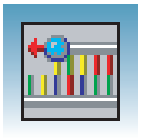
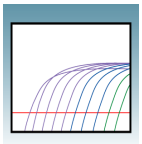
Applied Biosystems

Getting Started Guide

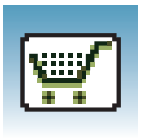


Before You Begin



Designing Primers and Probes for Quantification Assays

Designing Primers and Probes for Allelic Discrimination Assays



Ordering Primers and Probes

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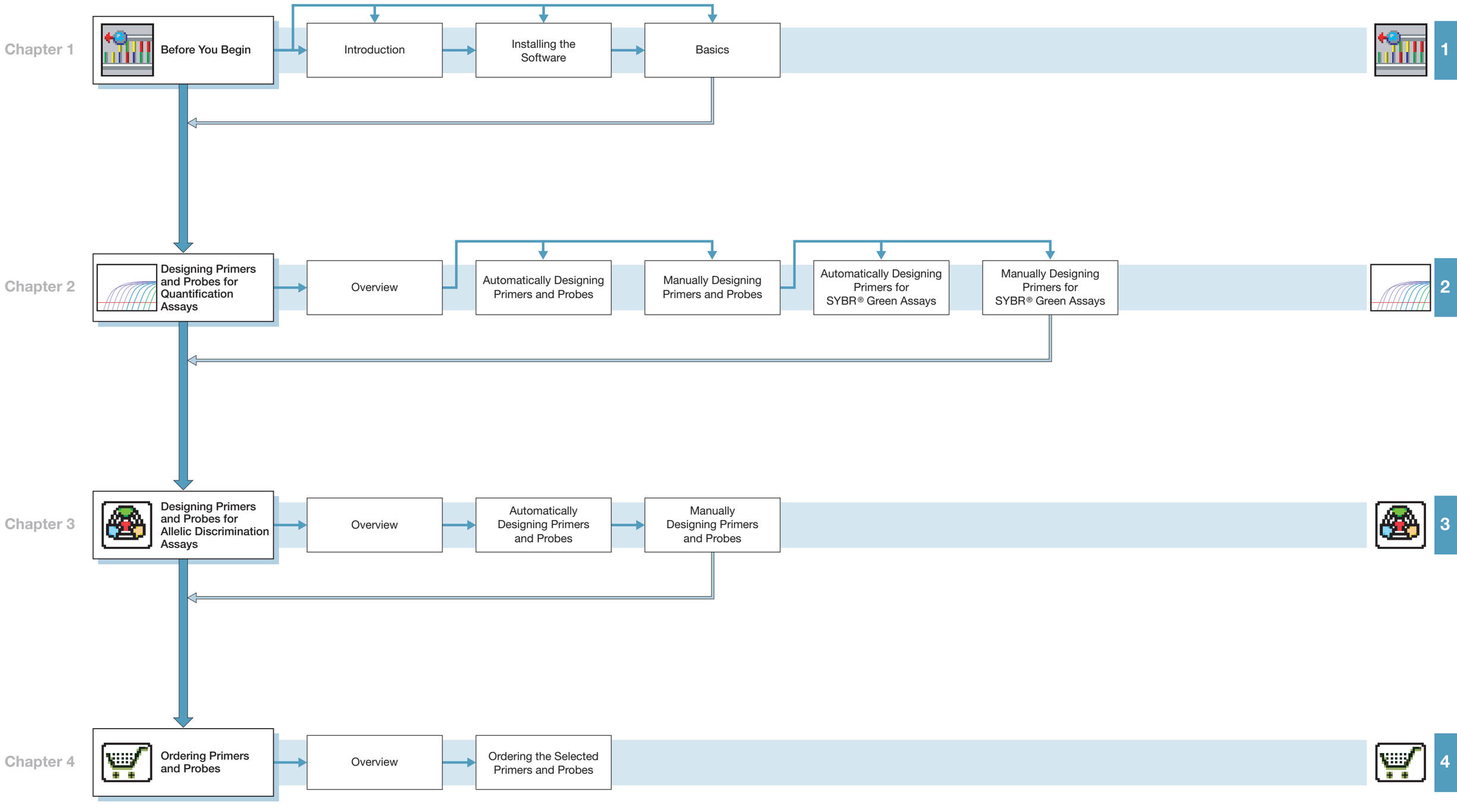
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Preface

How to Use This Guide

Purpose of This Guide

The *Primer Express® Software Version 3.0 Getting Started Guide* provides instructions for automating the primer and probe design for Quantification and Allelic Discrimination assays. It also explains how to manually annotate sequences and design customized primer/prober sets.

Audience

This guide is written for principal investigators and laboratory staff with general knowledge of PCR and realtime-PCR terminologies and applications.

Assumptions

This guide assumes that you have:

- A working knowledge of the assays
- Knowledge of primer and probe definitions
- Familiarity with Microsoft® Windows® XP operating systems

Text Conventions

- **Bold** indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow bracket (>) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File > Open > Spot Set**.

User Attention Words

Two user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note: Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! Provides information that is necessary for proper instrument operation, accurate chemistry kit use, safe use of a chemical, or proper software use.

Examples of the user attention words appear below:

Note: The size of the column affects the run time.

IMPORTANT! To verify your client connection to the database, you need a valid Oracle user ID and password.

How to Obtain More Information

For more information about using Primer Express Software, refer to the comprehensive on line help system, which includes context-sensitive help and detailed procedures for performing tasks. The help system can be invoked by pressing the F1 key anywhere in the software.

