# **PRISE 2.0**

# **User Manual**

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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 <a href="http://alglab1.cs.ucr.edu/OFRG/PRISE.php">http://alglab1.cs.ucr.edu/OFRG/PRISE.php</a>.

#### 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 <u>http://www.imore.com/how-open-apps-unidentified-developer-os-x-mountain-lion.</u>

#### 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.

	PRISE2 (PRImer SElector 2)	-		×
				7
Step 1.1	Identify Seed Sequences and Create Hit Table			
Step 1.2	Select Target and Non-target Sequences			
Step 2	Design Primers/Probes			
				_
	Manual Tutorial Ab	out PR	ISE2	

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 (<u>http://www.ncbi.nlm.nih.gov/BLAST/</u>) 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<sup>1</sup>.

#### 2.3 Create hit table using local BLAST application and database

<sup>&</sup>lt;sup>1</sup> **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.

		Wizard		?
Choose one optior	1.			
I have BLAST	on my machine and want	to run it locally.		
Provide BLAST pat	h ([blast root dir] \bin)			
C:\dev\blast-2.2	29+\bin			Browse
Provide path to da	tabase folder			
C:\dev\blast-2.2	29+\db			Browse
○ I will obtain th	e hit table from NCBI BLA	NST website.		
			Next >	Cancel

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.

Upload seed sequence	es in FASTA format file	
C:/dev/prise/sample	files/PochoniaSeed.txt	browse
Search Set		
Databases	other_genomic nt	
Entrez query (optiona	0	
Program Selection		
• Highly similar sequ	ences (megablast)	
O More dissimilar se	quences (discontiguous megablast)	
🔘 Somewhat similar	sequences (blastn)	
O Blastn optimized f	or sequences shorter than 50 bases (blastn-short)	

#### 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.

General Parameters
General Parameters
Max target 500 🗸
Expect threshold 10
Word size 28 💌
Scoring Parameters
Match/mismatch 1, -2 💌
Gap costs Linear 💌
Filter and Masking
Filter 🔽 Low complexity regions
Mask Mask lower case letters
< <u>B</u> ack OK Cancel

#### 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.

Blast Result an	d Error Message	?	×
Output	Warning and Error		
#Please wait while blast is running #When blast is done, you will see a popup message. ====================================			
	Save Output as Hit table file	Close	2

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**".

Dutput			Warning and Error
# BLASTN 2.2.29+		^	
# Ouery: PochoniaSeed			
# Database: other_genomic nt			
# Fields: query id, subject id, % identity, alignment length, n	nismatches, gap	opens, c	
# 510 hits found			
PochoniaSeed gi   533735124   dbj   AB709846.1   99.60	499	2	
PochoniaSeed gi   533735121   dbj   AB709845.1   99.60	499	2	
PochoniaSeed gi   533735118   dbj   AB709844.1   99.60	499	2	
PochoniaSeed gi 533735114 dbj AB709843.1  99.60	499	2	
PochoniaSeed gi 533735111 dbj AB709842.1 99.60	499	2	
PochoniaSeed gi 395439691 gb 3Q647436.1 99.60	499	2	
PochoniaSeed gi 27884304 dbj AB 100362.1 99.60	499	2	
PochoniaSeed gi 13160418 emb AJ292397.1 99.60	499	2	
PochoniaSeed gi 11933096 emb AJ291800.1 99.60	499	2	
PochoniaSeed gi 11933098 emb AJ291801.1 99.60	499	2	
PochoniaSeed gi 533735127 dbj AB709847.1 99.40	500	2	
PochoniaSeed gi 385663561 gb JQ433954.1 99.40	499	3	
PochoniaSeed gi 118627604 emb AM412780.1 99.40	500	2	
PochoniaSeed gi 49424884 gb AY 555964.1 99.60	496	2	
PochoniaSeed gi 430769399 gb KC291612.1 99.40	499	2	
PochoniaSeed gi 442774206 gb KC007316.1 99.40	499	2	
PochoniaSeed gi 441037499 gb KC171356.1 99.40	499	2	
PochoniaSeed gi 257286119 dbj AB378544.1 99.40	499	2	
PochoniaSeed gi 257286123 dbj AB378548.1  99.40	499	2	
PochoniaSeed gi 257286118 dbj AB378543.1  99.40	499 499	2	
PochoniaSeed gi 66796173 dbj AB214654.1 99.40	499 499	2	
PochoniaSeed gi   59797389   gb   AY912487.1   99.40 PochoniaSeed gi   58892741   gb   AY903605.1   99.40	499	2	
PochoniaSeed gi 58892741 gb AY903605.1  99.40 PochoniaSeed gi 13508841 emb AJ303054.1  99.40	499	2 ~	
2	499	_	
<		>	

### 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 <u>Sequence Alignment Settings for Pairwise Identity Analysis</u>, 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).

Load Seed Sequence and Hit Table	? ×
1. Load seed sequence(s)	GenBank sequence annotations
Enter accession for seed sequence(s)one line per accession number	Extract simple annotations (faster)
<b>^</b>	GI, ACCESSION, LENGTH, DEFINITION, ORGANISM
	Extract complete annotations (slower)
	All above plus SOURCE, FEATURES, AUTHORS, and TITLE
Or load seed sequence(s) in FASTA format	
Browse	
2. Load hit table and (or) FASTA file sequence(s)	
Load hit table	
Browse	
AND/OR	
Load FASTA sequence(s) Browse	
	OK Cancel

#### 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 "<u>-Select Target and Non-Target Sequences</u>." 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 nontarget 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

File	Mark/Unmark Move/Delete Clear Find Re-ali	gnment Compare Seq Lists	Help	
	Load Seed Sequence and Hit Table			
	Load Sequence List (.sequence File)			
	Save Sequence List (.sequence File)			
	Save Sequence List as Tab Delimited File (Excel File)			
	Save FASTA Sequences as			
	Add FASTA Sequences to (An Existing File)			
	Exit			

- 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 tabdelimited 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

	Se	elect Target and Non-target Sequences		
Fil	e		Find Re-Alignment Compare Seq Lists Help	
		Mark Sequences Unmark Sequences		
		Reverse Marked and Unmarked		

- 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 nontarget 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.

Mark All Sequences	Mark	All Selected Sequences	
Mark Sequences Satisfying Sele	cted Conditions:		
All sequences	© Sele	ted sequences only	
Sequence length betwee	n	and	-
	AND		
Solution Note: Not	ochoniaSeed 🔹 between	and	
	AND		
Sequence definition	<ul> <li>contains</li> <li>does not contain</li> </ul>		
	AND		
Sequence organism	<ul> <li>contains</li> <li>does not contain</li> </ul>		
	AND		
N. A	<ul> <li>contains</li> <li>does not contain</li> </ul>		
	AND		
N. A	<ul> <li>contains</li> <li>does not contain</li> </ul>		

#### *3.4 Move/Delete menu*

📰 S	Select Target and Non-target Sequences									
File	Mark/Unmark	Move/Delete Clear Find Re-Alignment Compare Seq Lists Help								
		Move Marked Sequences to FASTA Sequence Box Delete Marked Sequences Delete Selected Sequences								

- 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
-----	------	------

I	🗈 Select Target and Non-target Sequences									
	File Mark/Unmark Move/Delete Clear	Find Re-alignment Compare Seq Lists Help								
		Find Sequence								
		Find <u>N</u> ext								

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

Find Sequences	? <mark>×</mark>
Find next sequence satisfying selected conditions	
Sequence length between	and
AND	
% Identity for F11-Seed  between	and
AND	
<ul> <li>Sequence definitionn</li> <li>does not contain</li> </ul>	
AND	
<ul> <li>Sequence organism</li> <li>does not contain</li> </ul>	
AND	
N. A	
AND	
Octains     Octains     Octains     Octains	
	OK Cancel

• *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

File	Mark/Unmark	Move/Delete	Clear	Find	Re-Alignment	Compare Seq Lists Help		
<u> </u>						equence Alignment Settings	1	
					Update %	Identity for All Sequences		

- 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.

Sequence Alignment Settings for P	airwise Identity Analysis	? <mark>- ×</mark>	
When aligning two sequences		When computing identity percentage of two sequences	
Reward for a match Penalty for a mismatch Internal gap open penalty Internal gap extension penalty End gap penalty	(+) 1 (-) 0 (-) 2 (-) 1 (-) free	<ul> <li>Count end and internal gaps</li> <li>Count internal gap only</li> <li>Don't count gaps</li> </ul> Set All Parameters as Default	
		OK Cancel	H

#### 3.8 Compare Seq Lists menu

File	Mark/Unmark	Move/Delete	Clear	Find	Re-alignment	Compare Seq Lists Help
						Load GenBank (.gb) File to be Compared to Current List Display Sequences not in Sequence List Display Sequences not in GenBank File

- 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

ile	Mark/	Jnmark Move/D	elete Clear Fi	nd Re-Alignment	Compa	are Seq Lists Help	
	Seq #	NCBI GI#	Accession #	% Identity	Length	Definition	
1		SEED Pochonia		100	499	PochoniaSeed	
2		27884304	AB100362	99.5992	2327	Cordyceps chlamydosporia genes for 18S rRNA, ITS1,	Eukaryo
3		13160418	A J29	00 5000		Verticillium chlamydosporium var. chlamydosporium	Eukaryo
4		11933096	AJ29.	ay Pairwise Alignm	ent	Verticillium chlamydosporium 18S rRNA gene (partial	Eukaryo
5		11933110	AJ29: Insta	nt Blast		Verticillium chlamydosporium 18S rRNA gene (partial	Eukaryo
6		11933098	AJ291801	99.5992	634	Verticillium chlamydosporium, 18S rRNA gene (partial	Eukaryo
7		118627604	AM412780	99 5992	573	Verticillium chlamydosporium partial 185 rRNA gene	Fukaryo

• *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.

Pairwise Alig	inment
	ence PochoniaSeed V.S. subject sequence AJ292397: 99.5992%% identity. 497; Mismatch: 2; Internal gaps: 0; End gaps: 55.
Sed 1 Sbj 1	CATGTGAACTTATACCATTTACAACCGTTGCTTCGGCGGGTTCTCGCCCGGGCTTTACA
Sed 74 Sbj 101	CGGCCCGCCGGGGACCCAAACTCTAGATTTTATTTTGGCATGTCTGAGTGGAATCATTACAAAATGAATCAAAACTTTCAACAAC
Sed 174	CTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATGTGAATTGCAGAACTCAGTGAATCATCGAATCTTTGAACGCACATTG
Sed 274	TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCCAGCGGTTTGGTGTGGGGACCGGCGAGTACAGAGGCTTTGG TCTGGCGGGATGCCTGTTCGAGCGTCATTTCAACCCTCCAAGCCCCAGCGGTTGGTGTGGGGACCGGCGAGTACAGAGGCTTTGG
Sed 374 Sbj 401	TTCCCTCGGCGCCCCCCGAATGAATTGGCGGTCTCGTCGCGGCCTCCTCTGCGTAGTAGCACAACCTCGCATCAGGAGCGGGC
Sed 474 Sbj 501	TARARCGCCCARCTTTTTTTTTTTTTTARGAG         499           IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII
•	III

• *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.)

