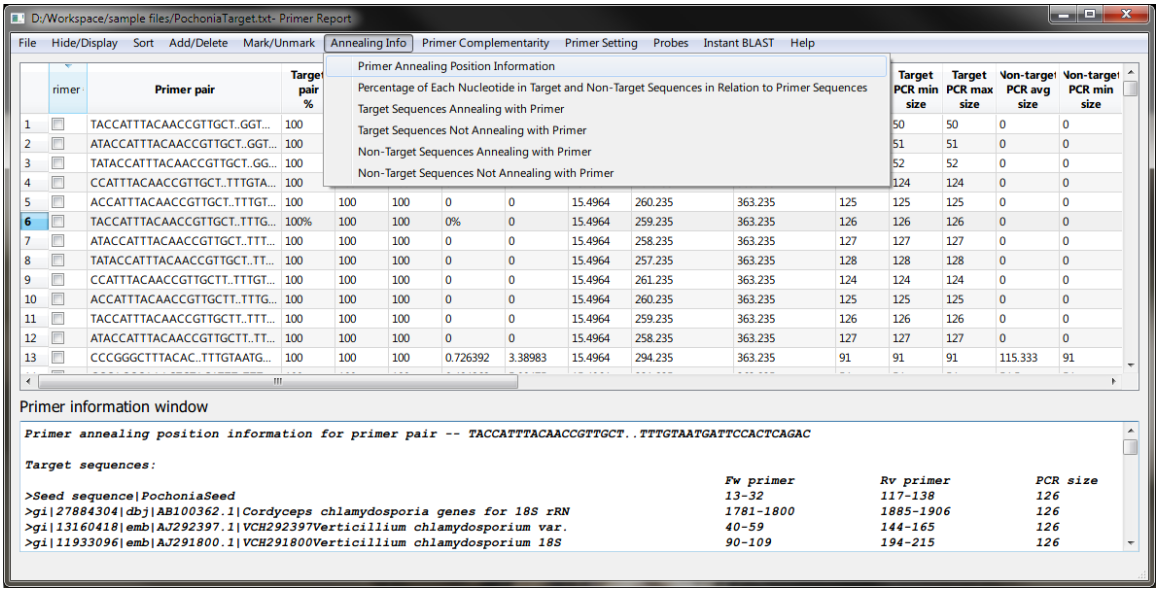
Note that selected primers are designated by their rows being highlighted in blue. Primers can be selected by clicking on any part of the row except the boxes in the second column. Standard key commands such as shift and control can be used with the selection function, allowing groups of primer pairs to be selected.

- *Unmark Selected Primer Pairs:* Allows selected primer pairs to be unmarked.

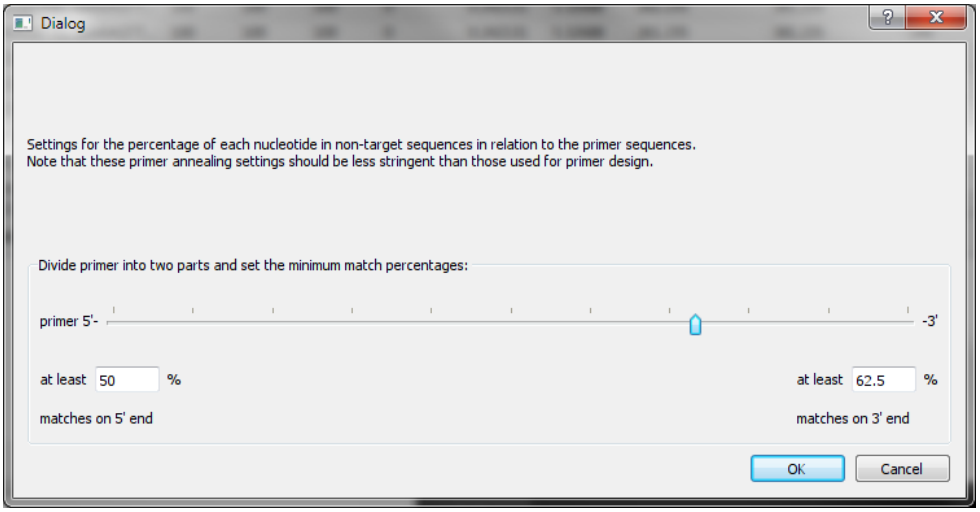
4.7 Annealing Info menu

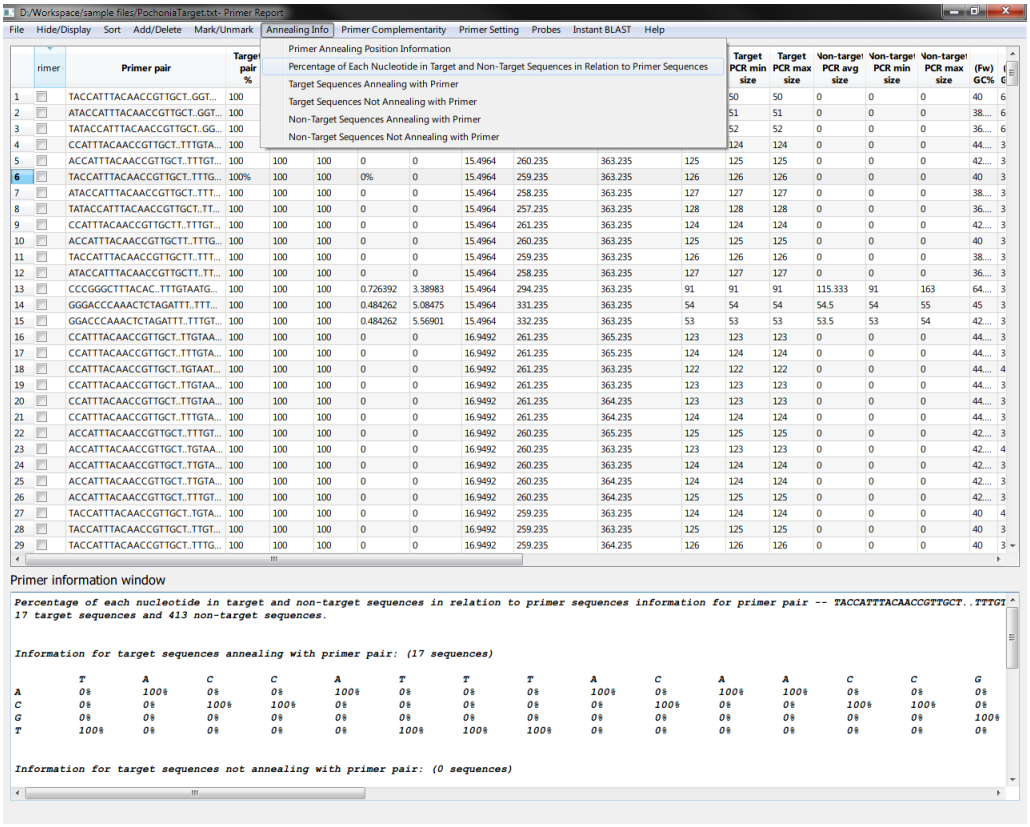
All of the functions below need to be performed on one primer pair. Before the function is performed, exactly one primer pair must be selected. Selected primers are designated by their rows being highlighted in blue. Primer pairs can be selected by clicking on any part of the row except the boxes in the second column.

- *Primer Annealing Position Information:* Provides information on where the primers anneal to the target and non-target sequence.

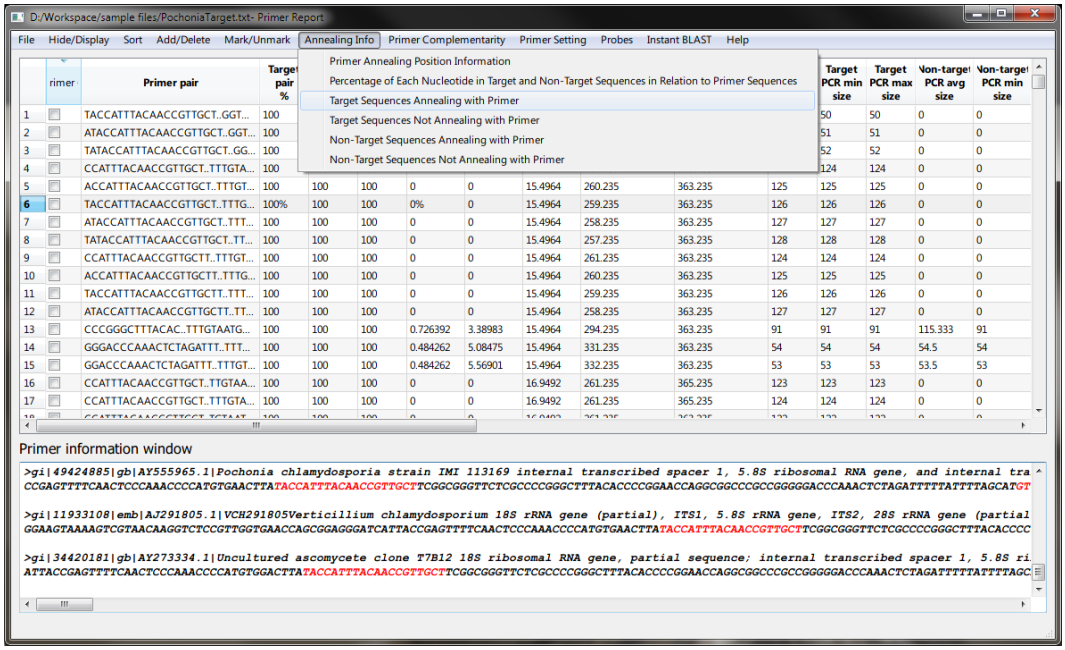


- *Percentage of Each Nucleotide in Target and Non-Target Sequences in Relation to Primer Sequences:* Provides the percentage of each nucleotide, at each position in the target and non-target sequences, in relation to the nucleotides in each position of the primers.