Send Us Your Comments

Applied Biosystems welcomes your comments and suggestions for improving its user documents. You can e-mail your comments to:

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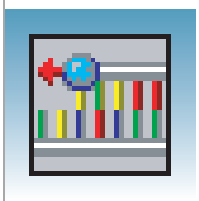
How to Obtain Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches



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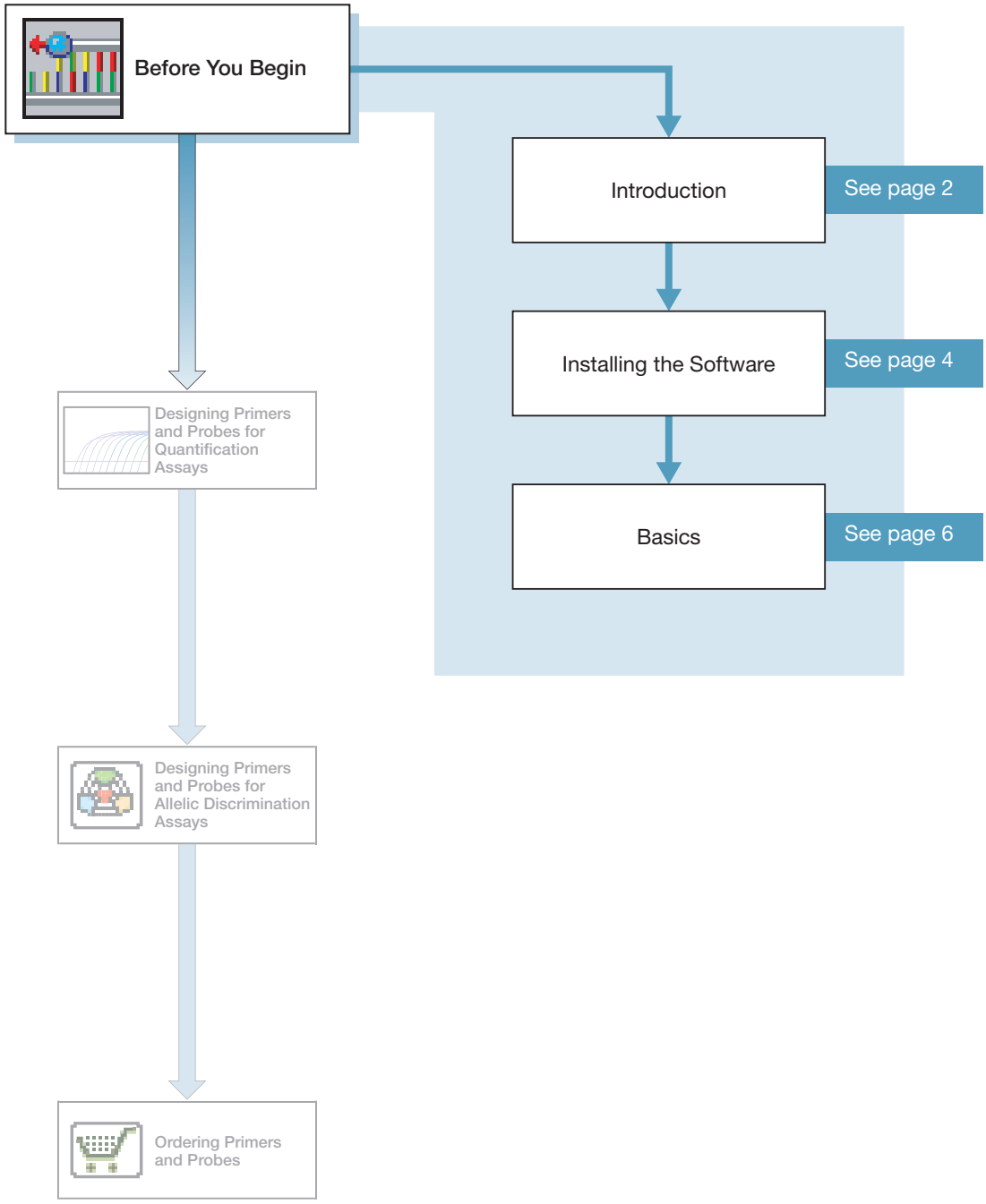


Notes

Introduction

About Primer Express® Software

Primer Express software is a primer and probe design tool made specifically for use with the following instruments:

- Applied Biosystems 7900HT Fast Real-Time PCR System
- Applied Biosystems 7500 Fast Real-Time PCR System
- Applied Biosystems 7500 Real-Time PCR System
- Applied Biosystems 7300 Real-Time PCR System

Primer Express software lets you independently design oligonucleotides (oligos) for PCR applications using a customized application specific document for each of the following assay types:

- Absolute/Relative Quantification
- Allelic Discrimination

When using Primer Express software, keep in mind the Applied Biosystems Rapid Assay Development Guidelines that contain the following important components:

- Design of primers and probes using Primer Express software
- Selection of the appropriate reagent configuration (TaqMan® Universal PCR Master Mix or SYBR® Green PCR Master Mix)
- Use of universal thermal cycling parameters
- Use of default primer and probe concentrations (or optimizing, if necessary)

IMPORTANT! These components provide a rapid and reliable system for assay design and optimization only when used in their entirety. Due to the interdependence of many of the individual components, the system must be adopted as a whole in order to achieve the highest level of success.

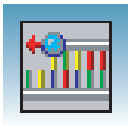
Terms You Need to Know

Allelic Discrimination Assay – An assay that discriminates between two alleles of single nucleotide polymorphisms (SNPs). TaqMan® allelic discrimination assays use two probes specific for the two possible SNP variants.

Anti-Sense Strand – In double-stranded DNA, the strand that does not code for the RNA, and is not translated into proteins. Also referred to as anti-coding, negative, or reverse strand. The Primer Express Software designs primers and probes using the sense strand, not the anti-sense strand.

Document – In the Primer Express Software, a container used to hold sequences, generate candidate primer and probe designs, and order candidate primer and probe designs. The four document types available are TaqMan MGB Quantification, TaqMan Quantification, TaqMan MGB Allelic Discrimination, and TaqMan Allelic Discrimination.