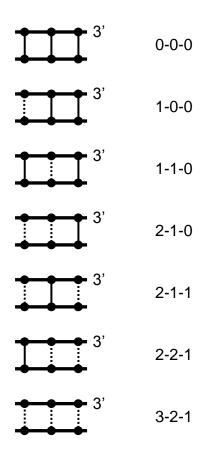


Figure 2: Scoring scheme for the sequence-selectivity component of the Design Primers module. On the left side are depictions of the last three 3' nucleotides of a primer and its corresponding template. The primer is the top strand. Base-paired nucleotides are designated by solid lines. Non-based paired 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, 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 <u>Load or Design New List</u> page, where users can load a previously created primer list file or initiate a new primer design project. Next is the <u>Input Target/Non-Target Sequences</u> 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.

Input target and non-target sequences (FASTA file format) Target sequence	)	
D:/Workspace/sample file/PochoniaTarget_mac.txt		Browse
Non-target sequence		
D:/Workspace/sample file/PochoniaNonTarget_mac.txt		Browse
Duplicate sequences		
Remove duplicate sequences		
Remove and dump to file		Browse
Sequence length range		
Sequences must have length between 60	and 4000	nucleotides
Remove sequences not within this length range		
Remove and dump to file		Browse

The next page is the <u>Extract/Load Primer Candidates</u> 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.

Primer/Probe Design Wizard	? <mark>x</mark>
Extract/Load Primer Candidates	
Extract primer candidates from target sequences or load user's primer candidates	
Design primers based on target and non-target sequences	
Load user's primer candidates	Browse
Note: If you provide your own primer candidates, the program will not consider the primer candidates extracted from target sequences	
< <u>B</u> ack <u>N</u> ext > Cancel	<u>H</u> elp

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 <u>Primer/Probe Design Settings</u>, the user can select (i) **Use all default** settings, (ii) **Use previous settings**, or (iii) **Show/change settings**.

Primer/Probe Design Wizard	? ×
Primer Design Settings	
This primer design wizard will help you to choose most of the settings for designing primers	
Select from the following choices	
Use all default settings (and start designing primers now)	
Use previous settings	
Show/change settings	
< <u>Back</u> <u>Next</u> Cancel	Help

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:

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

Primer/Probe Design Wizard	[?] <mark></mark> ■×
Primer Properties Settings	
Primer length range 14 to 22	Primer Tm 48 to 58 C
Minimum % of target sequences containing candidate primers	[Na+] concentrations     0.1     M       Max primer Tm difference     2     C
Only consider top 100 % (single) candidate primers	Primer GC% 25 % to 75 %
PCR product size range 50 to 200	Max primer inter-complementarity 10
✓ Avoid continuous 4 A's or C's or T's	Max primer 3' inter-complementarity 4 on last 8 bases at 3' end
✓ Avoid continuous 4 G's	Max primer intra-complementarity 10
Image: Avoid more than         3         C+G at last 5 bases from 3' end	Max primer 3' intra-complementarity 4 on last 8 bases at 3' end
	< Back Next > Cancel Help

#### 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 <u>Basic Primer Selectivity Settings</u> 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.



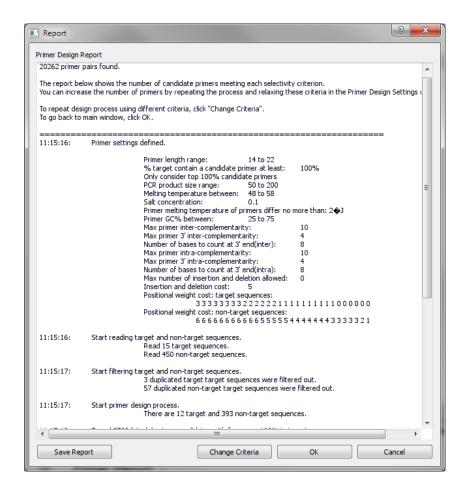
As explained earlier, theoretically, highly selective primers should be obtained when both target settings are set to *high* and both non-Target settings are set to *low*. However, when making primers from conserved sequences, such as rRNA genes, such settings may not produce PCR primers that meet these 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 <u>Advanced Primer Selectivity Settings</u> 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 m The l	nisma arge	tche	d nu	cleo	tides	in p	rime	r-ten	pen nplat proc	e du	plex.	* 		A C C I G I T I			5		Ma	x#g	n/dele apsa n/del	llowe	ł						0		T
B Positi	inary: onal r	Less	likely	to pe owan	enalize	e amb	iguou				-	t/non on-ta	-			es															
5'-	30 3	29 3	28 3		26 3	25 3			22 2			19 2	18 2		16 1	15 1	14 1	13 1				9 1	8	7	6 0	5 0	4 0	3 0	2 0	1	-3'
	n-tai	get	sequ	ence																											
No	n-tai			27	26	25	24	23	22	21	20	19	18	17	16 5	15 5	14	13	12	11	10	9	8	7	6	5	4	3	2	1	-3'

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".



#### IV. Primer Report

After the design process is finished, a dialog titled <u>Display Primer List</u> 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 "<u>- Primer Report.</u>" 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 nontarget 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.

		Primer pair	Target pair %	Target forward %	Target reverse %	Non-target pair %	Non-target forward %	reverse	Target forward annealing avg pos	Target reverse annealing avg pos	Target PCR avg size	Target PCR min size		Non-ta PCR a size
L		GCGAGTACAGAGGCTTTTGATGC	100.00	100.00	100.00	1.78	3.31	23.92	672.25	763.25	109.00	109	109	10
		CGAGTACAGAGGCTTTGTGATGC	100.00	100.00	100.00	1.27	4.33	23.92	673.25	763.25	108.00	108	108	10
		GAGTACAGAGGCTTTGGTGATGC	100.00	100.00	100.00	1.27	5.09	23.92	674.25	763.25	107.00	107	107	1
		ACCGGCGAGTACAGATGATGCGA	100.00	100.00	100.00	5.09	24.94	23.92	668.25	763.25	113.00	113	113	4
		CCATTTACAACCGTTGCTTTGTAA	100.00	100.00	100.00	1.27	1.78	35.88	345.25	449.25	123.00	123	123	1
		CCATTTACAACCGTTGCTTTTGTA	100.00	100.00	100.00	1.27	1.78	35.88	345.25	449.25	124.00	124	124	1
		ACCATTTACAACCGTTGCTTTTGT	100.00	100.00	100.00	1.27	1.78	35.88	344.25	449.25	125.00	125	125	1
		CCATTTACAACCGTTGCTTTTTGT	100.00	100.00	100.00	1.27	1.78	35.88	345.25	449.25	124.00	124	124	1
		CATTTACAACCGTTGCTTTGTAAT	100.00	100.00	100.00	1.27	6.87	35.88	346.25	449.25	122.00	122	122	1
0		CATTTACAACCGTTGCTTTTGTAA	100.00	100.00	100.00	1.27	6.87	35.88	346.25	449.25	123.00	123	123	1
1		CATTTACAACCGTTGCTTTTGTAA	100.00	100.00	100.00	1.27	6.87	35.88	346.25	449.25	122.00	122	122	1
2		CATTTACAACCGTTGCTTTTTGTA	100.00	100.00	100.00	1.27	6.87	35.88	346.25	449.25	123.00	123	123	1
3		GCGAGTACAGAGGCTTTTGATGC	100.00	100.00	100.00	1.78	3.31	39.19	672.25	764.25	109.00	109	109	1
4		CGAGTACAGAGGCTTTGTGATGC	100.00	100.00	100.00	1.27	4.33	39.19	673.25	764.25	108.00	108	108	1
5		GAGTACAGAGGCTTTGGTGATGC	100.00	100.00	100.00	1.27	5.09	39.19	674.25	764.25	107.00	107	107	1
6		CATTTACAACCGTIGCT TTIGTAA	100.00	100.00	100.00	1.27	6.87	39.44	346.25	451.25	123.00	123	123	1
rin	ner 1	Information Window												

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

File	:/Workspace/sample file/PochoniaTarget_mac.tx Hide/Display Sort Add/Delete Mark/Un		er Report Annealing	Info Pi	imer Compl	ementarity	Primer Sett	ing Probes Inst	ant BLAST Help		-		• ×
	Load Primer List (.primer File) Save Primer List (.primer File) Save Primer List as Tab Delimited File (Excel)	rget air %	Target forward %	Target reverse %	Non-target pair %	Non-target forward %	Non-target reverse %	Target forward annealing avg pos	Target reverse annealing avg pos	Target PCR avg size	Target PCR min size	Target PCR max size	Non-ta PCR a size
	Save Primer Information Window Content	00.00	100.00	100.00	1.78	3.31	23.92	672.25	763.25	109.00	109	109	10
	Save Primer Pairs Only	100.00	100.00	100.00	1.27	4.33	23.92	673.25	763.25	108.00	108	108	10
	Exit	00.00	100.00	100.00	1.27	5.09	23.92	674.25	763.25	107.00	107	107	10
4		100.00	100.00	100.00	5.09	24.94	23.92	668.25	763.25	113.00	113	113	41

- 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)<sup>2</sup>
  - +  $\frac{1}{2}$  (% of non-target sequences estimated to anneal with forward primer)<sup>2</sup>
  - +  $\frac{1}{2}$  (% of non-target sequences estimated to anneal with reverse primer)<sup>2</sup>.