- *Target Sequences Annealing with Primer:* Shows the target sequences that anneal to the primer, using the user-selected primer design criteria.

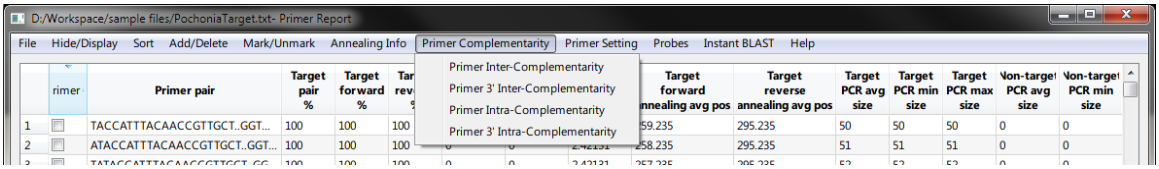


- *Target Sequences Not Annealing with Primer:* Shows the target sequences that do not anneal to the primer, using the user-selected primer design criteria.

- *Non-Target Sequences Annealing with Primer:* Shows the non-target sequences that anneal to the primer, using the user-selected primer design criteria.

- *Non-Target Sequences Not Annealing with Primer:* Shows the non-target sequences that do not anneal to the primer, using the user-selected primer design criteria.

4.8 Primer Complementarity menu



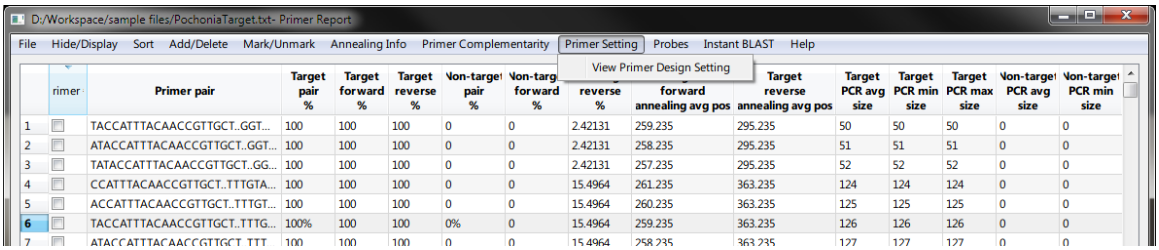
- *Primer Inter-complementarity:* Provides information on the inter-complementarity of the entire primer.

- *Primer 3' Inter-complementarity:* Provides information on the inter-complementarity of the last eight 3' primer nucleotides. Note that this value can be customized in the Standard Primer Property Settings window.

- *Primer Intra-complementarity:* Provides information on the intra-complementarity of the entire primer.

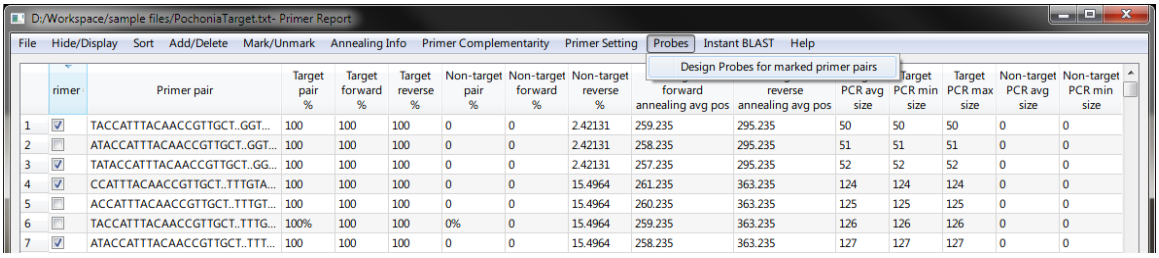
- *Primer 3' Intra-complementarity:* Provides information on the intra-complementarity of the last eight 3' primer nucleotides. Note that this value can be customized in the Standard Primer Property Settings window.

4.9 Primer Setting menu



- *View Primer Design Setting:* Show all settings used for current primer list, but users will not be able to change the settings at this time.

4.10 Probe menu



	rimer	Primer pair	Target pair %	Target forward %	Target reverse %	Non-target pair %	Non-target forward %	Non-target reverse %	forward annealing avg pos	reverse annealing avg pos	PCR avg size	Target PCR min size	Target PCR max size	Non-target PCR avg size	Non-target PCR min size
1	☑	TACCATTTACAACCGTTGCT..GGT...	100	100	100	0	0	2.42131	259.235	295.235	50	50	50	0	0
2	☐	ATACCATTTACAACCGTTGCT..GGT...	100	100	100	0	0	2.42131	258.235	295.235	51	51	51	0	0
3	☑	TATACCATTTACAACCGTTGCT..GG...	100	100	100	0	0	2.42131	257.235	295.235	52	52	52	0	0
4	☑	CCATTTACAACCGTTGCT..TTTGTA...	100	100	100	0	0	15.4964	261.235	363.235	124	124	124	0	0
5	☐	ACCATTTACAACCGTTGCT..TTTGT...	100	100	100	0	0	15.4964	260.235	363.235	125	125	125	0	0
6	☐	TACCATTTACAACCGTTGCT..TTTG...	100%	100	100	0%	0	15.4964	259.235	363.235	126	126	126	0	0
7	☑	ATACCATTTACAACCGTTGCT..TTT...	100	100	100	0	0	15.4964	258.235	363.235	127	127	127	0	0

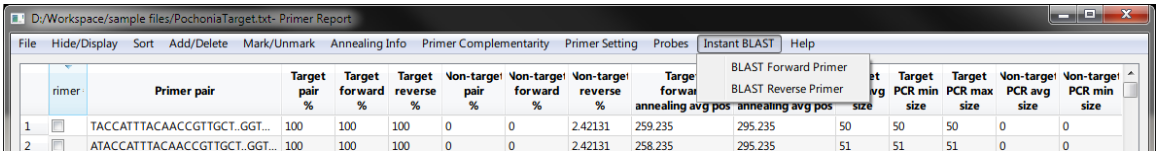
- *Design Probes for marked primer pairs:* To design probes for selected primer pairs (for TaqMan type assays, for example), users can mark some primer pairs and then continue to design probes for these primer pairs. The intention is that all three sequences (two primers and one probe) should bind to same target sequences. We note that probes can also be designed for hybridization-based assays such as FISH, by simply ignoring the primers from the primer-probe sets.

After clicking this option, a wizard will pop up to help users to generate probes. The settings and the designing process are very similar to those for primer pairs. There are two differences, however:

1. Nucleotide mismatches in probes are more destabilizing in the middle than the ends. So the selectivity setting process is different. For probes, we do alignment from the center of probe toward both ends.

See the next section for more details.

4.11 Instant BLAST menu



- *Blast Forward Primer:* Allows a single forward primer to be subjected to a BLAST analysis, by opening the BLAST page at NCBI and loading the primer. Note that this function only works when one primer pair is selected.

- *Blast Reverse Primer:* Allows a single reverse primer to be subjected to a BLAST analysis, by opening the BLAST page at NCBI and loading the primer. Note that this function only works when one primer pair is selected.

4.12 Help menu

- *PRISE2 Manual:* Opens this PRISE2 Manual.

- *PRISE2 Tutorial:* Opens the PRISE2 Tutorial, which provides a step-by-step protocol showing how the software was used to create sequence-selective PCR primers or primer-probe sets for a specific fungal rRNA gene.

5. Step 2: Design Primers/Probes (Choosing Probes)

After choosing the desired primer pairs, the user can select probes for each primer pair. The three sequences: the forward primer, the reverse primer, and the probe are referred to in the program as a *primer-probe set*. While designing probes, similar as in the primer design process, PRISE2 allows the user to select a number of parameters, such as the length of gaps between the primers and the probe, the GC content, the probe length, complementarity properties, and other. In the current version of the program, designing probes for FISH analyses requires primers to be designed first, even though they will not be used.

The criteria for probe selectivity are quite different than those for the primers. For example, for probes, the nucleotide mismatches near the center of the probe are more destabilizing than near the ends. Thus in the probe design wizard, users can specify the threshold value for the number of matches in both directions from the center of the probe that are required for the probe to be considered to match the template (either a target or a non-target sequence).

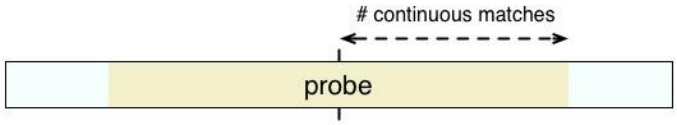


Figure 3: Illustration of selectivity setting for probes. The shaded part shows is where the exact match is required to occur.

Figure 3 illustrates this feature. The larger the number of required continuous matching bases, the fewer template sequences will be considered to match by the probe. In the default setting, these numbers are set to the probe length for target sequences and to a small value for non-target sequences. With this setting the program will look for probes that bind to target sequences perfectly, while minimizing the likelihood of it binding to non-target sequences. If no probes are found to meet such stringent criteria, the user can relax them by lowering the threshold for the matches for target sequences and/or increase the threshold for non-target sequences.