Notes



Primer – A complementary oligonucleotide that initiates amplification of a target region of DNA. A forward primer anneals to the anti-sense strand. A reverse primer anneals to the sense strand.

Probe – A short oligonucleotide sequence that anneals specifically to a target sequence and serves as a fluorescence monitoring system for DNA amplification.

TaqMan® MGB Probe – An oligonucleotide with a reporter fluorescent dye attached to the 5′ end and a non-fluorescent quencher attached to the 3′ end. The probe is coupled with a minor groove binder (MGB), which increases its Tm. When the probe is cleaved by the DNA polymerase during the PCR reaction, reporter dye fluorescence increases proportional to the quantity of the target sequence.

TaqMan Probe – An oligonucleotide with a reporter fluorescent dye attached to the 5′ end and a quencher fluorescent dye (usually TAMRA™) attached to the 3′ end. When the probe is cleaved by the DNA polymerase during the PCR reaction, reporter dye fluorescence increases proportional to the quantity of the target sequence.

Quantification Assay – An assay that determines the relative or absolute quantity of target sequence within a sample. Relative quantification measures the change in the expression of the target gene in a test sample, relative to a calibrator sample. Absolute quantification uses a standard curve to calculate the quantity of an unknown target sequence.

Sense Strand – In double-stranded DNA, the strand that codes for the RNA that is translated into proteins. Also referred to as coding, forward, or positive strand. The Primer Express Software designs primers and probes using the sense strand.

System Requirements

The following table lists the hardware and software requirements and recommendations for installing and using Primer Express version 3.0 software.

Item	Minimum Requirements	Recommendations
Computer	• Intel® Pentium® III processor • 540 MHz	• Intel® Pentium IV® processor faster than 2GHz
Monitor	• 17-inch monitor • 800 x 600 pixels resolution	• 19-inch or larger monitor • 1024 x 768 pixels or higher pixels resolution
Hard Drives	• 256 MB RAM • 20 MB free hard disk space	• 512 MB RAM • 10 GB EIDE hard drive
Network Adaptors	• 10/100 NIC with RWV (internal)	
Printer	• Any PC-compatible printer.	
Operating System	• Windows® XP Professional, Service Pack 1 or later	• Windows® XP Professional, Service Pack 1 or later

Operating Systems Not Supported

- Microsoft® Windows® NT and 2000
- Macintosh®

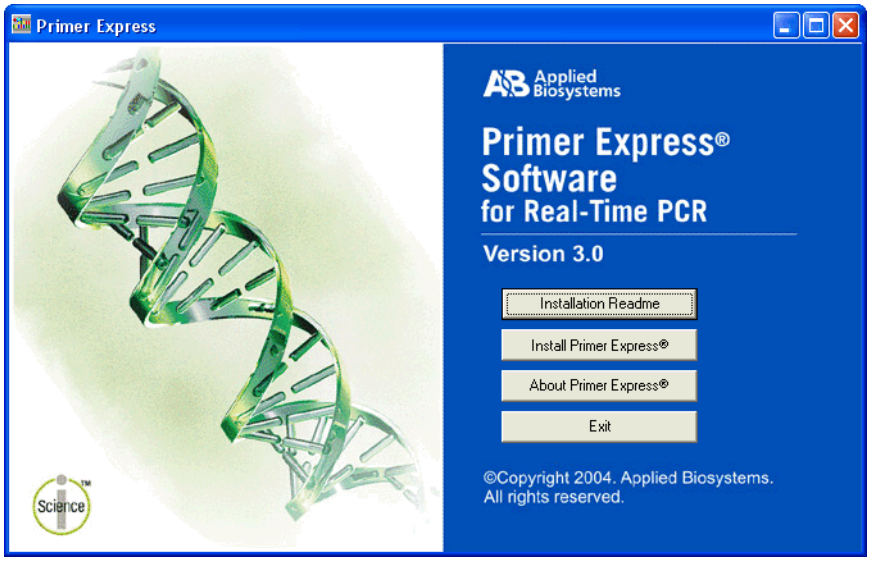
Notes

Installing the Software

Installing Primer Express Software Version 3.0

Note: Applied Biosystems recommends that you disable any virus protection software enabled on your computer before installing Primer Express software version 3.0. You can enable the virus protection software after installation is complete.

1. Insert the Primer Express 3.0 software CD into your CD drive.
2. If the Primer Express Installer does not start automatically, in Windows Explorer, locate and then double-click the *Setup.exe* file. The Primer Express software displays the following window:



3. Click **Install Primer Express®** and follow the prompts to complete the installation.

IMPORTANT! Do not over write Primer Express Software version 2.0. The default installation location for Primer Express Software version 3.0 is in the Windows **Start > All Programs > Applied Biosystems** menu. If you change this default, verify that you are not installing it in the version 2.0 folder. You will need Primer Express Software version 2.0 to convert any older files. See "Converting Primer Express® Software Version 2.0 Documents" on page 5.

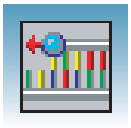
You can start using Primer Express software without restarting your computer.

To Uninstall Primer Express Software Version 3.0

To uninstall Primer Express Software:

1. On the taskbar, select **Start** > **Control Panel**. The Control Panel window opens.

Notes



2. In the **Name** column, double-click **Add or Remove Programs**.The Add or Remove Programs window opens.

3. In the **Currently installed programs** box, scroll down to, and then click **Primer Express 3.0**.

4. Click **Change/Remove**.

5. Follow the instructions on the Install Shield Wizard to remove all installed features.

Converting Primer Express® Software Version 2.0 Documents

In Primer Express software version 2.0, information about your oligonucleotide designs was stored in one or more archive files. In Primer Express software version 3.0, information about each oligonucleotide design is saved to its own separate *.pxd file.