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

Sort	A	? ×
Sort by		
Selectivity Formula	•	Ascending Descending
Then by		
Target pair %	•	Ascending Descending
Then by		
	•	Ascending Descending
Then by		
	•	Ascending Descending
	ОК	Cancel

#### 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.

ile	Hide/D	Display Sort	Add/Delete	Mark/U	Jnmark	Annealin	g Info Prin	ner Compler	nentarity	Primer Settin	ig Probes Instan	nt BLAST Help				
_	rimer	P	Delete F	mer Pair Primer Pa Marked P	airs Conc	litionally	Target reverse %	Non-target pair %	Non-target forward %	Non-target reverse %	forward	Target reverse annealing avg pos	Target PCR avg size	Target PCR min size	Target PCR max size	Non- PCF
1		TACCATTTAC		Seleted P			100	0	0	2.42131	259.235	295.235	50	50	50	0
2	<b>V</b>	ATACCATTTA				100	100	0	0	2.42131	258.235	295.235	51	51	51	0
3	<b>V</b>	TATACCATTTA	ACAACCGTTG	CTGG	100	100	100	0	0	2.42131	257.235	295.235	52	52	52	0
4		CCATTTACAA	CCGTTGCTT	TTGTA	100	100	100	0	0	15.4964	261.235	363.235	124	124	124	0
5	<b>V</b>	ACCATTTACA	ACCGTTGCT.	TTTGT	100	100	100	0	0	15.4964	260.235	363.235	125	125	125	0
6		TACCATTTAC	AACCGTTGCT	TTTG	100	100	100	0	0	15.4964	259.235	363.235	126	126	126	0
7	<b>V</b>	ATACCATTTA	CAACCGTTGC	TTTT	100	100	100	0	0	15.4964	258.235	363.235	127	127	127	0
8		TATACCATTTA	ACAACCGTTG	СТТТ	100	100	100	0	0	15.4964	257.235	363.235	128	128	128	0
9		CCATTTACAA	CCGTTGCTT	TTTGT	100	100	100	0	0	15.4964	261.235	363.235	124	124	124	0
10		ACCATTTACA	ACCGTTGCTT	TTTG	100	100	100	0	0	15.4964	260.235	363.235	125	125	125	0
11	<b>V</b>	TACCATTTAC	AACCGTTGCT	TTTT	100	100	100	0	0	15.4964	259.235	363.235	126	126	126	0
12	<b>V</b>	ATACCATTTA	CAACCGTTGC	TTTT	100	100	100	0	0	15.4964	258.235	363.235	127	127	127	0

• 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.

	rinde, e	isplay Sort	Add/Delete	Mark/L		Annealin	g Info Prin			Primer Settin	5	it BLAST Help				
	rimer	Ρ	Delete F	Primer Pa	Manually airs Cond rimer Pai	tionally	Target reverse %	Non-target pair %	Non-target forward %	Non-target reverse %	forward	Target reverse annealing avg pos		Target PCR min size	Target PCR max size	Non- PCF si
L		TACCATTTAC	Delete	Seleted P	rimer Pai	rs	100	0	0	2.42131	259.235	295.235	50	50	50	0
		ATACCATTTA	CAACCOTTOC	1	100	100	100	0	0	2.42131	258.235	295.235	51	51	51	0
		TATACCATTT	ACAACCGTTG	CTGG	100	100	100	0	0	2.42131	257.235	295.235	52	52	52	0
		CCATTTACAA	CCGTTGCTT	TTGTA	100	100	100	0	0	15.4964	261.235	363.235	124	124	124	0
		ACCATTTACA	ACCGTTGCT.	TTTGT	100	100	100	0	0	15.4964	260.235	363.235	125	125	125	0
		TACCATTTAC	AACCGTTGCT	TTTG	100	100	100	0	0	15.4964	259.235	363.235	126	126	126	0
		ATACCATTTA	CAACCGTTGC	TTTT	100	100	100	0	0	15.4964	258.235	363.235	127	127	127	0
		TATACCATTT	ACAACCGTTG	CTTT	100	100	100	0	0	15.4964	257.235	363.235	128	128	128	0
		CCATTTACAA	CCGTTGCTT	TTTGT	100	100	100	0	0	15.4964	261.235	363.235	124	124	124	0
0		ACCATTTACA	ACCGTTGCTT	TTTG	100	100	100	0	0	15.4964	260.235	363.235	125	125	125	0
1		TACCATTTAC	AACCGTTGCT	TTTT	100	100	100	0	0	15.4964	259.235	363.235	126	126	126	0
12		ATACCATTTA	CAACCGTTGC	TTTT	100	100	100	0	0	15.4964	258.235	363.235	127	127	127	0
13		CCCGGGCTTI	ACACTTTGT	AATG	100	100	100	0.726392	3.38983	15.4964	294.235	363.235	91	91	91	115.3

#### 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.

	*		Target	Prin	ner Annea	ling Position I	nformation					Target	Tornat	Non-target	Ven targe
ri	imer	Primer pair	pair %		-	Each Nucleot ces Annealing	-		rget Sequences	in Relation to Primer S	equences			PCR avg size	PCR min size
E	1	TACCATTTACAACCGTTGCTGGT	100	-		ces Not Anne	·					50	50	0	0
E	1	ATACCATTTACAACCGTTGCTGGT	100	-		quences Ann						51	51	0	0
1		TATACCATTTACAACCGTTGCTGG	100			quences Not						52	52	0	0
		CCATTTACAACCGTTGCTTTTGTA	100	INOT	1- larget Se	quences Not	Annealing v	vith Primer				124	124	0	0
	1	ACCATTTACAACCGTTGCTTTTGT	100	100	100	0	0	15.4964	260.235	363.235	125	125	125	0	0
		TACCATTTACAACCGTTGCTTTTG	100%	100	100	0%	0	15.4964	259.235	363.235	126	126	126	0	0
		ATACCATTTACAACCGTTGCTTTT	100	100	100	0	0	15.4964	258.235	363.235	127	127	127	0	0
		TATACCATTTACAACCGTTGCTTT	100	100	100	0	0	15.4964	257.235	363.235	128	128	128	0	0
		CCATTTACAACCGTTGCTTTTTGT	100	100	100	0	0	15.4964	261.235	363.235	124	124	124	0	0
		ACCATTTACAACCGTTGCTTTTTG	100	100	100	0	0	15.4964	260.235	363.235	125	125	125	0	0
		TACCATTTACAACCGTTGCTTTTT	100	100	100	0	0	15.4964	259.235	363.235	126	126	126	0	0
2		ATACCATTTACAACCGTTGCTTTT	100	100	100	0	0	15.4964	258.235	363.235	127	127	127	0	0
3		CCCGGGCTTTACACTTTGTAATG	100	100	100	0.726392	3.38983	15.4964	294.235	363.235	91	91	91	115.333	91
-															F
rime arge Seec	er a et s d se	formation window nnealing position informa requences: quence PochoniaSeed 4304 db] AB100362.1 Cordy		-	-				TTTGTAAT	GATTCCACTCAGAC Fw primer 13-32 1781-1800		Rv prime 117-138 1885-190		PCR 126 126	

• 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.

Dialog     Settings for the percentage of each nucleotide in non-target sequences in relation to the primer sequences. Note that these primer annealing settings should be less stringent than those used for primer design.	?
Divide primer into two parts and set the minimum match percentages:	· · · · · · · · · · · -3'
at least 50 % matches on 5' end	at least 62.5 % matches on 3' end
	OK Cancel