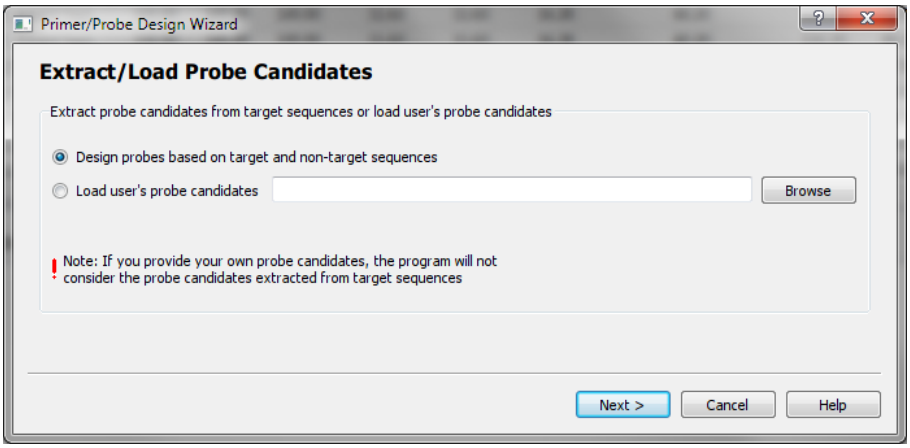
The remainder of this chapter explains the probe design process in more detail.

5.1 Using the module

I. Loading the Sequences

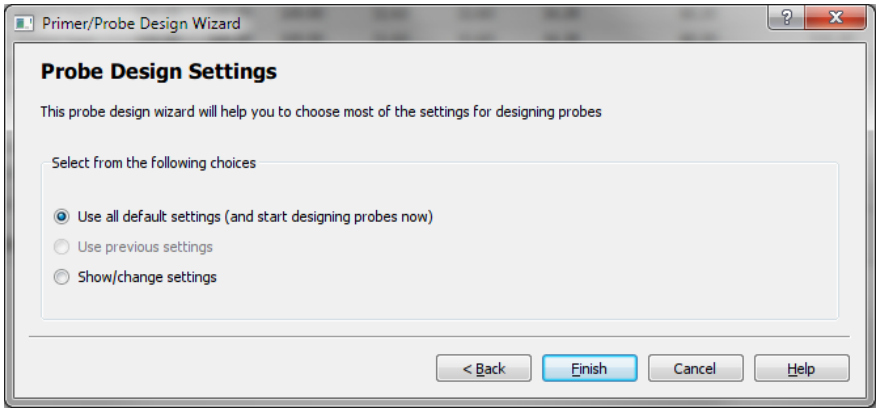
After marking the desired primer pairs and clicking on the "*Design Probes for Marked Primer Pairs",* a wizard window similar to that for the primer design process will appear.

The first page is titled *Extract/Load Probe Candidates*. Here, users can choose from (i) **Design probes based on the target and non-target sequences** or (ii) **Load user's probe candidates** to assess their properties in relation to the target and non-target sequences and user-defined probe criteria.

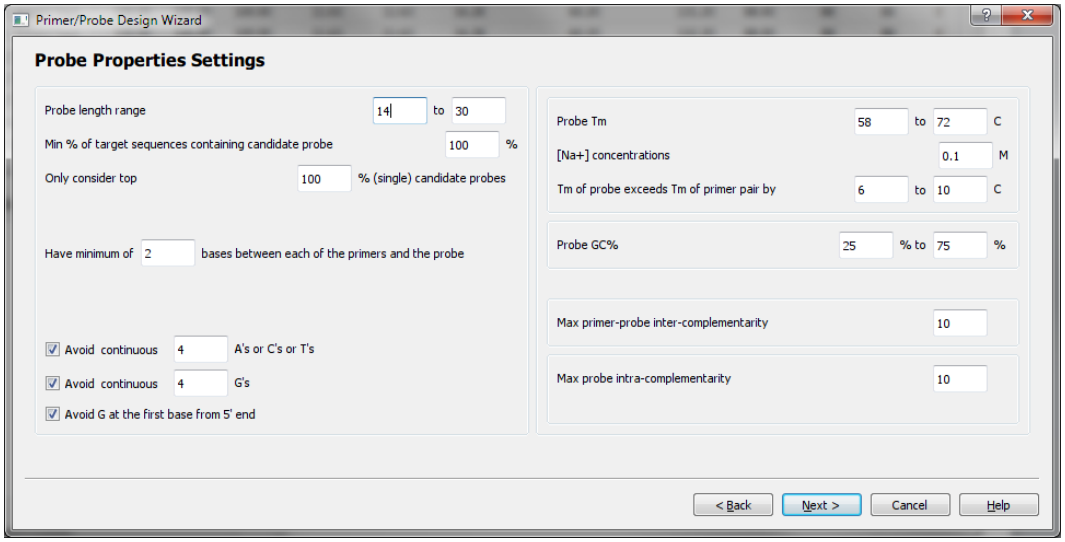


II. Probe Property Settings

In the next page, titled *Probe Design Settings*, the user can select (i) **Use all default settings**, (ii) **Use previous settings**, or (iii) **Show/change settings**.



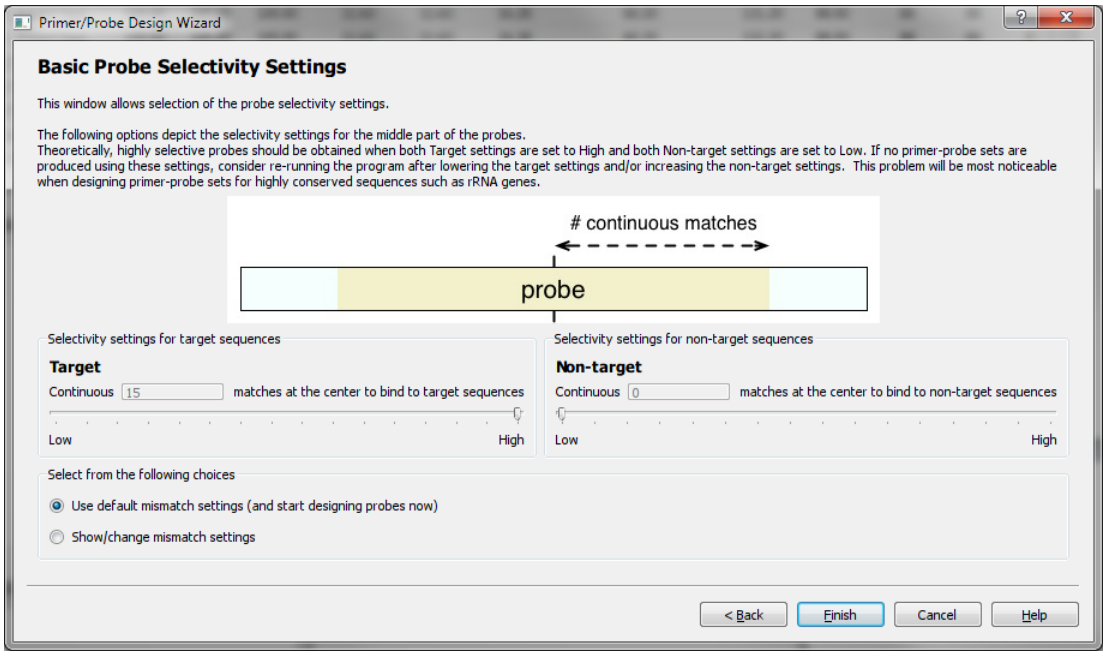
The last option allows users to review and change the current used probe settings; it opens the Probe Properties Settings window, showing various probe properties such as probe length, gap between the probe and the primers' binding positions, Tm range, Tm difference (between the primers and the probe), and complementary.



III. *Probe Selectivity Settings*

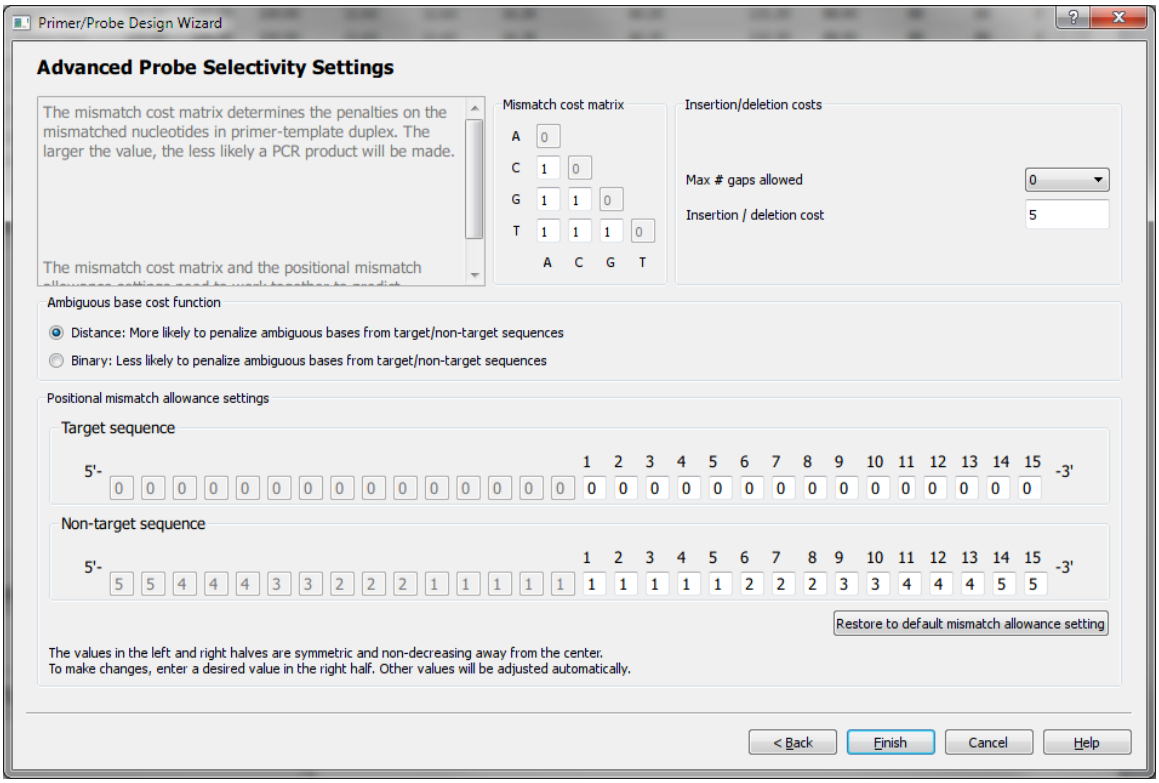
The probe selectivity settings are located in the next two windows. These two successive windows are ordered by increasing user complexity and control.