If you would like to use Primer Express version 2.0 documents in version 3.0, you must first convert the version 2.0 archive files. Use the Primer Express version 2.0 Export command to convert documents within *.pcr files to individual *.pex documents. For more information, see *Primer Express Software v2.0 User's Manual* (PN 4329500).

You can open version 2.0 *.pex files without converting to *.pxd.

Note: If the "Limit 3′ G+C" checkbox was unchecked in documents created in Primer Express Software version 2.0, this parameter (in the converted file) will be inconsistent with the setting from the version 2.0 document. Re-run the design with the "Max Primer 3′ GCs" set to -1 to obtain consistent results.

Notes

Basics

Starting and Exiting Primer Express Software

To start Primer Express software for the first time:

1. On your desktop, select **Start > Programs > Applied Biosystems > Primer Express > Primer Express 3.0**. After you start Primer Express 3.0 for the first time, the registration dialog box opens.

2. Enter your name, your organization, and your registration code, which is located on your Primer Express CD envelope and paper.

IMPORTANT! Be sure to store your Primer Express software registration code in a safe place. You will need it after the first installation and any re-installation. If it is lost, you must repurchase Primer Express Software.

3. Click **OK**.

To exit Primer Express software:

Select **File > Exit**.

Using Online Help

The Primer Express Software Online Help provides context-sensitive help for most windows in the software. It also provides more general information about the software and procedures for common tasks.

Press **F1** on the keyboard to display information about the window or dialog box you are viewing.

Select **Help > Contents and Index** to display the default help topic.

Refer to *Primer Express Software Version 3.0 Online Help* for more information on these Primer Express software functions:

- Annotating Sequences
- Exporting
- Printing
- Using the Batch Process Tool

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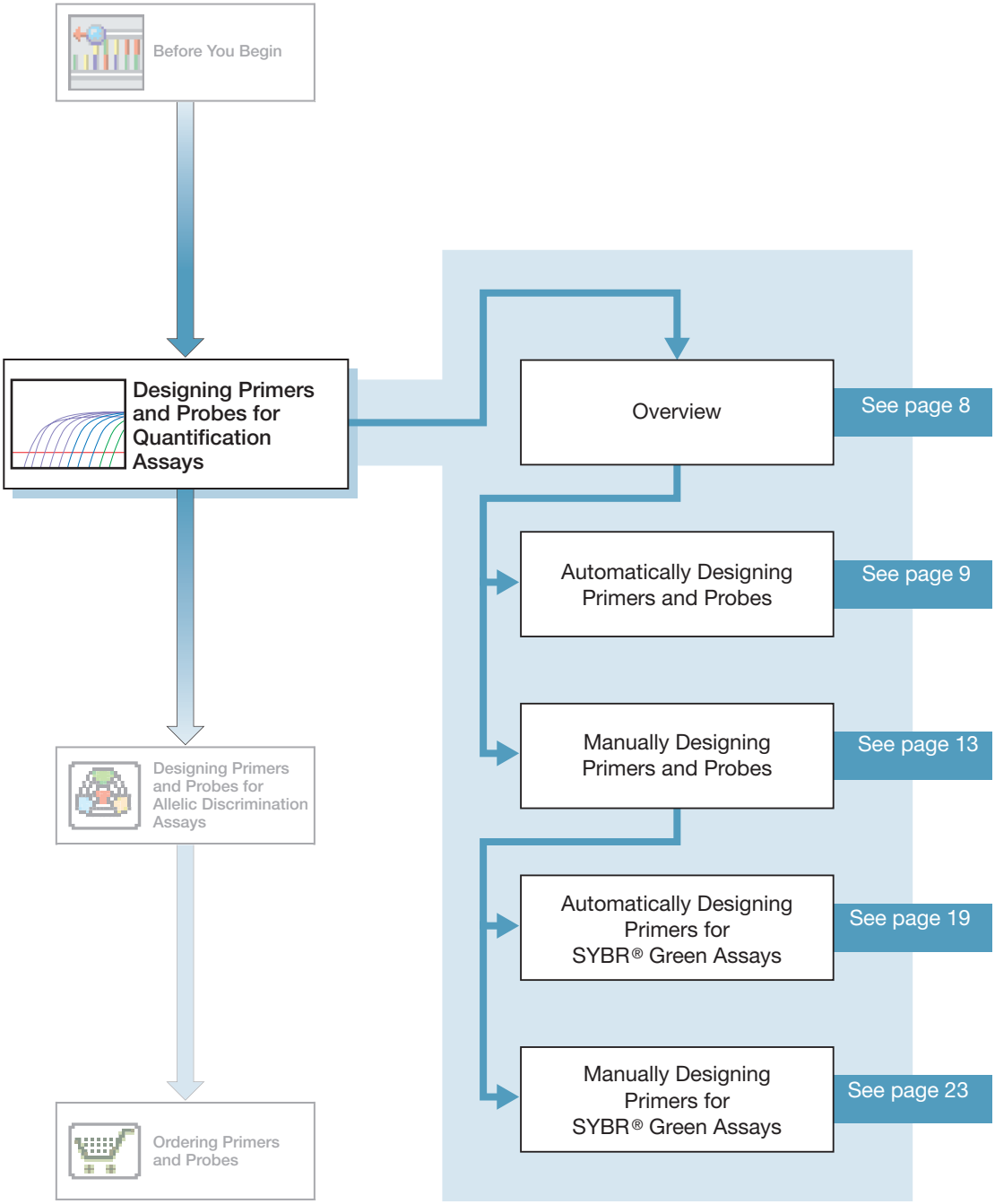
Designing Primers and Probes for Quantification Assays



Notes

Overview

About This Chapter

This chapter provides information on using Primer Express Software Version 3.0 to automatically design primers and probes for quantification assays (including SYBR® Green Dye Assays) using default parameters. It also includes information on how to manually design primers and probes to obtain customized results.