ACCGTTGCT.GGT AACCGTTGCT.GG GTTGCT.TTTGTA CGTTGCT.TTTGTA CGTTGCT.TTTG GTTGCT.TTTG GTTGCTT.TTG GTTGCTT.TTG CGTTGCTT.TTG CCGTTGCTT.TTG ACCGTTGCTT.TTG ACCGTTGCTT.TTG CCGTGCTT.TTG ACCGTTGCTT.TTG CCGTGCTT.TTG CCGTGCTT.TTG CCGTGCTT.TTG	100 100 100 100 100% 100 100 100 100 100	Non-	-Target Seq	es Not Anne uences Ann uences Not 0% 0% 0 0 0	ealing with I	Primer	260.235 259.235 258.235 257.235	363.235 363.235 363.235 363.235	125 126 127	127	50 51 52 124 125 126 127	0 0 0 0 0 0 0	0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	40 38 36 44 42 40 38	6
ACCGTTGCTTTGTA GTTGCTTTTGTA CCGTTGCTTTTGTA CCGTTGCTTTTGT CCGTTGCTTTT ACCCGTTGCTTTT GTTGCTTTTTG CCGTTGCTTTTTG CCGTTGCTTTTTG ACC.CTTTGCTTTT ACCTTTGTAATG TCTAGATTTTTT	100 100 100% 100% 100 100 100 100 100 10	Non- Non- 100 100 100 100 100 100	Target Seq Target Seq 100 100 100 100 100 100	uences Ann uences Not 0% 0% 0 0 0	0 0 0 0 0 0	Primer vith Primer 15.4964 15.4964 15.4964	259.235 258.235	363.235 363.235	126 127	52 124 125 126 127	52 124 125 126	0 0 0 0	0 0 0 0	0 0 0 0	36 44 42 40	6 3 3
GTIGCTTTIGTA CCGTIGCTTTIGTA ACCGTIGCTTTIGA ACCGTIGCTTTIGA GTIGCTTTTIGTACCGTIGCTTTTIGTACCGTIGCTTTTIGACCGTIGCTTTTIGTACTGACTGACTGACTGACTGACTGACTGACTGACTGAC	100 100% 100% 100 100 100 100 100 100 10	Non- 100 100 100 100 100 100	Target Seq 100 100 100 100 100	0 0% 0 0 0 0	Annealing v 0 0 0 0	15.4964 15.4964 15.4964	259.235 258.235	363.235 363.235	126 127	124 125 126 127	124 125 126	0 0 0 0	0 0 0 0	0 0 0 0	44 42 40	3
CGTIGCTTTIG CCGTIGCTTTIG ACCGTIGCTTTIG GTIGCTTTTIG CGTIGCTTTTIGT CGTIGCTTTTIGT ACCGTIGCTTTTIGT ACCGTIGCTTTT CACTTIGTAATG TCTAGATTTTT	100 100% 100 100 100 100 100 100	100 100 100 100 100 100	100 100 100 100 100	0 0% 0 0 0	0 0 0 0 0	15.4964 15.4964 15.4964	259.235 258.235	363.235 363.235	126 127	125 126 127	125 126	0	0	0	42 40	3
CCGTTGCTTTTG ACCGTTGCTTTT GTTGCTTTTGT CGTTGCTTTTGT CCGTTGCTTTTTG ACCGTTGCTTTTT ACCGTTGCTTTT CACTTTGTAATG TCTAGATTTTTT	100% 100 100 100 100 100 100	100 100 100 100 100	100 100 100 100	0% 0 0 0	0 0 0 0	15.4964 15.4964	259.235 258.235	363.235 363.235	126 127	126 127	126	0	0	0	40	
ACCGTTGCTTTTG GTTGCTTTTTGT CGTTGCTTTTTGT CCGTTGCTTTTTG ACCGTTGCTTTTT ACCGTTGCTTTT CCGTTGCTTTT CCGTTGCTTTT	100 100 100 100 100 100	100 100 100 100	100 100 100	0 0 0 0	0	15.4964	258.235	363.235	127	127				-		3
AACCGTTGCTTT.G. GTTGCTTTTTG CGTTGCTTTTTG CCGTTGCTTTT ACCGTTGCTTTT CCGTTGCTTTT CCGTTGCTTTT CCGTTGCTTTT	100 100 100 100 100	100 100 100	100 100	0 0	0						127	0	0	0	20	
GTTGCTTTTTG CGTTGCTTTTTG CCGTTGCTTTTT ACCGTTGCTTTT (ACTTTGTAATG ICTAGATTTTTT	100 100 100 100	100 100	100	0		15.4964	257.235	262,225					0	0	30	
CGTTGCTTTTTG CCGTTGCTTTTT ACCGTTGCTTTT ACTTTGTAATG ICTAGATTTTTT	100 100 100	100			0			303.235	128	128	128	0	0	0	36	
CCGTTGCTTTTT ACCGTTGCTTTT ACTTTGTAATG TCTAGATTTTTT	100 100		100		0	15.4964	261.235	363.235	124	124	124	0	0	0	42	
ACCGTTGCTTTT ACTTTGTAATG ICTAGATTTTTT	100	100		0	0	15.4964	260.235	363.235	125	125	125	0	0	0	40	1
ACTTTGTAATG			100	0	0	15.4964	259.235	363.235	126	126	126	0	0	0	38	
TCTAGATTTTTT	100	100	100	0	0	15.4964	258.235	363.235	127	127	127	0	0	0	36	
		100	100	0.726392	3.38983	15.4964	294.235	363.235	91	91	91	115.333	91	163	64	
	100	100	100	0.484262	5.08475	15.4964	331.235	363.235	54	54	54	54.5	54	55	45	
TAGATTTTTTGT	100	100	100	0.484262	5.56901	15.4964	332.235	363.235	53	53	53	53.5	53	54	42	
GTTGCTTTGTAA	100	100	100	0	0	16.9492	261.235	365.235	123	123	123	0	0	0	44	
GTTGCTTTTGTA	100	100	100	0	0	16.9492	261.235	365.235	124	124	124	0	0	0	44	
GTTGCTTGTAAT		100	100	0	0	16.9492	261.235	363.235	122	122	122	0	0	0	44	
GTTGCTTTGTAA		100	100	0	0	16.9492	261.235	363.235	123		123	0	0	0	44	
GTTGCTTTGTAA		100	100	0	0	16.9492	261.235	364.235	123	123	123	0	0	0	44	
												•	•	•		
CCGHGCL.THG			100	0	0	16.9492	259.235	304.235	120	120	120	0	0	0	40	ł
	CGTTGCTTTGTA CGTTGCTTGTAA CGTTGCTTTGTA CGTTGCTTTGTA CGTTGCTTTGTA CGTTGCTTTGTA CGTTGCTTTGTA	GTTGCTTTTGTA         100           CGTTGCTTTGTA         100           CGTTGCTTTGTA         100           CGTTGCTTTGTA         100           CGTTGCTTTGTA         100           CGTTGCTTTGTA         100           CGTTGCTTGTGTA         100           CGTTGCTTGTGTA         100           CGTTGCTTGTGTA         100           CGTTGCTTGTGTA         100           CGTTGCTTGTGTA         100           CGTTGCTTTGT         100	CGTTGCTTTTGT         100         100           CGTTGCTTGTAA         100         100           CGTTGCTTTGTA         100         100           CCGTTGCTTTGTA         100         100           CCGTTGCTTTGTA         100         100	CGTTGCLTTGTA         00         100         100           GTTGCL.TGTAA         100         100         100           GTTGCL.TGTAA         100         100         100           CGTTGCL.TTGTA         100         100         100	Cartract.rtract         000         100         100         0           GTIGCL.TGTAA         100         100         100         0           GTIGCL.TGTAA         100         100         100         0           GTIGCL.TGTAA         100         100         100         0           GTIGCL.TGTA         100         100         100         0           SCHTGCL.TIGTA         100         100         100         0           CGTIGCL.TIGTA         100         100         100         0           CGTIGCL.TIGT         100         100         100         0           CGTIGCL.TIGT         100         100         100         0	CGTIGCL.TIGTA.         100         100         100         0         0           GTIGCL.TIGTA.         100         100         100         0         0         0           GTIGCL.TIGTA.         100         100         100         0         0         0         0           GTIGCL.TIGTA.         100         100         100         0	CGTIGCT,TTIGT         100         100         100         0         16.9992           CGTIGCT,TIGTA         100         100         0         0         16.9992           CGTIGCT,TIGTA         100         100         0         0         16.9992           CGTIGCT,TIGTA         100         100         0         0         16.9492           CGTIGCT,TIGTA         100         100         0         0         16.9492	CGTIGCT.TTGTA         100         100         0         6.64982         662255           CGTIGCT.TTGTAA         100         100         0         0         16.6492         260.235           CGTIGCT.TTGTAA         100         100         0         0         16.6492         260.235           CGTIGCT.TTGTAA         100         100         0         0         16.6492         260.235           CGTIGCT.TTGTA         100         100         0         0         16.6492         259.235           CGTIGCT.TTGTA         100         100         0         0         16.6492         259.235           CGTIGCT.TTGTA         100         100         0         0         16.492         259.235	CGTIGCT,TTIGT         100         100         0         0         16.9492         26.0235         36.235           CGTIGCT,TIGTA         100         100         0         0         16.9492         26.0235         36.325           CGTIGCT,TIGTA         100         100         0         0         16.9492         26.0235         36.3256           CGTIGCT,TIGTA         100         100         0         0         16.9492         26.0235         364.235           CGTIGCT,TIGTA         100         100         0         0         16.9492         26.0235         364.235           CGTIGCT,TIGTA         100         100         0         0         16.9492         26.0235         364.235           CGTIGCT,TIGTA         100         100         0         0         16.9492         26.9235         364.235           CGTIGCT,TIGTA         100         100         0         16.9492         29.235         363.235           CGTIGCT,TIGTA         100         100         0         16.9492         29.235         364.235           CGTIGCT,TIGT         100         100         0         16.9492         29.235         364.235	CGTTGCT, TTGTA         100         100         100         0         16.9492         260.235         365.235         123           GGTTGCT, TGTAA         100         100         0         0         16.9492         260.235         382.235         123           GGTTGCT, TGTAA         100         100         0         16.9492         260.235         382.235         124           GGTTGCT, TTGTAA         100         100         0         16.9492         260.235         384.235         124           GGTTGCT, TTGTAA         100         100         0         0         16.9492         260.235         384.235         124           GGTTGCT, TTGTA         100         100         0         0         16.9492         260.235         384.235         124           GGTTGCT, TGTA         100         100         0         0         16.9492         259.235         384.235         124           CGTTGCT, TGTA         100         100         0         16.9492         259.235         384.235         124           CGTTGCT, TTGTA         100         100         0         16.9492         259.235         384.235         124           CGTTGCT, TTGT	CGTIGCT, TITGTA.         100         100         0         0         16.9492         260.235         365.235         125         125           CGTIGCT, TIGTAA.         100         100         0         0         16.9492         260.235         363.235         124         124           CGTIGCT, TIGTAA.         100         100         0         0         16.9492         260.235         363.235         124         124           CGTIGCT, TIGTAA.         100         100         0         0         16.9492         260.235         364.235         124         124           CGTIGCT, TIGTAA.         100         100         0         0         16.9492         260.235         364.235         124         124           CGTIGCT, TIGTAA.         100         100         0         0         16.9492         260.235         364.235         124         124           CGTIGCT, TIGTAA.         100         100         0         0         16.9492         269.235         364.235         124         124           CGTIGCT, TIGTA.         100         100         0         16.9492         259.235         363.235         124         124           CGTIGCT, TIGTA.         100	CGTIGCT,TTIGT         100         100         100         0         16.9492         260235         86.235         125         125         125           GTIGCT,TIGTA         100         100         0         0         16.9492         260235         36.325         124         124         124         124           GTIGCT,TIGTA         100         100         0         0         16.9492         260235         36.325         124         124         124           CITIGCT,TIGTA         100         100         0         0         16.9492         260235         364.235         124         124         124           CITIGCT,TIGTA         100         100         0         0         16.9492         260235         364.235         125         125         125         125           CITIGCT,TIGTA         100         100         0         0         16.9492         260235         364.235         128         125         125         125         125         125         125         125         125         126         126         126         126         126         126         126         126         126         126         126         126         126 </th <th>CGTTGCT,TTGTA         100         100         100         0         164942         260235         865235         125         125         126         0           GGTTGCT,TTGTA         100         100         0         0         164942         260235         863235         123         123         0           GGTTGCT,TTGTA         100         100         0         0         164942         260235         863235         124         124         124         0           GGTTGCT,TTGTA         100         100         0         0         164942         260235         864235         124         124         124         0           GGTTGCT,TTGTA         100         100         0         0         164942         260235         864235         125         125         125         0           GGTTGCT,TTGTA         100         100         0         0         164942         260235         864235         125         125         125         0           CGTTGCT,TTGTA         100         100         0         0         164942         259235         864235         124         124         0           CGTTGCT,TTGTA         100         <td< th=""><th>CGTTGCT,TTGTA         100         100         100         0         169992         260235         365.235         125         125         126         0         0           GGTGCT,TTGTA         100         100         100         0         0         169492         260235         363.235         123         123         123         0</th><th>CGTTGCT,TTGTA         100</th><th>CGTTGCT, TTGTA.         100         100         100         0         16.9492         260.235         365.235         125         125         0.7         0         0         42.           GGTGCT, TGTAA.         100         100         0         0         16.9492         260.235         362.35         124         124         124         0         0         0         42.           GGTGCT, TGTAA.         100         100         0         16.9492         260.235         362.35         124         124         124         0         0         0         42.           CGTGCT, TGTAA.         100         100         0         0         16.9492         260.235         362.355         124         124         124         0         0         0         42.           CGTGCT, TGTAA.         100         100         0         0         16.9492         260.235         362.355         124         124         124         0         0         0         42.           CGTGCT, TGTAA.         100         100         0         0         16.9492         259.235         362.355         125         125         126         0         0         0         42.</th></td<></th>	CGTTGCT,TTGTA         100         100         100         0         164942         260235         865235         125         125         126         0           GGTTGCT,TTGTA         100         100         0         0         164942         260235         863235         123         123         0           GGTTGCT,TTGTA         100         100         0         0         164942         260235         863235         124         124         124         0           GGTTGCT,TTGTA         100         100         0         0         164942         260235         864235         124         124         124         0           GGTTGCT,TTGTA         100         100         0         0         164942         260235         864235         125         125         125         0           GGTTGCT,TTGTA         100         100         0         0         164942         260235         864235         125         125         125         0           CGTTGCT,TTGTA         100         100         0         0         164942         259235         864235         124         124         0           CGTTGCT,TTGTA         100 <td< th=""><th>CGTTGCT,TTGTA         100         100         100         0         169992         260235         365.235         125         125         126         0         0           GGTGCT,TTGTA         100         100         100         0         0         169492         260235         363.235         123         123         123         0</th><th>CGTTGCT,TTGTA         100</th><th>CGTTGCT, TTGTA.         100         100         100         0         16.9492         260.235         365.235         125         125         0.7         0         0         42.           GGTGCT, TGTAA.         100         100         0         0         16.9492         260.235         362.35         124         124         124         0         0         0         42.           GGTGCT, TGTAA.         100         100         0         16.9492         260.235         362.35         124         124         124         0         0         0         42.           CGTGCT, TGTAA.         100         100         0         0         16.9492         260.235         362.355         124         124         124         0         0         0         42.           CGTGCT, TGTAA.         100         100         0         0         16.9492         260.235         362.355         124         124         124         0         0         0         42.           CGTGCT, TGTAA.         100         100         0         0         16.9492         259.235         362.355         125         125         126         0         0         0         42.</th></td<>	CGTTGCT,TTGTA         100         100         100         0         169992         260235         365.235         125         125         126         0         0           GGTGCT,TTGTA         100         100         100         0         0         169492         260235         363.235         123         123         123         0	CGTTGCT,TTGTA         100	CGTTGCT, TTGTA.         100         100         100         0         16.9492         260.235         365.235         125         125         0.7         0         0         42.           GGTGCT, TGTAA.         100         100         0         0         16.9492         260.235         362.35         124         124         124         0         0         0         42.           GGTGCT, TGTAA.         100         100         0         16.9492         260.235         362.35         124         124         124         0         0         0         42.           CGTGCT, TGTAA.         100         100         0         0         16.9492         260.235         362.355         124         124         124         0         0         0         42.           CGTGCT, TGTAA.         100         100         0         0         16.9492         260.235         362.355         124         124         124         0         0         0         42.           CGTGCT, TGTAA.         100         100         0         0         16.9492         259.235         362.355         125         125         126         0         0         0         42.