In the *Basic Probe Selectivity Settings* page, users can select to either use the default settings or adjust the binding criteria (described earlier) for both target and non-target sequences.



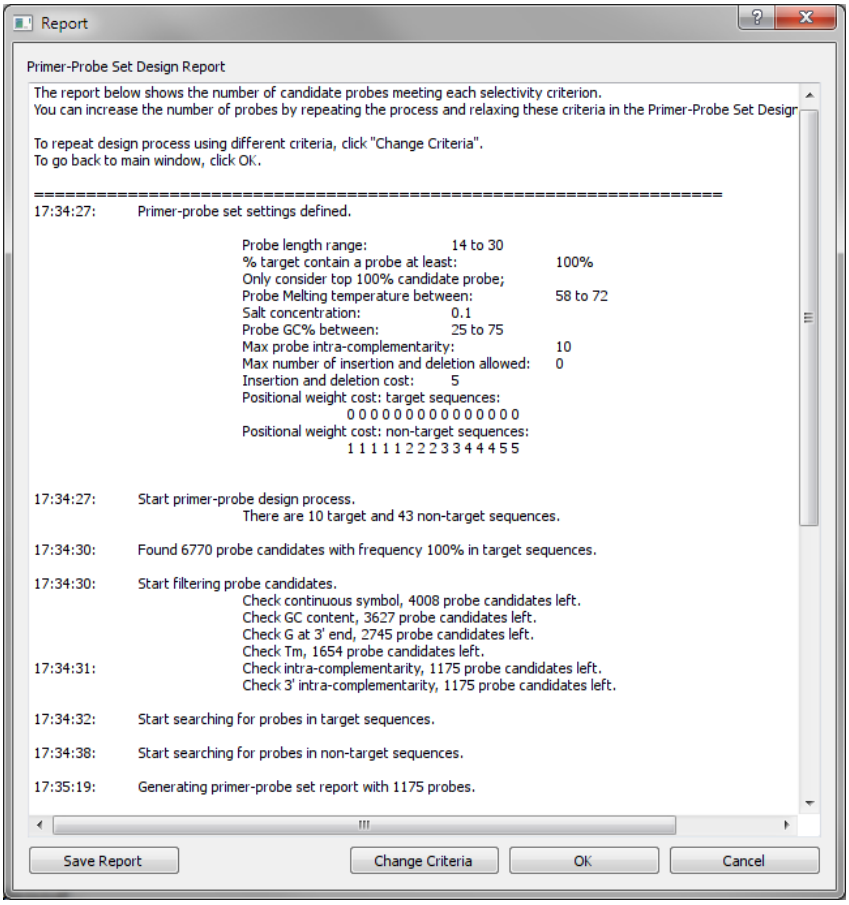
Theoretically, highly selective probes should be obtained when target setting is *high* and non-Target setting is *low*. If no primer-probe sets are found, these criteria can be relaxed to increase the likelihood of finding primer-probe sets.

In the *Advanced Probe Selectivity Settings* page, the user can adjust the scoring function for ambiguous bases, mismatch cost matrix and Insertion/Deletion costs. These features are similar to those for the primers, except that for probes the compound mismatch values are counted starting from the center, with the left and right directions symmetric (so the changes are only allowed on the left-hand side; the right-hand side will be adjusted automatically). In the default setting shown below, the probe is considered to match the target sequence if all its bases match perfectly those in the target sequence. To match a non-target sequence, one mismatch is allowed in the first 5 bases to the right of the center, two mismatches in the first 8 bases to the right from the center, and so on, and symmetrically on the left-hand side.



The designing process could take minutes to hours, depending on the size and complexity of the sequences in the target and non-target files. After the designing process is finished, a report dialog will pop up, showing detailed information of this designing process such as how many candidate probes were left after each single step. This information is useful for finding which selection criteria may be too stringent, causing many probe candidates to be filtered out.

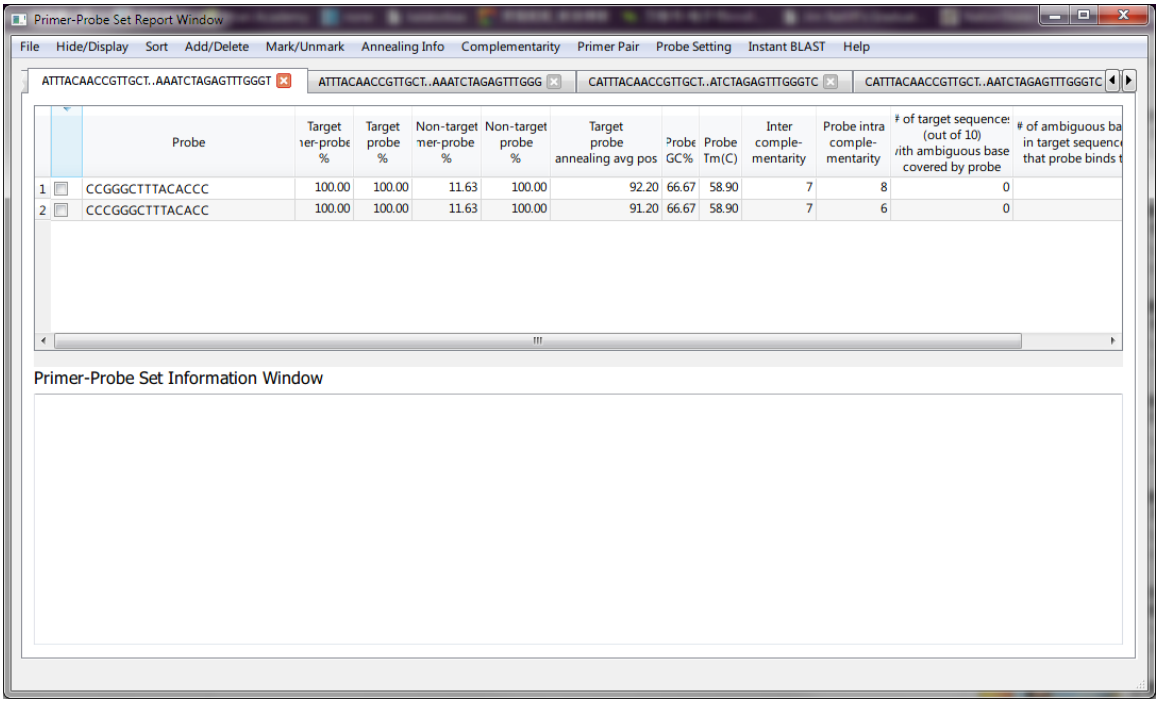
If no probes are found, or if the user is not satisfied with the found probes, clicking on “Change criteria” button will allow users to change criteria and restart the designing process again. Otherwise users can continue to see the current result by clicking “OK” or go back to Primer Report Window by clicking “Cancel”.



IV. *Probe Report*

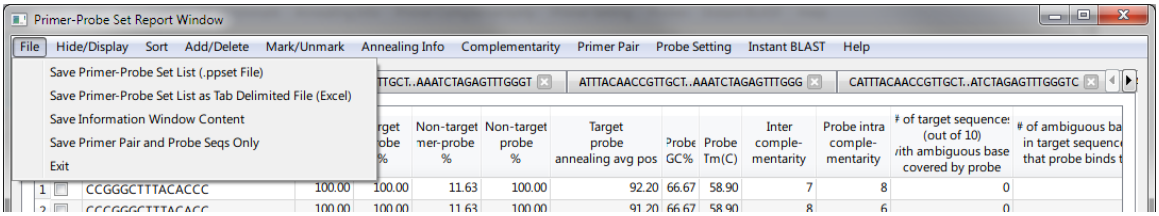
After clicking on OK, the next window shows the primer-probe sets. The title of this window will be "*Primer-Probe Set Report Window.*" This report window lists primer pair sequences in the tabs near the top of the window. For each tab, the table below displays the corresponding probes and the properties of the whole primer-probe set, including the percentage of target and non-target sequences predicted to be amplified, PCR product size, etc.

To assist the process of selecting optimal probes, the probes in the table can be sorted by their parameters and by a formula that identifies probes that are most likely to amplify target but not non-target sequences (the "Selectivity Formula"). In addition, probes can be sorted by clicking on the column headings. This module also provides tools enabling the user to obtain detailed information about the primer-probe sets. These data include the percent of each nucleotide, at each position, in the target and non-target sequences in relation to the nucleotides in each position of the probes. In addition, the user can identify the target and non-target sequences that should or should not be amplified by each probe. He/she can also load additional probes, not necessarily created by PRISE2, enabling the properties of these probes to be examined in relation to the target and non-target sequences and compared to the PRISE2-generated probes. The probes and their properties can be saved in a tab-delimited format, so that the user can import the data into other programs such as spreadsheet software.



Below is a description of all of the functions in this module, organized by the pull down menu they reside in. Note that some of the functions are also available by right clicking on a row.

5.2 File menu



- *Save Primer-Probe Set List:* Allows primer-probe set lists to be saved in the format used by the PRISE2 software.

- *Save Primer-Probe Set List as Tab Delimited File:* Allows primer-probe set lists to be saved in a tab-delimited format, which can be used in standard spread sheet software.

- *Save Information Window Content:* Saves information in the Primer-Probe Set Information Window as a text file.

- *Save Primer Pair and Probe Seqs Only:* Saves primer-probe sets as a text file. Such files can be used for a variety of purposes, including being loaded in the Extract / Load Probe Candidates window (see above) in future experiments.