Workflow

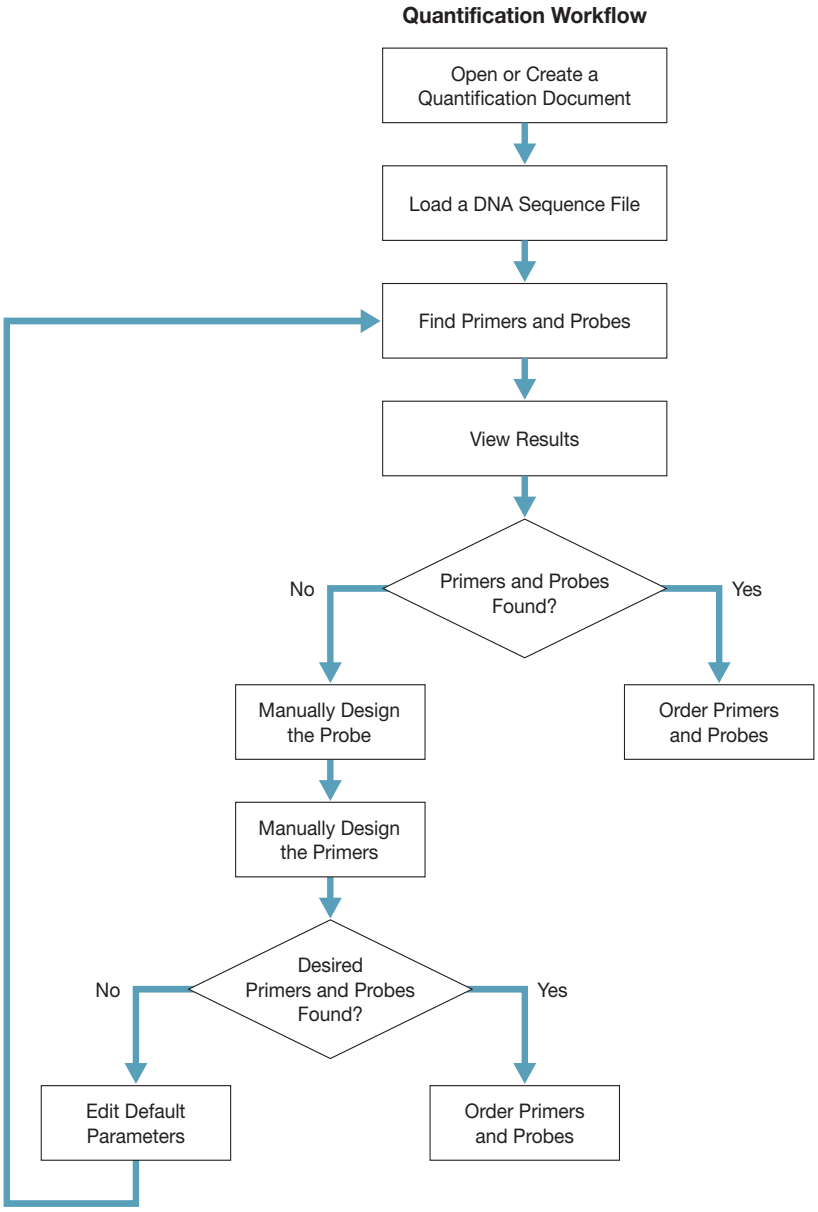
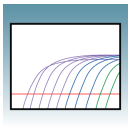


Figure 1. Quantification workflow

Notes



Automatically Designing Primers and Probes

This section describes automatically designing primers and probes for one sequence. You can automatically design quantification primers and probes for multiple sequences using the Batch Process Tool. For more information, see *Primer Express Software Version 3.0 Online Help*.

Creating a Quantification Document

To create a new quantification document:

1. Select **File** > **New**. The New dialog box opens.
2. In the **Type** list, select **TaqMan® MGB Quantification** or **TaqMan® Quantification**.
3. Click **OK**.

The document window opens to the Sequence tab.

Loading a DNA Sequence File

A sample sequence, *NM_002217*, is located in the sample sequences folder within the Primer Express folder. You can use this sample file to experiment with the software and design your primers and probes.

To load a sequence file:

1. Select **Tools** > **Add DNA File** ( ). Note you can also copy and paste or type your sequence file in the Sequence tab.
2. At the Add DNA File dialog box, navigate to and select the desired file. For information on the various file formats supported, see *Primer Express Software Version 3.0 Online Help*.
3. Click **Add**. Primer Express software loads the nucleotide sequence from the file and displays the sense strand in the Sequence tab (see "Figure 2. Sequence tab" on page 10). The sequence serves as the starting point for primer and probe design.