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

	rimer	Primer pair	Target pair %	Pero	centage of	ling Position I Each Nucleot	tide in Targe		arget Sequences	in Relation to Prime	r Sequences	Target PCR min size		Non-targe PCR avg size	Non-targe PCR min size
		TACCATTTACAACCGTTGCTGGT	100	Taro	aet Sequer	nces Not Anne	aling with F	rimer				50	50	0	0
		ATACCATTTACAACCGTTGCTGGT	100		· ·	equences Ann						51	51	0	0
		TATACCATTTACAACCGTTGCTGG	100			equences Not						52	52	0	0
		CCATTTACAACCGTTGCTTTTGTA	100	INO	n- larget 5	equences Not	Annealing	with Primer				124	124	0	0
		ACCATTTACAACCGTTGCTTTTGT	100	100	100	0	0	15.4964	260.235	363.235	125	125	125	0	0
		TACCATTTACAACCGTTGCTTTTG	100%	100	100	0%	0	15.4964	259.235	363.235	126	126	126	0	0
		ATACCATTTACAACCGTTGCTTTT	100	100	100	0	0	15.4964	258.235	363.235	127	127	127	0	0
		TATACCATTTACAACCGTTGCTTT	100	100	100	0	0	15.4964	257.235	363.235	128	128	128	0	0
		CCATTTACAACCGTTGCTTTTTGT	100	100	100	0	0	15.4964	261.235	363.235	124	124	124	0	0
		ACCATTTACAACCGTTGCTTTTTG	100	100	100	0	0	15.4964	260.235	363.235	125	125	125	0	0
		TACCATTTACAACCGTTGCTTTTT	100	100	100	0	0	15.4964	259.235	363.235	126	126	126	0	0
		ATACCATTTACAACCGTTGCTTTT	100	100	100	0	0	15.4964	258.235	363.235	127	127	127	0	0
		CCCGGGCTTTACACTTTGTAATG	100	100	100	0.726392	3.38983	15.4964	294.235	363.235	91	91	91	115.333	91
		GGGACCCAAACTCTAGATTTTTT	100	100	100	0.484262	5.08475	15.4964	331.235	363.235	54	54	54	54.5	54
		GGACCCAAACTCTAGATTTTTTGT	100	100	100	0.484262	5.56901	15.4964	332.235	363.235	53	53	53	53.5	53
		CCATTTACAACCGTTGCTTTGTAA	100	100	100	0	0	16.9492	261.235	365.235	123	123	123	0	0
		CCATTTACAACCGTTGCTTTTGTA	100	100	100	0	0	16.9492	261.235	365.235	124	124	124	0	0
	6000	CONTITUCANCECTTECT TOTANT	100	100	100	0	•	10 0400	261.225	262.225	100	100	100	^	<u>^</u>
in		formation window 24885   gb   AY555965 . 1   Pochor	nia chi	lamvdos	poria s	train IMI	113169	internal	transcrib	ed spacer 1. 5	5.85 ribos	omal RNA	деле.	and inte	rnal tr
Ţİ	1193	TTCAACTCCCAAACCCCATGTGAAC 33108   emb   AJ291805.1   VCH2 AAAGTCGTAACAAGGTCTCCGTTGG	01805V	erticil	lium ch	lamydospo	rium 18	S rRNA ge	ene (partia.	1), ITS1, 5.85	rRNA gen	e, ITS2,	285 rR	NA gene	(partia
GJ gi	AGTA		GAACCA	AGCGGAG ascomyc	GGATCA1	TACCGAGTI	TTCAACTO	CCCAAACCC	CATGTGAACT	TATACCATTTACAA	CCGTTGCTT	CGGCGGGI 1 transc	TCTCGCC	CCGGGCTI pacer 1,	5.85

- *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

File	Hide/	Display !	ort Add/Delete Mark/	Unmark	Annealing	nfo P	rimer Comple	mentarity	Primer Setting	Probes Instar	it BLAST Help					
	rimer		Primer pair	Target pair %	Target forward %	Tar rev	Primer 3' I	er-Complem nter-Comple ra-Complem	ementarity	Target forward nnealing avg pos	Target reverse annealing avg pos		Target PCR min size			Non-targe PCR min size
1		TACCAT	TACAACCGTTGCTGGT	100	100	100		ntra-Comple		59.235	295.235	50	50	50	0	0
2		ATACCA	TTACAACCGTTGCTGGT	100	100	100 -	0	o compie		258.235	295.235	51	51	51	0	0
-	[	TATACC	TTACAACCETTECT CE	100	100	100	0	0	2 42121	257 225	205 225	60	50	52	0	0

- *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.

le	Hide/D	Display Sort Add/Delete Mark/U	Inmark .	Annealing I	nto Prin	ner Compler	mentarity	Primer Settir	ng Probes Instan	nt BLAST Help					
-			Target	Target	Target	Non-target	Non-targ	View Pr	rimer Design Setting	Target	Target	Target	Target	Non-target	Non-target
	rimer	Primer pair	pair %	forward %	reverse %	pair %	forward %	reverse %	forward annealing avg pos	reverse annealing avg pos	PCR avg size	PCR min size	PCR max size	PCR avg size	PCR min size
		TACCATTTACAACCGTTGCTGGT	100	100	100	0	0	2.42131	259.235	295.235	50	50	50	0	0
		ATACCATTTACAACCGTTGCTGGT	100	100	100	0	0	2.42131	258.235	295.235	51	51	51	0	0
		TATACCATTTACAACCGTTGCTGG	100	100	100	0	0	2.42131	257.235	295.235	52	52	52	0	0
		CCATTTACAACCGTTGCTTTTGTA	100	100	100	0	0	15.4964	261.235	363.235	124	124	124	0	0
		ACCATTTACAACCGTTGCTTTTGT	100	100	100	0	0	15.4964	260.235	363.235	125	125	125	0	0
		TACCATTTACAACCGTTGCTTTTG	100%	100	100	0%	0	15.4964	259.235	363.235	126	126	126	0	0
,		ATACCATTTACAACCGTTGCT TTT	100	100	100	0	0	15 4964	258 235	363 235	127	127	127	0	0

#### 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

	Hide/D	Display Sort Add/Delete Mark/L	Jnmark i	Annealing I	nto Prin	ner Compler	nentarity I	Primer Settin	g Probes Instar	t BLAST Help		_			
	*		Target	Target	Target	Non-target	Non-target	Non-target	Design Pro	ign Probes for marked primer pairs		Target	Target	Non-target	Non-target
	rimer	Primer pair	pair %	forward %	reverse %	pair %	forward %	reverse	forward annealing avg pos	reverse annealing avg pos		PCR min size	PCR max size	PCR avg size	PCR min size
	<b>V</b>	TACCATTTACAACCGTTGCTGGT	100	100	100	0	0	2.42131	259.235	295.235	50	50	50	0	0
2		ATACCATTTACAACCGTTGCTGGT	100	100	100	0	0	2.42131	258.235	295.235	51	51	51	0	0
	V	TATACCATTTACAACCGTTGCTGG	100	100	100	0	0	2.42131	257.235	295.235	52	52	52	0	0
	<b>V</b>	CCATTTACAACCGTTGCTTTTGTA	100	100	100	0	0	15.4964	261.235	363.235	124	124	124	0	0
		ACCATTTACAACCGTTGCTTTTGT	100	100	100	0	0	15.4964	260.235	363.235	125	125	125	0	0
		TACCATTTACAACCGTTGCTTTTG	100%	100	100	0%	0	15.4964	259.235	363.235	126	126	126	0	0
1		ATACCATTTACAACCGTTGCTTTT	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

💷 D:	/Worksp	ace/sample files/PochoniaTarget.txt	Primer Re	port												- 🗆 <mark>- X</mark>
File	Hide/D	Display Sort Add/Delete Mark/	Unmark	Annealing I	info Prin	ner Compler	mentarity	Primer Settin	g Probes	Insta	ant BLAST Help					
	rimer	Primer pair	Target pair %	Target forward %		Non-target pair %	Non-target forward %	Non-target reverse %	forwar		BLAST Forward Prim BLAST Reverse Prime annealing avg pos	er vg	Target PCR min size		Non-target PCR avg size	Von-target ^ PCR min size
1		TACCATTTACAACCGTTGCTGGT	100	100	100	0	0	2.42131	259.235		295.235	50	50	50	0	0
2		ATACCATTTACAACCGTTGCTGGT.	. 100	100	100	0	0	2.42131	258.235		295.235	51	51	51	0	0

- *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).

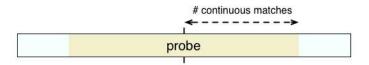


Figure 3: Illustration of selectivity setting for probes. The shaded part shows is where the exact match is required to occur.

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 <u>Extract/Load Probe Candidates</u>. 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.

Primer/Probe Design Wizard		? ×
Extract/Load Probe Candidates		
Extract probe candidates from target sequences or load user's probe candidates		
<ul> <li>Design probes based on target and non-target sequences</li> </ul>		
🔘 Load user's probe candidates		Browse
Note: If you provide your own probe candidates, the program will not consider the probe candidates extracted from target sequences		
	Next > Cancel	Help

II. Probe Property Settings

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

Primer/Probe Design Wizard
Probe Design Settings
This probe design wizard will help you to choose most of the settings for designing probes
Select from the following choices
Use all default settings (and start designing probes now)
Use previous settings
Show/change settings
< <u>B</u> ack <u>Finish</u> Cancel <u>H</u> elp

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.