- *Exit:* Closes the Design Probe module.

5.3 Hide/Display menu

- *Display All Columns:* Allows all data columns to be viewed. This function is only needed if the user had previously hidden columns.

- *Hide/Display Columns:* Allows selected data columns to be hidden or displayed.

- *Hide/Display Primer-Probe Sets:* Allows selected sets to be hidden or displayed.

5.4 Sort menu

- *Sort Primer-Probe Set List:* Allows the probes in the list to be sorted by a variety of user-selected criteria. One parameter that we find particularly useful is the Selectivity Formula, which is

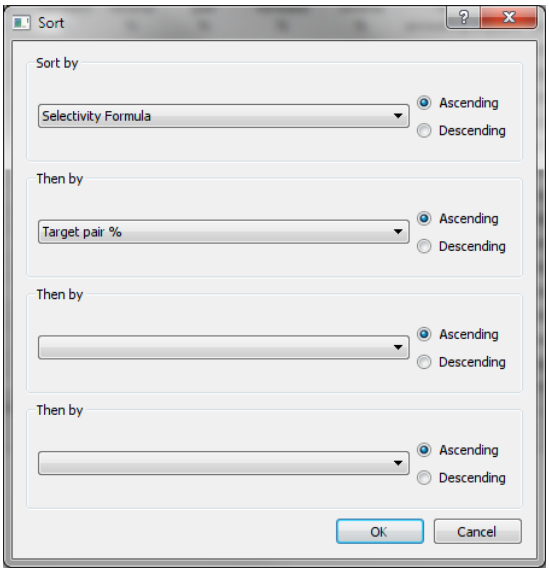
  (100 - % of target sequences estimated to anneal with whole primer-probe set)^2

  +(% of non-target sequences estimated to anneal with whole primer-probe set)^2

  + ½ (100 - % of non-target sequences estimated to anneal with probe)^2

  + 0.25 (% of non-target sequences estimated to anneal with probe)^2.

  The smaller the value generated by the Selectivity Formula, the more likely the primer-probe set will amplify target sequences and not amplify non-target sequences.

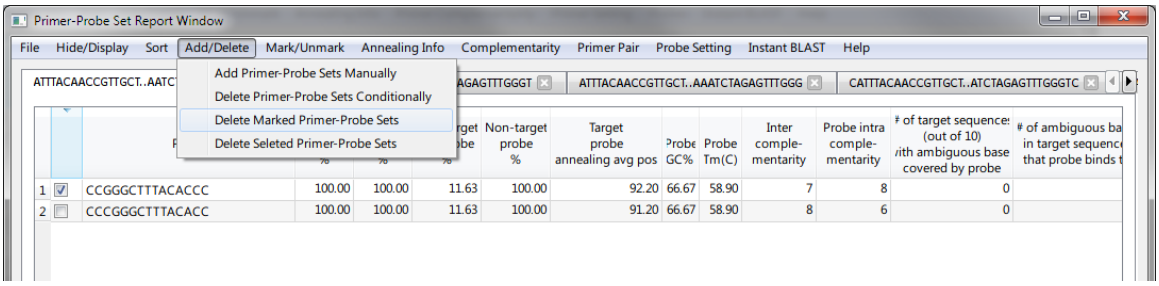


5.5 Add/Delete menu

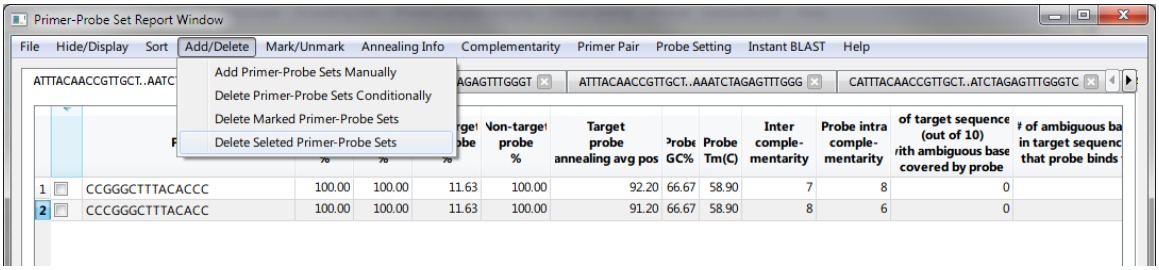
- *Add Primer-Probe Sets Manually:* Allows an individual probe to be added to the list of probes for the selected primer pair, and its properties determined in relationship to the target and non-target sequence and user-defined primer-probe set design settings.

- *Delete Primer-Probe Sets Conditionally:* Allows primer-probe sets to be deleted from the list by user-specified criteria.

- *Delete Marked Primer-Probe Sets:* Allows marked primer-probe set to be deleted. Marked sets are designated by a check mark in the second column (and a highlighted row). Primer-probe sets can be marked by clicking on the boxes in the second column or by using the Mark/Unmark functions below.



- *Delete Selected Primer-Probe Sets:* Allows selected primer-probe sets to be deleted. Selected sets are designated by their rows being highlighted in blue. Primer-probe sets can be selected by clicking on any part of the row except the boxes in the second column. Standard key commands such as shift and control can be used with this function, allowing groups of primer-probe sets to be selected.



5.6 Mark/Unmark menu

- *Mark Selected Primer-probe Sets:* Allows selected sets to be marked. Marked primer-probe sets are designated by a check mark in the box in the second column (and a yellow-highlighted row). Marked sets can be saved in the PRISE2 program format or tab-delimited format using options in the File menu.

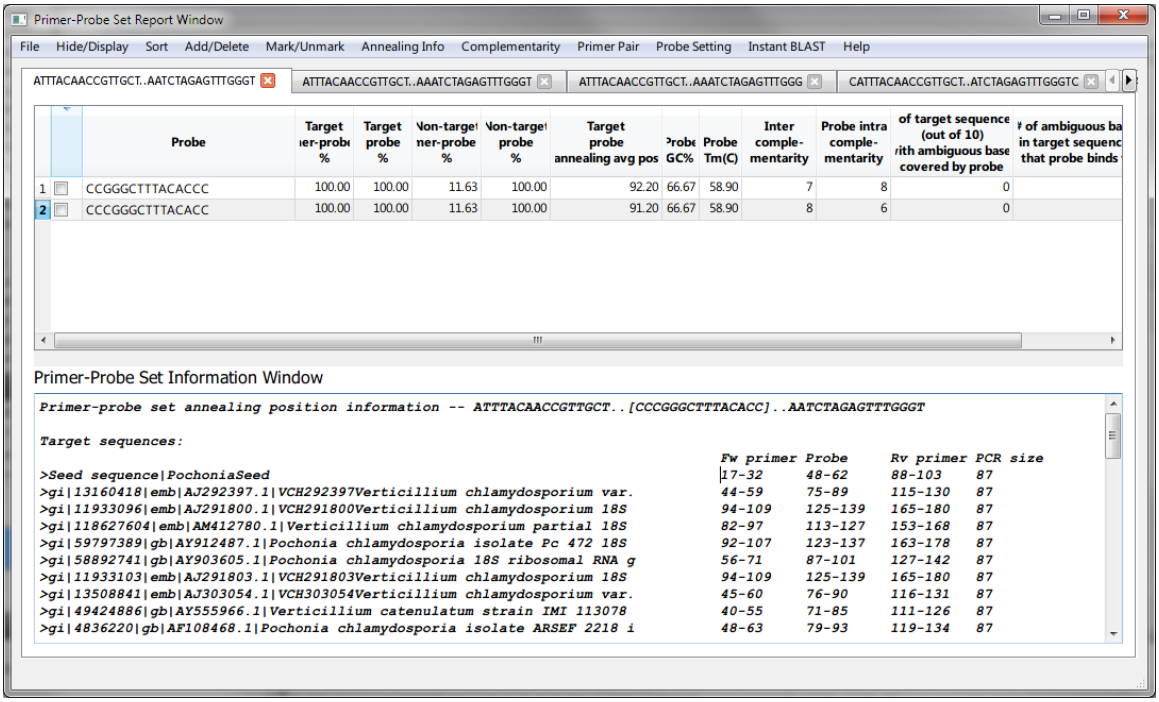
Note that selected primer-probe sets are designated by their rows being highlighted in blue. Primer-probe sets can be selected by clicking on any part of the row except the boxes in the second column. Standard key commands such as shift and control can be used with the selection function, allowing groups of primer-probe sets to be selected.

- *Unmark Selected Primer Pairs:* Allows selected primer-probe sets to be unmarked.