Notes

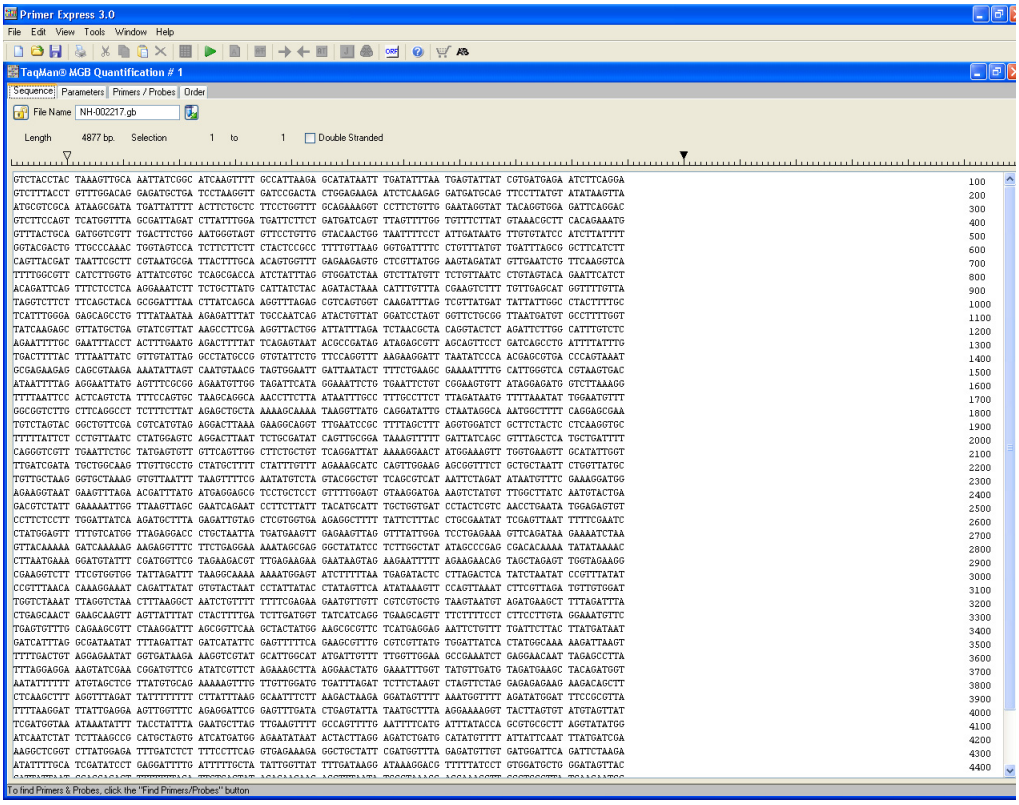


Figure 2. Sequence tab

Note: If you select the Double-Stranded checkbox in the Sequence tab, both sense and anti-sense strands will be displayed. However, primers and probes are designed using the sense strand sequence only.

Finding Primers and Probes

To find primers and probes:

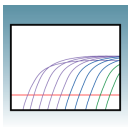
Select **Tools > Find Primers/Probes** ( ). Primer Express software performs its calculations based on default parameter values.

The status bar, located at the bottom of the window, displays information about the progress of the calculations as the software searches for primer/probe sets. If primers and probes are found, go to "Viewing Results" on page 11.

If primers and probes were not found:

If the software does not find primers and probes using default parameters, a pop-up will appear stating that no acceptable primer pairs were found and that you can see the Interim Results window. For more information on Interim Results, see *Primer Express Software Version 3.0 Online Help*.

Notes



At this point, you can:

- Manually design primers and probes as described in "Manually Designing Primers and Probes" on page 13.
- Design using the complementary sequence. For more information, see *Primer Express Software Version 3.0 Online Help*.

Viewing Results

Primer Express software automatically displays the Primers/Probes tab, if it finds primers and probes. The Primers/Probes tab displays the Candidate Primers & Probes table that contains information about the candidate primers, probes, and amplicons (see "Figure 3. Primers/Probes tab displaying candidate primers and probes"). The forward primer sequences are displayed using the left-to-right 5′-to-3′ convention, and reverse primer sequences are displayed using the right-to-left 5′-to-3′ convention.