Probe length range 14 to 30	Probe Tm 58 to	72 C
Min % of target sequences containing candidate probe 100 %	[Na+] concentrations	0.1 M
Only consider top 100 % (single) candidate probes	Tm of probe exceeds Tm of primer pair by 6	10 C
Have minimum of 2 bases between each of the primers and the probe	Probe GC% 25 % to	75 %
Avoid continuous     4     A's or C's or T's	Max primer-probe inter-complementarity	10
Avoid continuous     4     G's	Max probe intra-complementarity	10
✓ Avoid G at the first base from 5' end		

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

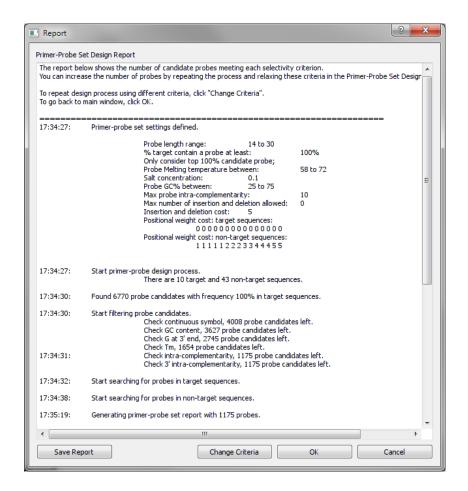
Primer/Probe Design Wizard	2 <mark>- × -</mark>
Basic Probe Selectivity Settings	
This window allows selection of the probe selectivity settings.	
The following options depict the selectivity settings for the middle part of the probes. Theoretically, highly selective probes should be obtained when both Target settings are produced using these settings, consider re-running the program after lowering the targ when designing primer-probe sets for highly conserved sequences such as rRNA genes	et settings and/or increasing the non-target settings. This problem will be most noticeable
	# continuous matches
pi pi	robe
Selectivity settings for target sequences	Selectivity settings for non-target sequences
Target           Continuous         15         matches at the center to bind to target sequences                       Low         High	Non-target           Continuous
Select from the following choices <ul> <li>Use default mismatch settings (and start designing probes now)</li> <li>Show/change mismatch settings</li> </ul>	
	< Back Einish Cancel Help

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 <u>Advanced Probe Selectivity Settings</u> 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 mismatch cost matrix determines the penalties on the mismatched nucleotides in primer-template duplex. The arger the value, the less likely a PCR product will be made.	*		0 1 1	0	0	0	м	sertio ax # ( sertio	gaps a	llowe	d						0	•
The mismatch cost matrix and the positional mismatch	-		A	с	G	т												
Ambiguous base cost function																		
<ul> <li>Distance: More likely to penalize ambiguous bases from target/non-target</li> <li>Binary: Less likely to penalize ambiguous bases from target/non-target</li> <li>Positional mismatch allowance settings</li> <li>Target sequence</li> </ul>	get se	quenc	es		2	3	4	5	6	7	8	9 1	0 11	12	13	14	15	
5'- 0 0 0 0 0 0 0 0 0 0 0 0	0 0	0 0	0		0	0	0	0	0	0	0	9 1 0 (		0	0	0	0	-3'
Non-target sequence																		
<b>5</b> '- <b>5 5 4 4 4 3 3 2 2 2 1 1</b>	1 1	L 1	1	1	2	3	4	5	6 2	7	8 2	9 1 3 3	0 11	12 4	13 4	14 5	15 5	-3'
The values in the left and right halves are symmetric and non-decreasin to make changes, enter a desired value in the right half. Other values w	ig awa	ay from	the ed au	cente	r. tically							Restor	e to de	fault i	mismal	tch all	owand	te setting

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.

ATTIA	TTACAACCGTTGCTAAATCTAGAGTTTGGGT 🗵	ATTTAC	AACCGTTG	CTAAATCTA	GAGTTTGGG		сөттөс	TATCTA	GAGTTTGGGTC		TACAACCGTTGCTAATC	TAGAGTTTGGGTC
	Probe	Target ter-probe %		Non-target mer-probe %	Non-target probe %	Target probe annealing avg pos		Probe Tm(C)		Probe intra comple- mentarity	<pre># of target sequence:</pre>	# of ambiguous b in target sequen that probe binds
1	CCGGGCTTTACACCC	100.00	100.00	11.63	100.00	92.20	66.67	58.90	7	8	0	
2	CCCGGGCTTTACACC	100.00	100.00	11.63	100.00	91.20	66.67	58.90	7	6	0	
< Prim	mer-Probe Set Information Windo	ow			III							,
	mer-Probe Set Information Windo	ow			m							,
	mer-Probe Set Information Windo	ow			m							,
	mer-Probe Set Information Windo	ow			m							,

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

	imer-Probe Set Report Window											
File	Hide/Display Sort Add/Delete M	lark/Unmark	Annealin	g Info Cor	mplementari	ty Primer Pair F	Probe S	etting	Instant BLAS	ST Help		
	Save Primer-Probe Set List (.ppset File) Save Primer-Probe Set List as Tab Delimit	ed File (Excel)	ттост.	.AAATCTAGA	аттадат 🗵	ATTTACAACCGT	TGCTA	AATCTAG	SAGTTTGGG	CATTTA	CAACCGTTGCTATCTAG	AGTTTGGGTC 🗵 🖣
	Save Information Window Content Save Primer Pair and Probe Seqs Only		rget obe	Non-target mer-probe	Non-target probe	Target probe	Probe	Probe	Inter comple-	Probe intra comple-	¥ of target sequence: (out of 10) /ith ambiguous base	# of ambiguous ba in target sequence
	Exit		%	%	%	annealing avg pos	GC%	Tm(C)	mentarity	mentarity	covered by probe	that probe binds t
1	CCGGGCTTTACACCC	100.00	100.00	11.63	100.00	92.20	66.67	58.90	7	8	0	
1		100.00	100.00	11.63	100.00	91.20	66.67	58.90	8	6	0	

- 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 userselected 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)<sup>2</sup>
    - +(% of non-target sequences estimated to anneal with whole primer-probe set)<sup>2</sup>
    - +  $\frac{1}{2}$  (100 % of non-target sequences estimated to anneal with probe)<sup>2</sup>
    - + 0.25 (% of non-target sequences estimated to anneal with probe)<sup>2</sup>.

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

I Sort	? 💌
Sort by	
Selectivity Formula	Ascending     Oescending
Then by	
Target pair %	Ascending     Oescending
Then by	
	Ascending     Descending
Then by	
	Ascending     Oescending
	OK Cancel

#### 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.

le ATI		ACCGTTGCTAATC		Mark/Unmark ner-Probe Sets rimer-Probe Se	Manually	Ā	Complementar	,		AAATCTA	Instant BLA		CAACCGTTGCTATCTAG	AGTTTGGGTC 🗵
	Ť			1arked Primer-F eleted Primer-P 70			get Non-target be probe %	Target probe annealing avg pos		Probe Tm(C)	Inter comple- mentarity	Probe intra comple- mentarity	<ul> <li>* of target sequence: (out of 10)</li> <li>/ith ambiguous base covered by probe</li> </ul>	# of ambiguous b in target sequen that probe binds
1	1	CCGGGCTTTAC	ACCO	100.0	0 100.00	11	.63 100.00	92.20	66.67	58.90	7	8	0	
2		CCCGGGCTTTA	САСС	100.0	0 100.00	11	.63 100.00	91.20	66.67	58.90	8	6	0	

• 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.

le	Hide	e/Display So	rt 🗛	dd/Delete	Mark/Unmark	Annealing Ir	nfo Cor	mplementari	ity Primer Pair P	Probe S	etting	Instant BLAS	ST Help		
AT	TTACA	ACCGTTGCTAA	πC		imer-Probe Sets M Primer-Probe Sets	1 A A		GTTTGGGT 🗵	ATTTACAACCGT	TGCT	AATCTA	GAGTTTGGG	CATTTA	CAACCGTTGCTATCTAG	AGTTTGGGTC 🗵 🖣
			F		Marked Primer-Pro Seleted Primer-Pro 70		rge1	Non-target probe %	Target probe annealing avg pos		Probe Tm(C)	Inter comple- mentarity	Probe intra comple- mentarity	(out of 10) vith ambiguous base	# of ambiguous b in target sequent that probe binds
1		CCGGGCTTT	ACAC	сс	100.00	100.00	11.63	100.00	92.20	66.67	58.90	7	8	covered by probe	
2		CCCGGGCTT	TACA	cc	100.00	100.00	11.63	100.00	91.20	66.67	58.90	8	6	0	

#### 5.6 Mark/Unmark menu

• *Mark Selected Primer-probe Sets:* Allows selected sets to be marked. Marked primerprobe sets are designated by a check mark in the box in the second column (and a yellowhighlighted row). Marked sets can be saved in the PRISE2 program format or tabdelimited 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.