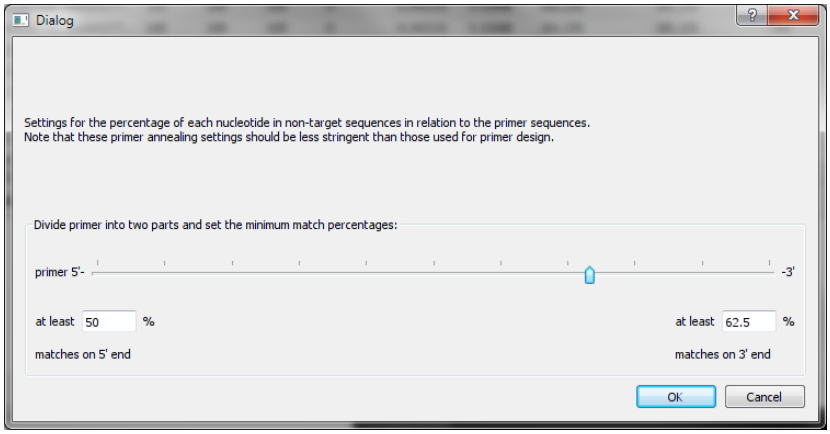
5.7 Annealing Info menu

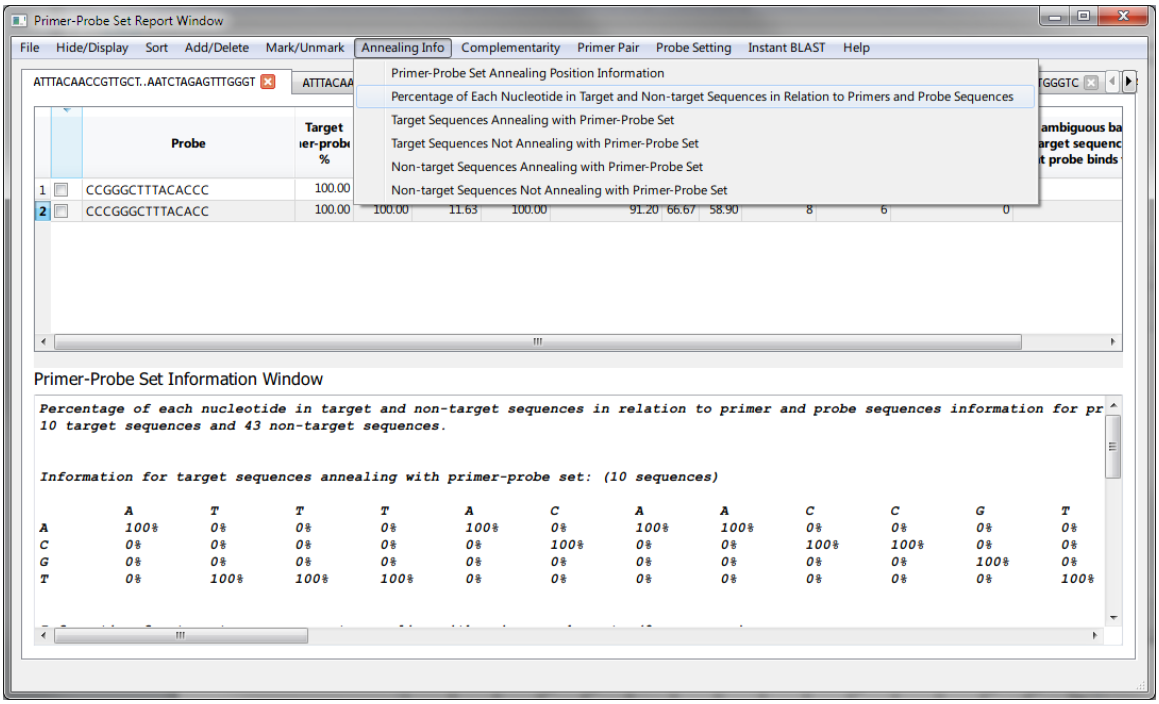
All of the functions below need to be performed on one set. Before the function is performed, exactly one set must be selected, by choosing the tab with the primer pair and selecting one probe in the table. Selected probes are designated by their rows being highlighted in blue. Probes can be selected by clicking on any part of the row except the boxes in the second column.

- *Primer-Probe Set Annealing Position Information:* Provides information on where the primer-probe set anneal to the target and non-target sequence.

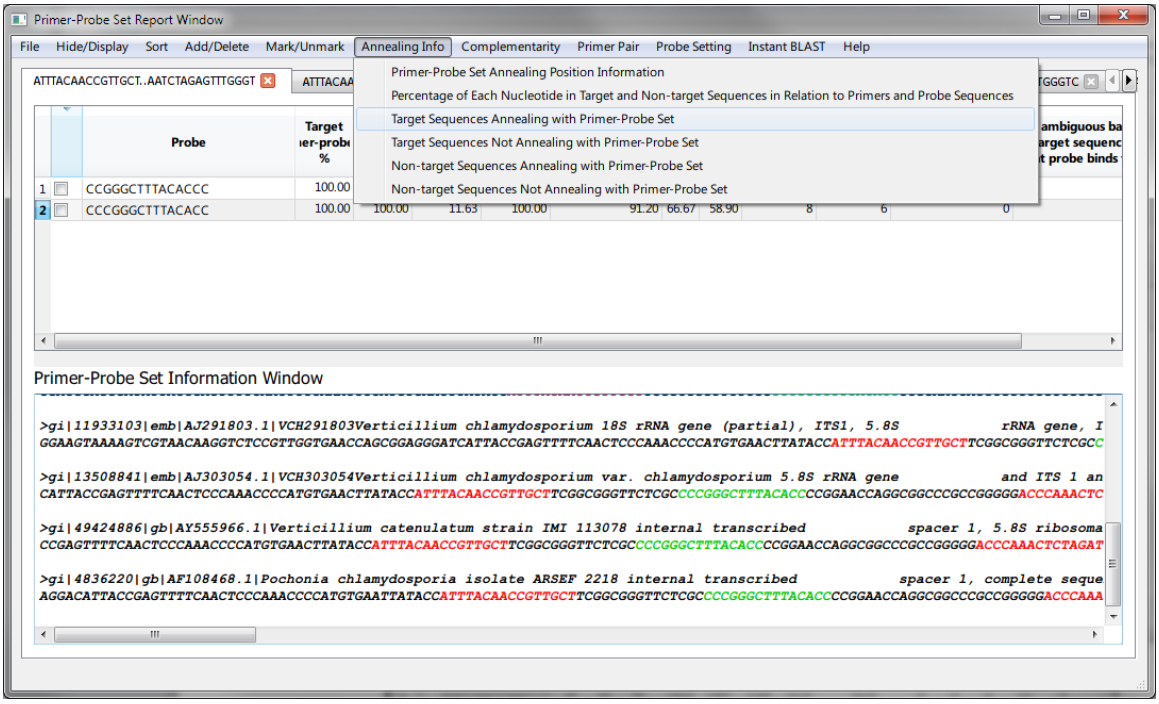


- *Percentage of Each Nucleotide in Target and Non-Target Sequences in Relation to Primers and Probe Sequences:* Provides the percentage of each nucleotide, at each position in the target and non-target sequences, in relation to the nucleotides in each position of the primers and probe.





- *Target Sequences Annealing with Primer-Probe Set:* Shows the target sequences that anneal to the whole primer-probe set, using the user-selected design criteria.

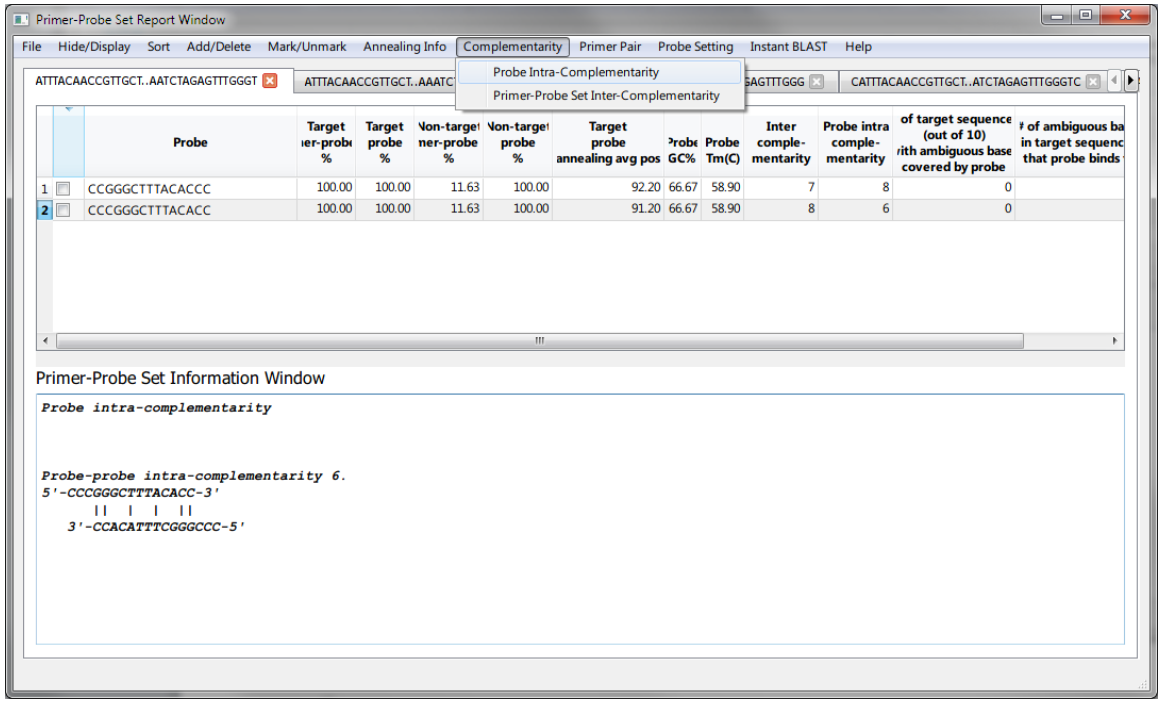


- *Target Sequences Not Annealing with Primer-Probe Set:* Shows the target sequences that do not anneal to the whole primer-probe set, using the user-selected design criteria.

- *Non-Target Sequences Annealing with Primer-Probe Set:* Shows the non-target sequences that anneal to the whole primer-probe set, using the user-selected design criteria.

- *Non-Target Sequences Not Annealing with Primer-Probe Set:* Shows the non-target sequences that do not anneal to the whole primer-probe set, using the user-selected design criteria.