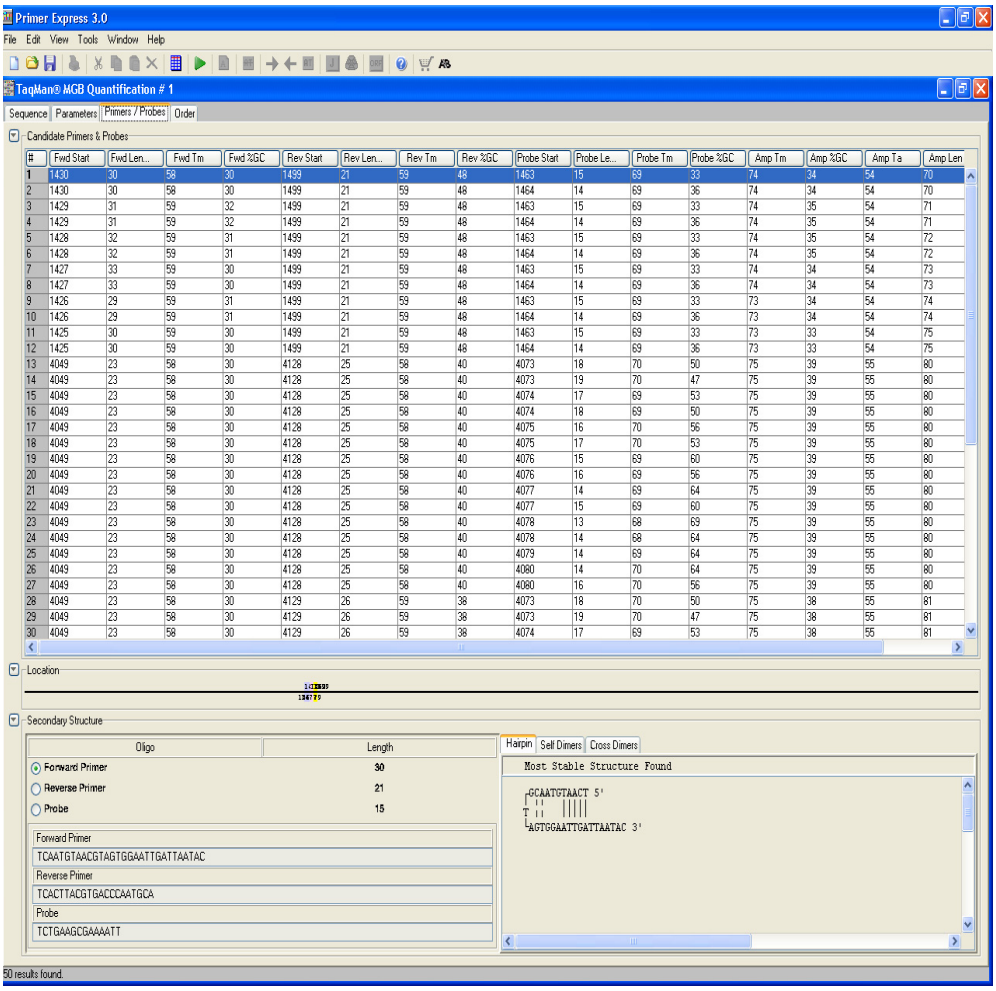


Figure 3. Primers/Probes tab displaying candidate primers and probes

Notes

Evaluating the candidate primer and probe sets:

The Location section of the Primers/Probes tab illustrates the location of the primers and probes within the sequence. The number above the line is the starting base; the number below the line is the ending base. Note that you can also see the corresponding location of a selected candidate Primer/Probe set in the Sequence tab.

In the Sequence tab, the probe will be highlighted in pink, the forward primer in blue, and the reverse primer in yellow. These default color designations can be changed by clicking **Tools > Options**. If you place your cursor over any of these annotations, a tool tip will appear showing the name of the annotation (Probe, Forward Primer, Reverse Primers) start and end locations, Tm and %GC.

As a general guideline, select the primer/probe sets with a low Penalty score and a low amplicon length (if the Penalty score and Amplicon Length fields are not displayed, scroll to the right in the table). However, all primer/probe sets generated using default parameters meet primer and probe guidelines. For more information regarding Penalty scores, see *Primer Express Software Version 3.0 Online Help*.

Note: After the software finds primers and probes, the sequence box is locked. To edit the sequence, click to unlock.

Saving the Document

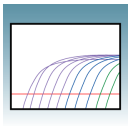
Select **File > Save As** to save the document for future use.

Ordering Primers and Probes

To order your selected primers and probes, refer to Chapter 4, "Ordering Primers and Probes."

IMPORTANT! Before running your samples, Applied Biosystems recommends that you run control samples to verify the performance of the selected primers and probes.

Notes



Manually Designing Primers and Probes

You may choose to manually design primers and probes for various reasons:

- Automated primer/probe design did not find primers and probes.
- To design a probe over an exon junction.
- To design a probe for DNA sequence homologs.
- To design primers and probes according to your own specifications.

Creating a Quantification Document

Create a Quantification document and load a sequence file as you would for automatic primer/probe set design. See "Creating a Quantification Document" on page 9.

Manually Designing the Probe

1. Select a putative probe region containing at least 25 bases.
2. Copy (**Ctrl+C**) the sequence.

IMPORTANT! The Primer Probe Test Tool eliminates non-ATCG bases. Before copying a sequence, change any non-ATCG bases, or select a different region of the sequence.

3. Select **Tools > Primer Probe Test Tool**. The Primer Probe Test Tool dialog box appears (see "Figure 4. Primer Probe Test Tool dialog box" on page 14).

Notes

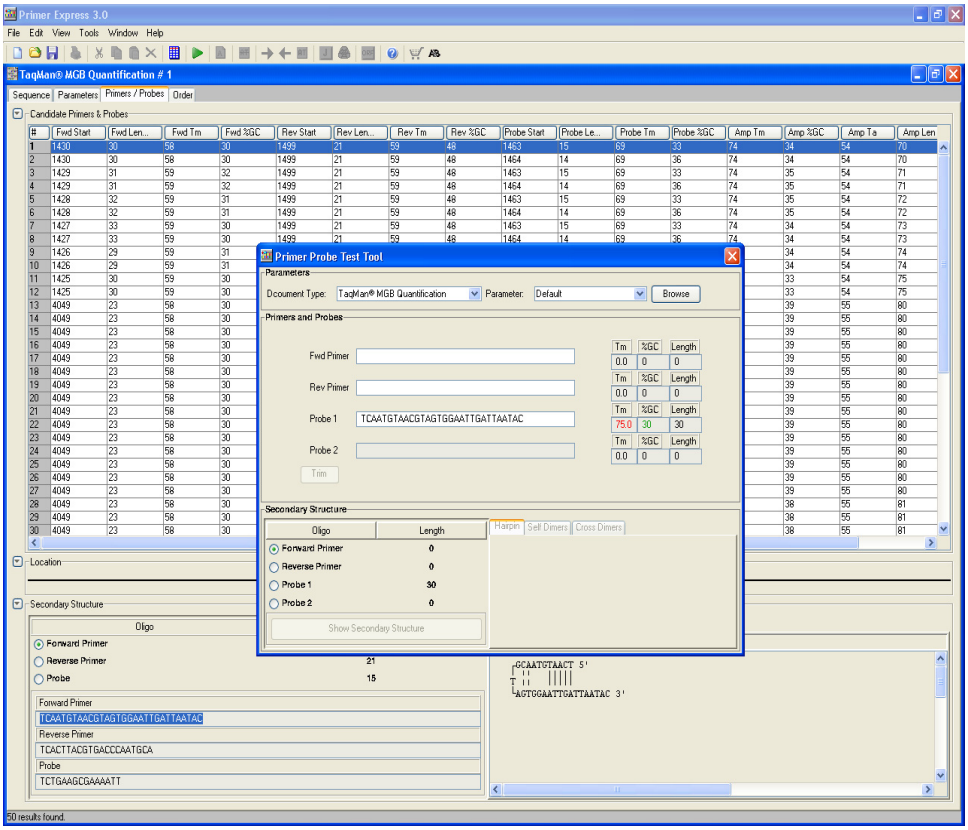


Figure 4. Primer Probe Test Tool dialog box

4. From the Document Type drop down menu, select the desired document type. Verify that the Parameter box is set to **Default**. For more information about changing parameters, see *Primer Express Software Version 3.0 Online Help.*

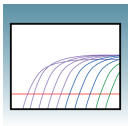
5. Paste (**Ctrl+V**) the putative sequence in the Probe 1 field. The Primer Probe Test Tool displays the Tm, %GC, and the oligonucleotide length to the right of the Probe 1 field.