TITACAACCGTTGCTAATCTAGAGTTTGGGT 🔀	ATTTACAA	ссөттөст	.AAATCTAGA	GTTTGGGT 🗵	ATTTACAACCGT	IGCTA	AATCTA	GAGTTTGGG		CAACCGTTGCTATC	CTAGAGTTTGGGTC
Probe	Target Ier-probi %	Target probe %	Non-target ner-probe %	Non-targe1 probe %	Target probe annealing avg pos		Probe Tm(C)	Inter comple- mentarity	Probe intra comple- mentarity	of target seque (out of 10) rith ambiguous covered by pro	in target seque
1 CCGGGCTTTACACCC	100.00	100.00	11.63	100.00	92.20	66.67	58.90		7 8	3	0
	100.00	100.00	11.63	100.00	91.20	66.67	58.90		8 6	i	0
				III							
Primer-probe set annealing po		nformat	tion A		CGTTGCT[CCC	GGGC1	TTTAC	ACC]AA	TCTAGAGTT:	IGGGT	
Primer-probe set annealing po		nformat	tion A		CGTTGCT[CCC	GGGC1		ACC]AA		TGGGT Rv primer P(	CR size
Primer-probe set annealing po Target sequences: >Seed sequence PochoniaSeed	sition i			TTTACAAC		GGGC1	Fw 17-	primer . -32	Probe 48-62	Rv primer P0 88-103 8	7
Primer-probe set annealing po Target sequences: >Seed sequence PochoniaSeed gi 13160418 emb AJ292397.1 V	sition i. CH292397	Vertici	llium ch	<i>TTTACAAC</i>	porium var.	GGGC1	Fw 17- 44-	primer -32 -59	Probe 48-62 75-89	Rv primer P0 88-103 8 115-130 8	7 7
Primer-probe set annealing po Target sequences: >Seed sequence PochoniaSeed gi 13160418 emb AJ292397.1 V gi 11933056 emb AJ291800.1 V	sition i CH292397 CH291800	Vertici Vertici	llium ch llium ch	TTTACAAC lamydosp lamydosp	porium var. porium 185	GGGC1	Fw 17- 44- 94-	primer . -32 -59 -109	Probe 48-62 75-89 125-139	Rv primer P0 88-103 8 115-130 8 165-180 8	7 7 7
Primer-probe set annealing po Target sequences: >Seed sequence PochoniaSeed ygi 13160418 emb AJ292397.1 V >gi 118627604 emb AJ291800.1 V ygi 118627604 emb AM412780.1	sition i CH292397 CH291800 Verticil	Vertici Vertici lium ch	llium ch llium ch lamydosp	TTTACAAC lamydosp lamydosp porium pa	oorium var. oorium 185 ortial 185	GGGC1	Fw 17- 44- 94- 82-	primer . -32 -59 -109 -97	Probe 48-62 75-89 125-139 113-127	Rv primer P0 88-103 8 115-130 8 165-180 8 153-168 8	7 7 7
Primer-probe set annealing po Target sequences: >Seed sequence PochoniaSeed qi 13160418 emb AJ292397.1 V ygi 11933096 emb AJ291800.1 V ygi 119627604 emb AM412780.1  qi 159797389 gb AY212487.1 Po	sition i CH292397 CH291800 Verticil chonia c	Vertici Vertici lium ch hlamydd	llium ch llium ch lamydosp osporia i	TTTACAAC lamydosp lamydosp orium pa solate P	oorium var. oorium 185 rtial 185 2c 472 185	GGGC1	Fw 17- 44- 94- 82- 92-	primer . -32 -59 -109 -97 -107	Probe 48-62 75-89 125-139 113-127 123-137	Rv primer P0 88-103 8 115-130 8 165-180 8 153-168 8 163-178 8	7 7 7 7
Primer-probe set annealing po Target sequences: >Seed sequence PochoniaSeed ggi13160418 emb AJ292397.1 V ggi1193306[emb AJ291800.1 V ggi118627604 emb AM412780.1 ggi5892741]gb AY912487.1 Po ggi158892741]gb AY903605.1 Po	sition i CH292397 CH291800 Verticil chonia c chonia c	Vertici Vertici lium ch hlamydd hlamydd	llium ch llium ch lamydosp osporia i osporia 1	TTTACAAC lamydosp lamydosp vorium porium solate P 85 ribos	oorium var. oorium 185 irtial 185 c 472 185 somal RNA g	GGGC1	Fw 17- 44- 94- 82- 92- 56-	primer . -32 -59 -109 -97 -107 -71	Probe 48-62 75-89 125-139 113-127	Rv primer P0 88-103 8 115-130 8 165-180 8 153-168 8	7 7 7 7 7
Primer-probe set annealing po Target sequence: >Seed sequence PochoniaSeed sqi13160418 emb AJ292397.1 V >gi118627604 emb AJ291800.1 V sqi118627604 emb AW412780.1  sqi158992741 gb AY912487.1 Po >gi158892741 gb AY912487.1 Po sqi11933103 emb AJ291803.1 V	cH292397 CH291800 Verticil chonia c. chonia c. CH291803	Vertici Vertici lium ch hlamydd hlamydd Vertici	llium ch llium ch alamydosp osporia i osporia 1 llium ch	ITTTACAAC lamydosp lamydosp oorium pa solate P 88 ribos lamydosp	oorium var. Norium 185 Irtial 185 De 472 185 Iomal RNA g Norium 185	GGGC	Fw 17- 44- 94- 82- 92- 56- 94-	primer -32 -59 -109 -97 -107 -71 -109	Probe 48-62 75-89 125-139 113-127 123-137 87-101	Rv primer P( 88-103 8 115-130 8 165-180 8 153-168 8 163-178 8 127-142 8	7 7 7 7 7 7
rimer-Probe Set Information Wir Primer-probe set annealing po Target sequences: >Seed sequence PochoniaSeed >gi 13160418 emb AJ292397.1 v >gi 13827604 emb AV412780.1 v >gi 193797389 gb AY912487.1 Po >gi 58892741 gb AY912487.1 Po >gi 1393103 emb AJ291803.1 v >gi 13508841 emb AJ303054.1 v >gi 13935966.1 v	CH292397 CH291800 Verticil. chonia c. CH291803 CH303054	Vertici Vertici lium ch hlamydd hlamydd Vertici Vertici	llium ch llium ch alamydosp osporia i sporia l llium ch	TTTACAAC lamydosp lamydosp vorium pa solate P 88 ribos lamydosp lamydosp	oorium var. oorium 185 rtial 185 oo 472 185 oorium 185 oorium 185 oorium var.	GGGC	Fw 17- 44- 94- 92- 56- 94- 45-	primer . -32 -59 -109 -97 -107 -71 -109 -60	Probe 48-62 75-89 125-139 113-127 123-137 87-101 125-139	Rv primer P0 88-103 8 115-130 8 165-180 8 153-168 8 163-178 8 127-142 8 165-180 8	7 7 7 7 7 7 7

• 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.

Dialog	? <mark>×</mark>
Settings for the percentage of each nucleotide in non-target sequences in relation to the primer sequences. Note that these primer annealing settings should be less stringent than those used for primer design.	
Divide primer into two parts and set the minimum match percentages:	· · · · · · · · · · · · · · · · · · ·
at least 50 % matches on 5' end	at least 62.5 % matches on 3' end
	OK Cancel

Hide/[	Display Sort /	Add/Delete M	/lark/Unmark	Annealing Info	Complement	arity Primer P	air Probe Set	ting Instar	t BLAST He	Р		
ПТАСААС	COTTOCTAATCTA		ATTTACAA	Primer-Pr	obe Set Annealin	ng Position Infor	mation					
TIACAAC			AITIACAA	Percentag	e of Each Nucleo	otide in Target a	nd Non-target	Sequences in	Relation to Pri	mers and Probe	e Sequences	
×	Pro	obe	Target Ier-probi %	Target Sec	uences Annealir uences Not Ann et Sequences An	nealing with Prin	ner-Probe Set					ambiguous arget seque t probe bin
	CCGGGCTTTACAG	cc	100.00	Non-targe	t Sequences No	t Annealing wit	h Primer-Probe	Set				
2 🗖 🤇	CCCGGGCTTTACA	ACC	100.00	100.00	11.63 100.0	00	91.20 66.67	58.90	8	6	0	
	Probe Set Inf					"			and probe		in formatio	
Percen	Probe Set Inf stage of each get sequence	n nucleoti	de in targ		target sequ		elation to	o primer	and probe	sequences	informatic	on for pr
Percen 10 tar	atage of each get sequence mation for ta	n nucleoti es and 43 arget sequ	de in targ non-target ences anne	sequences. aling with	target sequ primer-prol	uences in r be set: (10	) sequences	;)	-	·		
Percen 10 tar	atage of each get sequence mation for ta A	n nucleoti es and 43 arget sequ T	de in targ non-target ences anne T	sequences. aling with T	target sequ primer-prol A	uences in r be set: (10 C	) sequences A	5) A	с	с	G	n for pr T O%
Percen 10 tar	atage of each get sequence mation for ta	n nucleoti es and 43 arget sequ	de in targ non-target ences anne	sequences. aling with	target sequ primer-prol	uences in r be set: (10	) sequences	;)	-	·		Т
Percen 10 tar Inform	ation for ta A 100% 0% 0%	n nucleoti es and 43 arget sequ T O% O% O%	de in targ non-target ences anne T O% O% O%	sequences. aling with T O% O% O%	target sequ primer-prol A 100% 0% 0%	uences in r be set: (10 0% 100% 0%	) sequences A 100% 0% 0%	A 100% 0% 0%	C 0% 100% 0%	С 0% 100% 0%	G 0% 0% 100%	T 0% 0% 0%
Percen 10 tar Inform	atage of eacl aget sequence nation for ta A 100% 0%	n nucleoti es and 43 arget sequ T 0% 0%	de in targ non-target ences anne T O% O%	sequences. aling with T O% O%	target sequ primer-prol A 100% 0%	uences in 1 be set: (10 C 0% 100%	) sequences A 100% 0%	5) A 100% 0%	С 0% 100%	С 0% 100%	G 0 % 0 %	T 08 08

• *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.

ITTACAACCGTTGCT.AATCTAGAGTTTGGGT       ATTTACAA         Primer-Probe Set Annealing Position Information       Percentage of Each Nucleotide in Target and Non-target Sequences in Relation to Primers and Probe Sequences       Immediate and Non-target Sequences Annealing with Primer-Probe Set         Immediate       Probe       Immediate and Non-target Sequences Annealing with Primer-Probe Set       Immediate and Non-target Sequences Annealing with Primer-Probe Set       Immediate and Non-target Sequences Non-ta		r-Probe Set Report Window de/Display Sort Add/Delete Ma	rk/Unmark	Annealing Info Complementarity Primer Pair Probe Setting Instant BLAST Help			
Probe       Target Sequences Annealing with Primer-Probe Set       ambiguous arget seque t probe bin         1       CCCGGGCTTTACACCC       100.00       11.63       100.00       91.20       66.67       58.90       8       6       0         2       CCCGGGCTTTACACC       100.00       11.63       100.00       91.20       66.67       58.90       8       6       0         4       III       IIII       CCCGGGCTTTACACC       100.00       11.63       100.00       91.20       66.67       58.90       8       6       0         4       IIII       IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII				Primer-Probe Set Annealing Position Information	IGGGTC 🗶		
Probe       ier-probi       Target Sequences Not Annealing with Primer-Probe Set       probe bind         1       CCCGGGCTTTACACCC       100.00       100.00       11.63       100.00       91.20       66.67       58.90       8       0         2       CCCGGGCTTTACACCC       100.00       11.63       100.00       91.20       66.67       58.90       8       0         4       III       IIII       IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	v						
Image: CCCGGGCTTTACACCC       100.00       Non-target Sequences Not Annealing with Primer-Probe Set         Image: CCCGGGCTTTACACCC       100.00       11.63       100.00       91.20       66.67       58.30       8       6       0         Image: CCCGGGCTTTACACCC       100.00       11.63       100.00       91.20       66.67       58.30       8       6       0         Image: CCCGGGCTTTACACCC       100.00       11.63       100.00       91.20       66.67       58.30       8       6       0         Image: CCCGGGCTTTACACCC       100.00       11.63       100.00       91.20       66.67       58.30       8       6       0         Image: CCCGGGCTTTACACCC       100.00       11.63       100.00       91.20       66.67       58.30       8       6       0         Image: CCCGGGCTTTACACCC       100.00       11.63       100.00       91.20       66.67       58.30       8       6       0         Image: CCCGGGCTTTACACCCC       100.00       11.63       100.00       11.63       100.00       91.20       66.67       58.30       8       6       0         Image: CCCGGCTTTACACCCCGGCGCTTTACACCCCGGCGCTTGCGCCGGGGGACCAAACCCCCGGGGGGACCCAAACCCCCGGGGGG		Probe		Target Sequences Not Annealing with Primer-Probe Set			
CCCCGGGCTTTACACC       100.00       11.63       100.00       91.20       66.67       58.90       8       6       0         Image: Sequences represented and the sequences of t			%				
""         rimer-Probe Set Information Window         rgi 11933103 emb AJ291803.1 VCH291803Verticillium chlamydosporium 185 rRNA gene (partial), ITS1, 5.85 rRNA gene, I         rgAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCAAAACCCCATGTGAACTTAACAACCGTTGCTTCGGCGGGATCCTCGCC         rgi 13508841 emb AJ303054.1 VCH303054Verticillium chlamydosporium var. chlamydosporium 5.85 rRNA gene and ITS 1 an         rattaccGAGTTTTCAACTCCCAAACCCCCATGTGAACTATACCATTTACAACCGTTGCTTCGGCGGGGTTCTCGCCCCGGGGCTTTACACCCCGGAACCAGGCGGCCCGCCGGGGGGACCCAAACTC         rgi 49424886 gb AY555966.1 Verticillium catenulatum strain IMI 113078 internal transcribed spacer 1, 5.85 ribosoma         rcGaGTTTTCAACTCCCCAAACCCCATGTGAACTTATACCATTTACAACCGTTGCTCGGCCGGGTTCTCGCCCCGGGCTTTACACCCGGGGCCCCGCGGGGGACCCAAACTCTAGAT         rgi 4836220 gb AF108468.1 Pochonia chlamydosporia isolate ARSEF 2218 internal transcribed spacer 1, complete seque		CCGGGCTTTACACCC	100.00	Non-target Sequences Not Annealing with Primer-Probe Set			
rimer-Probe Set Information Window  primer-Probe Set Information  primer-Probe Set Informa	2	CCCGGGCTTTACACC	100.00	100.00 11.63 100.00 91.20 66.67 58.90 8 6 0			
GAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCAAACCCCATGTGAACTTATACCATTTACAACCGTTGCTTCGGCGGGGTTCTCGCC catil 13508841 emb AJ303054.1 VCH303054Verticillium chlamydosporium var. chlamydosporium 5.85 rRNA gene and ITS 1 an catTACCGAGTTTTCAACTCCCAAACCCCATGTGAACTTATACCATTTACAACCGTTGCTTCGGCGGGGTCTCGGCCCGGGGCTTTACACCCCGGAACCAGGCGGCCCGCGGGGGACCCAAACTC catTACCGAGTTTTCAACTCCCAAACCCCATGTGAACTTATACCATTTACAACCGTTGCTTCGGCGGGGTCTCGGCCCGGGGCTTTACACCCCGGAACCAGGCGGCCCGCCGGGGGACCCAAACTC catgi 49424886 gb AY555966.1 Verticillium catenulatum strain IMI 113078 internal transcribed spacer 1, 5.85 ribosoma ccGAGTTTTCAACTCCCAAACCCCATGTGAACTTATACCATTTACAACCGTTGCTTCGGCGGGGTTCTCGCCCCGGGCTTTACACCCCGGAACCAGGCGGCCCGCCGGGGGACCCAAACTCTAGAT ccGAGTTTTCAACTCCCCAAACCCCATGTGAACTTATACCATTTACAACCGTTGCTTCGGCGGGGTTCTCGCCCCGGGGCTTTACACCCCGGAACCAGGCGGCCCCCCAACTCTAGAT ccGAGTTTTCAACTCCCCAAACCCCATGTGAACTTATACCATTTACAACCGTTGCTTCGGCGGGGTTCTCGCCCCGGGGTTTACACCCCGGAACCAGGCGGCCCCCCGGGGGACCCAAACTCTAGAT ccGAGTTTTCAACTCCCAAACCCCATGTGAACTTATACCATTTACAACCGTTGCTTCGGCCGGC	1			m			
	۲ rime	er-Probe Set Information Wi	ndow	III.			