5.8 Complementarity menu

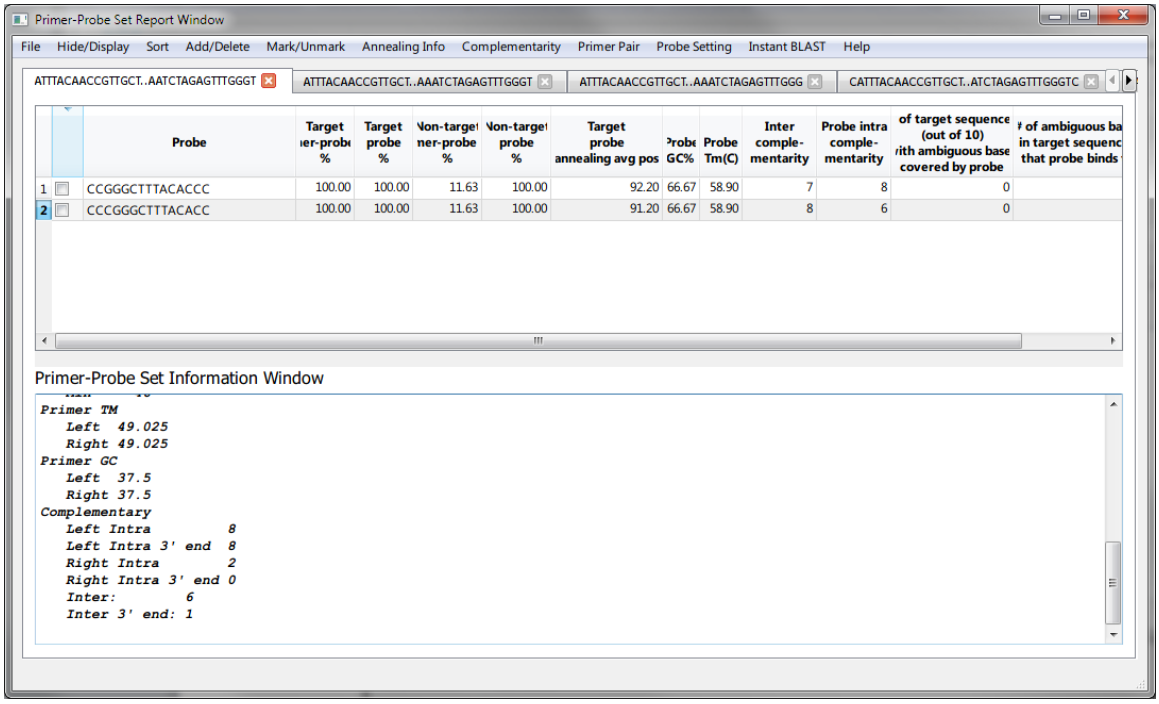


- *Probe Intra-complementarity:* Provides information on the intra-complementarity of the probe.

- *Primer-Probe Set Inter-complementarity:* Provides information on the inter-complementarity of the primers and probe.

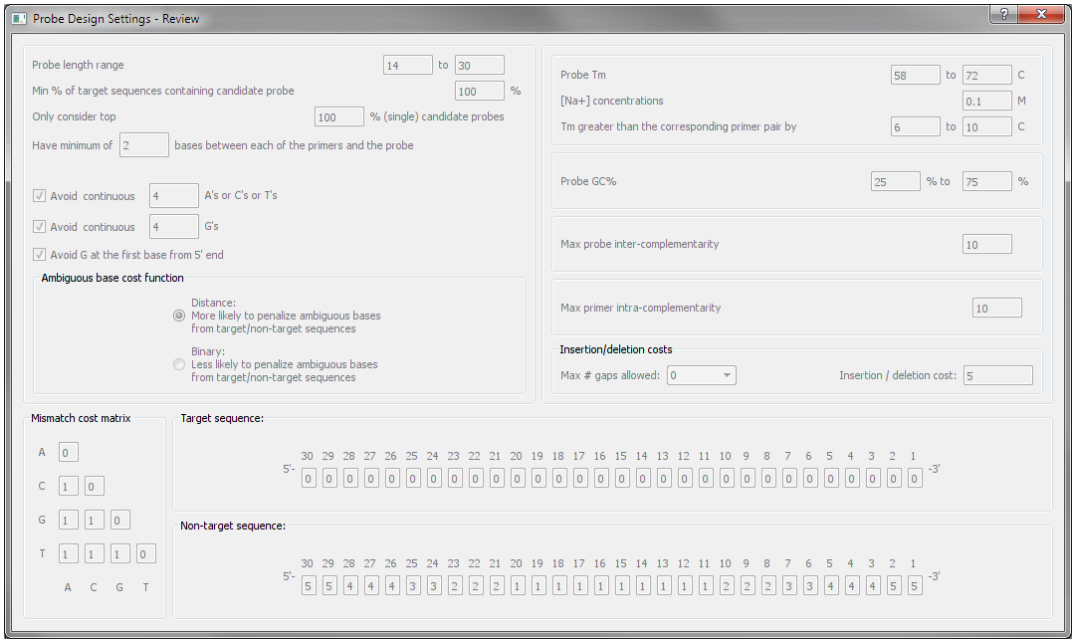
5.9 Primer Pair menu

- *Show Primer Pair Info:* Shows detailed information about the current primer pair.

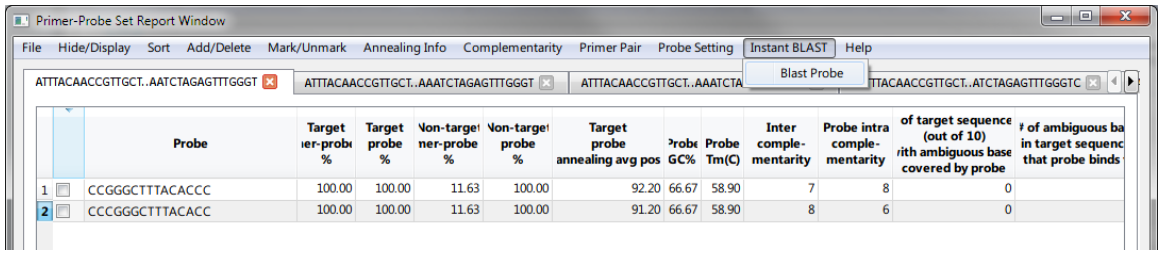


5.10 Probe Setting menu

- *View Probe Design Setting:* Show all settings used for computing the current collection of probes, but users will not be able to change the settings at this time. (To change these settings, the user needs to exit the window and redo the probe design process.)



5.11 Instant BLAST menu



- *Blast Probe:* Allows the selected probe to be subjected to a BLAST analysis, by opening the BLAST page at NCBI and loading the probe. Note that this function only works when one probe is selected.

5.12 Help menu

- *PRISE2 Manual:* Opens this PRISE2 Manual.

- *PRISE2 Tutorial:* Opens the PRISE2 Tutorial, which provides a step-by-step protocol showing how the software was used to create sequence-selective PCR primers or primer-probe sets for a specific fungal rRNA gene.

Appendix I: **Primer and Probe Selectivity Settings**

Mis-priming happens often in PCR experiments and it may or may not affect the PCR result. The efficiency of the polymerase to recognize and extend a mismatched duplex is not only sensitive to the number of mismatched nucleotide bases, but also to the nucleotide composition and location of the mismatches. Our *Primer Selectivity Settings* wizard pages are composed of the *mismatch cost matrix*, *positional mismatch allowance settings,* and two different *ambiguous base cost functions* to accurately evaluate the selectivity of a primer pair. Users can use default settings or customize the settings to suit their specific application. We now explain the fundamentals of our Primer Selectivity Settings.

1. Mismatch cost matrix: To capture various effects of mismatched nucleotides, the users are allowed to assign different penalties on the mismatched nucleotides in the mismatch cost matrix. Each entry in the matrix specifies the penalty level of the corresponding mismatch in the primer-template duplex. Here, the larger value of cost in the matrix, the more unlikely for a duplex with this mismatch to be predicted to be stable (and therefore a PCR to be made). The Mismatch Cost Matrix has entries for each nucleotide base A, C, G and T. The mismatch cost of ambiguous bases represented by IUPAC code, such as N, R and Y, etc., will be obtained automatically by the average of mismatch cost between the non-ambiguous bases represented by the corresponding ambiguous bases. For example, in IUPAC codes, ambiguous base R denotes {A,G}, and base Y denotes {T,C}, so the mismatch cost of R and Y can be calculated by the formula mc(R,Y) = (mc(A,T) + mc(A,C) + mc(G,T) + mc(G,C)) / 4.

2. Ambiguous base cost function: To deal with ambiguous bases in target/non-target sequences, users are allowed to choose from two different schemes to measure match/mismatch.

By choosing the Distance scheme, PRISE2 will calculate the mismatch cost using mismatch cost matrix described above. This way, ambiguous bases in target/non-target sequences are more likely to be penalized, since this scheme will penalize every two different bases even if they contain several common possible nucleotides.

For example, base N denotes all nucleotides {A,C,G,T}. When we consider two bases N and T, although T is a possible nucleotide in N, the cost 3/4 is still high (close to 1).

By choosing Binary, the function is simple: if two bases contain any common nucleotide, then they are considered match with cost 0, otherwise it's a mismatch with cost 1. This scheme guarantees that no possible binding will be missed. However, selectivity may be lost. Two bases R = {A,C} and B={C,G,T} are very different, but Binary scheme will consider them as a "match".

Since target/non-target sequences contain lots of 'N' bases that represent unknown nucleotides, we recommend using the Distance scheme in which only similar bases are considered a match.

3. Positional Mismatch Allowance Settings: This component captures the cost allowance of the insertion/deletion and mismatched nucleotides for position range in primer-template duplex. In the basic Primer Selectivity Settings, the exact Positional Mismatch Allowance for the three 3' end positions of primer can be specified for target and non-target sequences, respectively. If the

setting is set to xyz, then only primer-template pairs that satisfy this xyz match-mismatch configuration and those above will be considered as producing a PCR product. xyz is the maximum allowed accumulated number of mismatches counting from right hand side (i.e., 3’ end of primer).

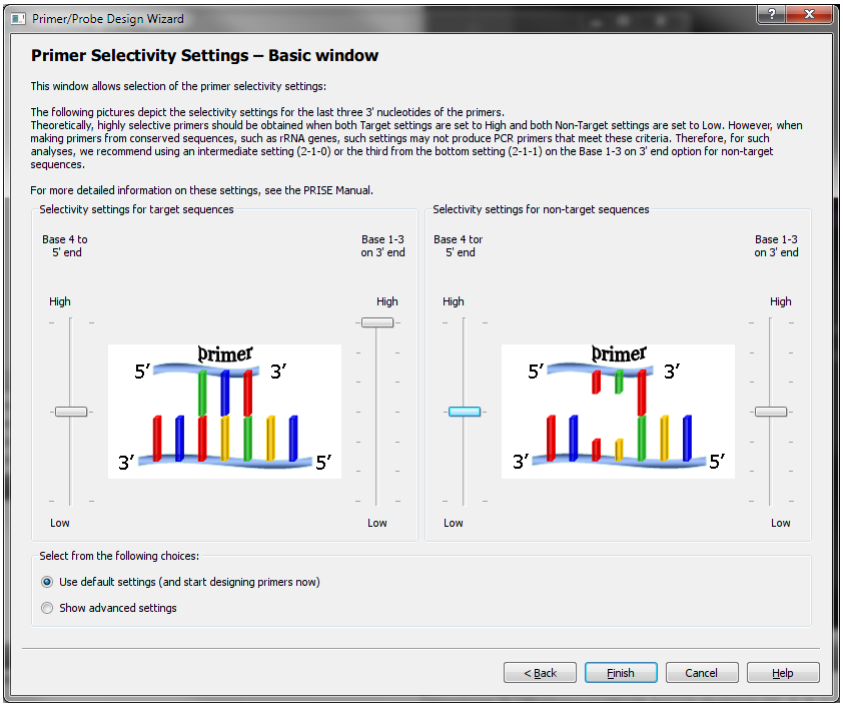


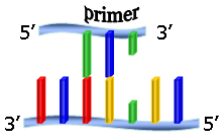
Figure 3. An example of basic Primer Selectivity Settings

An example of these settings for Primer Design is given in Figure 3, in which 0-0-0 setting is set for target sequences, and 2-1-0 setting is set for non-target sequences. This means that

- For target sequences, no mismatch is allowed on the three 3' end positions of primer. Thus only exact matches at all these three positions will be scored as creating a PCR product.
- For non-target sequences, there is no mismatch on the first base on 3’ end and at most two mismatches are allowed on the 2nd and 3rd bases on 3’ end of primer. Thus any primer-template pair with match-mismatch configurations of 0-0-0, 1-0-0, 1-1-0, and 2-1-0 for will be counted as producing a PCR product.

In this basic version of Primer Selectivity Settings, the approximate match/mismatch from the fourth base of primer's 3' end to 5' end can be specified, as well. This is illustrated by the example in Figure 3, in which high match percentage is required on the region from the fourth base to 5' end for target sequences, while medium match percentage is required on the segment from the 4th base to 5' end for non-target sequences. By moving the slider bars on the side of two pictures, these settings can be changed. Note that there are in total 8 different combinations of match-mismatch choices for the three 3’ end positions, but only 7 pictures can be shown in this window and they represent the settings: 0-0-0, 1-0-0, 1-1-0, 2-1-0, 2-1-1, 2-2-1, 3-2-1. The picture below, which represents the 1-1-1 setting, is left out because the 1-1-1 and 2-1-0 settings are not compatible. More specifically, all of the above 7 settings are ordered strictly from more to

less stringent in considering the likelihood of getting a PCR product. However, the 1-1-1 and 2-1-0 settings cannot be ordered by our Primer Selectivity Settings system.



1-1-1

Default positional mismatch allowance settings should be suitable for most applications, but they also can be customized using the advanced option. In this setting, the cumulative mismatch cost allowance for each primer position from 3' end can be specified. Each entry of positional mismatch array represents the maximum allowed cost for the region from 3' end to the corresponding point of the primer.

We give an example to describe the use of these advanced settings. Consider the mismatch cost matrix and the positional mismatch allowance settings for non-target sequences in Figure 4.

Figure 4. An example of Mismatch Cost Matrix and Positional Mismatch Allowance Setting

This combination setting can be interpreted as:

(1) No mismatch is allowed at the first base on 3' end;

(2) At most one C-A, G-C, T-A or T-G mismatch and no G-A or T-C mismatch is allowed on the second to the third base on 3' end;

(3) One T-C mismatch on the fourth base with no mismatch from the first to the third base on 3' end, or one G-A mismatch on the fourth base with at most one C-A, G-C, T-A or T-G mismatch is allowed on the second to the third base on 3' end.

Under this setting, the primer 5'-CTAACTACTGAGAA-3' will be predicted to amplify the sequence 5'-…CTAACTACTGGGAA...-3' (more precisely, anneal to the reverse complement strand of this sequence), since the cumulative positional cost is 5'-…,2,2,2,2,0,0,0 -3', which satisfies the Positional Mismatch Allowance Settings. Note that in this example we didn't count the effect of insertion/deletion costs. The calculations with these effect considered are similar.

According to the fixed Primer Selectivity Settings, PRISE2 performs a local alignment for the primer against each sequence in target and non-target group, and predicts the position in the sequence where this primer anneals (or does not anneal at all).

Users can use different Positional Mismatch Allowance settings for primer design and primer-probe set design processes. Actually, since the different sensitivity properties of primers and probes, two different settings should be applied.

A primer requires higher sensitivity on 3' end, which means it allows more mismatches on 5' end. For a probe, the sensitivity decreases from middle to both ends, since we prefer continuous matches in the middle. Once a probe can bind to target sequences with that fragment of continuous matches in the middle, some mismatches at two ends are tolerable and will not affect its function. Currently PRISE2 provides 16 sets of default settings for target and non-target selectivity each, corresponding to each possible number of continuous matches in the middle. Figure 5 shows the Basic Probe Selectivity Setting page and Figure 6 shows the default setting for probes. The allowed accumulated cost of mismatches is symmetric and calculated from the center of probe to both ends.

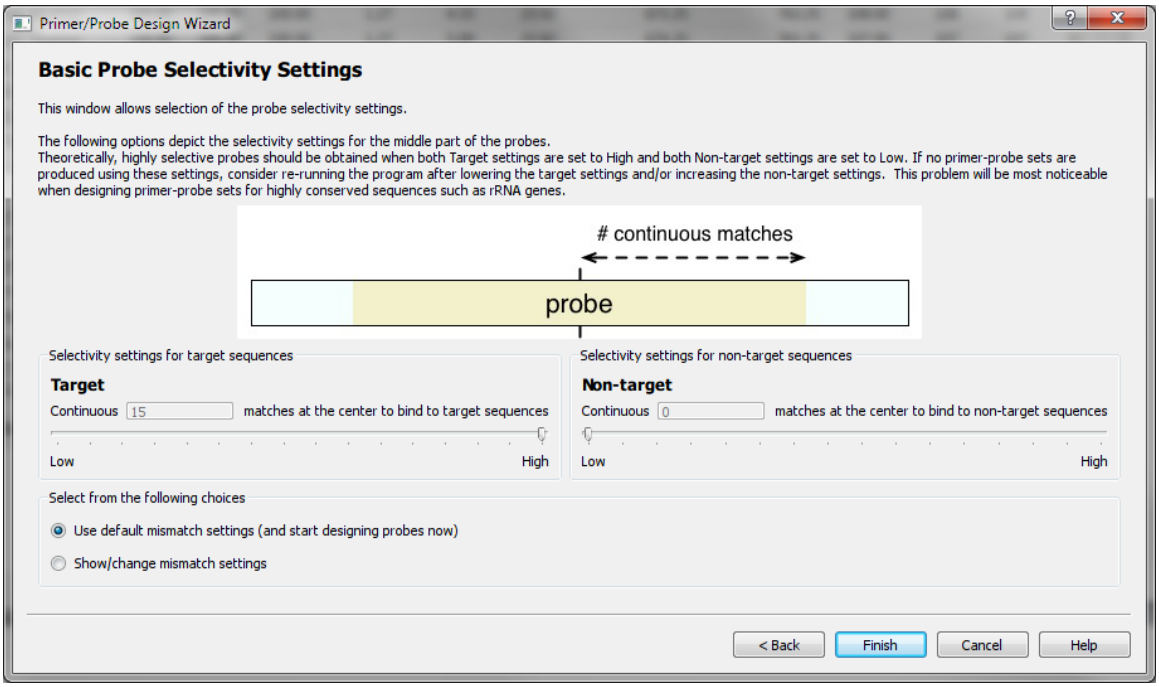


Figure 5. Basic Probe Selectivity Setting Page

Figure 6. Default Probe Selectivity Settings

A	0			
C	1	0		
G	2	1	0	
T	1	3	1	0
	A	C	G	T

With the same cost function as above, the setting for Non-target sequences can be interpreted similar to primer sensitivity:

(1) No mismatch is allowed at the 6 center bases (the 1st to 3th positions in the middle and their symmetric position)

(2) At most one C-A, G-C, T-A or T-G mismatch and no G-A or T-C mismatch is allowed on the 4th to 7th base from the center (4th , 5th, 6th, 7th positions and their symmetric positions);

(3) One G-A mismatch on the 10th to 13th base with no mismatch from the 1st to 6th base, or one C-A, G-C, T-A or T-G mismatch on the 1st to 9th base with at most one C-A, G-C, T-A or T-G mismatch are allowed on the 10th to 13th. The allowance at the left part is symmetric.

Similarly, PRISE2 performs a local alignment for the probe against each sequence in target and non-target group, and predicts the position in the sequence where it anneals (or does not anneal at all) according to the Probe Selectivity Settings.