- *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

	Display Sort Add/Delete Mar	k/Unmark	Annealin	g Info Co	mplementari	ty Primer Pair P	robe S	etting	Instant BLAS	ST Help				
TTACAAC	CGTTGCTAATCTAGAGTTTGGGT 🗵	ATTTACAA	ссөттөст.	.AAATC		a-Complementarity be Set Inter-Comple	menta	rity	SAGTTTGGG D	CATTTAC	CATTTACAACCGTTGCTATCTAGAGTTTGGGTC			
Ť	Probe	Target Ier-probi %		Non-target ner-probe %	Non-target probe %	Target probe annealing avg pos		Probe Tm(C)	Inter comple- mentarity	Probe intra comple- mentarity	of target sequence (out of 10) /ith ambiguous base covered by probe	<pre># of ambiguous in target seque that probe bine</pre>		
	CGGGCTTTACACCC	100.00	100.00	11.63	100.00	92.20	66.67	58.90	7	8	0			
2 🗌 🔿	CCGGGCTTTACACC	100.00	100.00	11.63	100.00	91.20	66.67	58.90	8	6	0			
robe .	intra-complementarity													
5'-CCC	probe intra-complementa GGGCTTTACACC-3'         CCACATTTCGGGCCC-5'	rity 6.												

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

#### 5.9 Primer Pair menu

	CCGTTGCTAATCTAGAGTTTGGGT 🗵	ATTTACAA	ссаттаст	AAATCTAGA	GTTTGGGT 🗵	ATTTACAACCGT	TGCTA	AATCTA	GAGTTTGGG	CATTTA	CAACCGTTGCTATCTAG	AGTTTGGGTC 🗵
*	Probe	Target ıer-probi %		Non-target ner-probe %	Non-target probe %	Target probe annealing avg pos			Inter comple- mentarity	Probe intra comple- mentarity	of target sequence (out of 10) rith ambiguous base covered by probe	¥ of ambiguous b in target sequer that probe bind
1	CCGGGCTTTACACCC	100.00	100.00	11.63	100.00	92.20	66.67	58.90	7	8	0	
2	CCCGGGCTTTACACC	100.00	100.00	11.63	100.00	91.20	66.67	58.90	8	6	0	
۲ Primer	Probe Set Information W	ndow			m							
Primer- Primer Lei	TM ft 49.025	ndow			m							
Primer Primer Let Rig	TM Ft 49.025 Tht 49.025	ndow			m							
Primer Primer Lei Rig Primer Lei	: TM Et 49.025 ht 49.025 : GC Et 37.5	ndow			m							
Primer Lei Rig Primer Lei Rig	TM t 49.025 th 49.025 t 37.5 th 37.5 th 37.5	ndow			m							
Primer Lei Rig Primer Lei Rig Comple	TM t 49.025 t 6C t 37.5 tht 37.5 mentary t Intra 8	ndow			m							
Primer- Lei Rig Primer Lei Rig Comple Lei	TM t 49.025 sGC t 37.5 sht 37.5 st 37.5 st 37.5 st 37.5 st 10.5 t Intra 8 t Intra 8 t Intra 8 t Intra 8	ndow										
Primer- Let Rig Primer Let Rig Comple Let Let	TM t 49.025 t 6C t 37.5 tht 37.5 mentary t Intra 8	ndow			m							

• 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.)

Probe Design Settings - Review	? <mark>×</mark>
Probe length range     14     to     30       Min % of target sequences containing candidate probe     100     %       Only consider top     100     % (single) candidate probes       Have minimum of     2     bases between each of the primers and the probe	Probe Tm         58         to         72         C           [Na+] concentrations         0.1         M           Tm greater than the corresponding primer pair by         6         to         10         C
Avoid continuous 4 A's or C's or T's	Probe GC% 25 % to 75 %
Image: Way of the second se	Max probe inter-complementarity
Ambiguous base cost function Distance: (i) More likely to penalize ambiguous bases from target/non-target sequences	Max primer intra-complementarity 10
Binary: Cless likely to penalize ambiguous bases from target/non-target sequences	Insertion/deletion costs Max # gaps allowed:       0     Insertion / deletion cost:
Mismatch cost matrix Target sequence:	
A 0 30 29 28 27 26 25 24 23 22 21 20 1 5'- 0 0 0 0 0 0 0 0 0 0	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$
G 1 1 0 Non-target sequence:	
	9 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 1 1 1 1 1 1 1 1 2 2 2 3 3 4 4 4 5 5 <sup>-3'</sup>

### 5.11 Instant BLAST menu

Hid	le/Display Sort Add/Delete Ma	rk/Unmark	Annealin	g Info Cor	nplementari	ity Primer Pair F	Probe S	etting	Instant BLAS	ST Help		
v	Probe	Target Ier-probi %		Von-targei ner-probe %	Von-target probe %	Target probe annealing avg pos		Probe Tm(C)	Inter comple- mentarity	Probe intra comple- mentarity		¥ of ambiguous b in target sequen that probe binds
1	CCGGGCTTTACACCC	100.00	100.00	11.63	100.00	92.20	66.67	58.90	7	8	0	
2	CCCGGGCTTTACACC	100.00	100.00	11.63	100.00	91.20	66.67	58.90	8	6	0	

• *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 <u>Primer Selectivity Settings</u> 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).

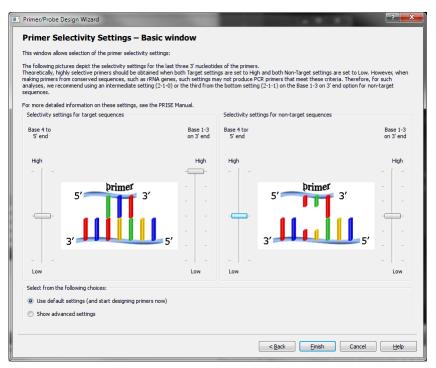


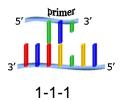
Figure 3. An example of basic Primer Selectivity Settings

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 2<sup>nd</sup> and 3<sup>rd</sup> 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.



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 primerprobe 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.

Primer/Probe Design Wizard	? <mark>- x -</mark>
Basic Probe Selectivity Settings	
This window allows selection of the probe selectivity settings.	
The following options depict the selectivity settings for the middle part of the probes. Theoretically, highly selective probes should be obtained when both Target settings are produced using these settings, consider re-running the program after lowering the targe when designing primer-probe sets for highly conserved sequences such as rRNA genes.	set to High and both Non-target settings are set to Low. If no primer-probe sets are at settings and/or increasing the non-target settings. This problem will be most noticeable
	# continuous matches
pr	obe
Selectivity settings for target sequences	Selectivity settings for non-target sequences
Target	Non-target
Continuous 15 matches at the center to bind to target sequences	Continuous 0 matches at the center to bind to non-target sequences
· · · · · · · · · · · · · · · · · · ·	Q
Low High	Low High
Select from the following choices	
Ose default mismatch settings (and start designing probes now)	
Show/change mismatch settings	
	< Back Finish Cancel Help

Figure 5. Basic Probe Selectivity Setting Page

Target sequence																
5'-00000000000000000	1 0	2	3 0	4 0	5 0	6 0	7 0	8 0	9 0	10 0	11 0	12 0	13 0	14 0	15 0	-3'
Non-target sequence																
<b>5</b> - <b>55444332211111</b>	1	2	3	4	5	6 2	7	8	9 3	10 3	11 4	12 4	13 4	14 5	15 5	-3'

Figure 6. Default Probe Selectivity Settings

А	0			
С	1	0		
G	2	1	0	
Т	1	3	1	0
	А	С	G	Т

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 1<sup>st</sup> to 3<sup>th</sup> 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 4<sup>th</sup> to 7<sup>th</sup> base from the center ( 4<sup>th</sup> , 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> positions and their symmetric positions);

(3) One G-A mismatch on the  $10^{th}$  to  $13^{th}$  base with no mismatch from the  $1^{st}$  to  $6^{th}$  base, or one C-A, G-C, T-A or T-G mismatch on the  $1^{st}$  to  $9^{th}$  base with at most one C-A, G-C, T-A or T-G mismatch are allowed on the  $10^{th}$  to  $13^{th}$ . 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.