6. If the Tm is not between 68 °C to 70 °C, highlight a section of the sequence to view the corresponding Tm, %GC, and oligonucleotide length. Once the highlighted region results in the desired Tm, click on **Trim** to delete the non-highlighted bases.

   Ensure the following guidelines are met (for more information on design guidelines, refer to *Primer Express Software Version 3.0 Online Help*):

   - **Amplicon Length** – 50 to 150 bases for optimum PCR efficiency.
   - **Probe Length** – 13 to 25 bases (13 to 30 bases if using conventional TaqMan probes)
   - **Tm** – 68 °C to 70 °C.
   - **% GC** – 30% to 80%.

Notes



- **5′ end** – Cannot be a G residue. A G residue adjacent to the reporter dye will quench the reporter fluorescence somewhat, even after cleavage.

Avoid the following motifs:

- **Repeating oligonucleotides**– Avoid runs of identical nucleotides. If repeats are present, there must be fewer than four consecutive G residues.
- **Consecutive A residues** – Avoid six consecutive A residues anywhere in the probe.
- **G residues on the 3′ end** – Avoid 5′-...GGG-MGB-3′ or 5′-...GGAG-MGB-3′
- **CC dinucleotides** – Avoid two or more CC dinucleotides in the middle of the probe (TaqMan MGB probes), which can sometimes reduce signal.
- **FAM™dye-labeled probes** – If ordering FAM™-dye labeled probes, avoid a G in the second position on the 5′ end.

For secondary structure design considerations, see *Primer Express Software Version 3.0 Online Help*.

Note: If you cannot achieve the recommended Tm, you can design using the complementary sequence. For more information, see *Primer Express Software Version 3.0 Online Help*.

7. Once the correct Tm is achieved, return to the Sequence tab and highlight the sequence found in the Probe 1 field of the Primer Probe Test Tool. To manually design primers, go to "Manually Designing the Primers" on page 16.

8. To automatically find primers after manually designing the probe, select **Edit** > **Annotate** > **Probe** ( ). The selected probe sequence text is displayed in green. For more information on annotating sequences, see *Primer Express Software Version 3.0 Online Help.*

9. Select **Tools > Find Primers/Probes** ( ). Primer Express software performs its calculations based on default parameter values. The status bar, located at the bottom of the window, displays information about the progress of the calculations as the software searches for primers based on the designed probe. If primers are found, go to "Viewing Results" on page 16.

If primers were not found:

If the software does not find primers using default parameters, a pop-up will appear stating that no acceptable primer pairs were found and that you can see the Interim Results window. For more information on Interim Results, see *Primer Express Software Version 3.0 Online Help*.

At this point, you can manually design primers described in "Manually Designing the Primers" on page 16.

Notes

Viewing Results

Primer Express software automatically displays the Primers/Probes tab, if it finds primers. The Primers/Probes tab displays the Candidate Primers & Probes table that contains information about the candidate primers, probes, and amplicons. The forward primer sequences are displayed using the left-to-right 5′-to-3′ convention, and reverse primer sequences are displayed using the right-to-left 5′-to-3′ convention.

Evaluating the candidate primer and probe sets:

The Location section of the Primers/Probes tab illustrates the location of the primers and probes within the sequence. The number above the line is the starting base; the number below the line is the ending base. Note that you can also see the corresponding location of a selected candidate Primer/Probe set in the Sequence tab.

In the Sequence tab, the probe will be highlighted in pink, the forward primer in blue, and the reverse primer in yellow. These default color designations can be changed by clicking **Tools > Options**. If you place your cursor over any of these annotations, a tool tip will appear showing the name of the annotation (Probe, Forward Primer, Reverse Primers) start and end locations, Tm and %GC.

As a general guideline, select the primer/probe sets with a low Penalty score and a low amplicon length (if the Penalty score and Amplicon Length fields are not displayed, scroll to the right in the table). However, all primer/probe sets generated using default parameters meet primer and probe guidelines. For more information regarding Penalty scores, see *Primer Express Software Version 3.0 Online Help*.

Note: After the software finds primers and probes, the sequence box is locked. To edit the sequence, click to unlock.

Manually Designing the Primers

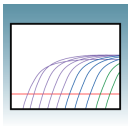
To design the Forward Primer:

1. Select a sequence (at least 25 bases) to the left of the probe. The sequence should be as close to the probe as possible without overlapping it.
2. Copy (**Ctrl+C**) the sequence.

IMPORTANT! The Primer Probe Test Tool eliminates non-ATCG bases. Before copying a sequence, change any non-ATCG bases, or select a different region of the sequence.

3. On the Primer Probe Test Tool dialog box, paste (**Ctrl+V**) the sequence into the Fwd Primer field. The Primer Probe Test Tool displays the Tm, %GC, and the oligonucleotide length to the right of the Fwd Primer field.

Notes



4. If the Tm is not between 58 °C to 60 °C, highlight a section of the sequence to view the corresponding Tm, %GC, and oligonucleotide length as if those highlighted bases were deleted. Once the highlighted region results in the desired Tm, click on **Trim** to delete the non-highlighted bases.

   Ensure the following guidelines are met (for more information on design guidelines, refer to *Primer Express Software Version 3.0 Online Help*):

   - **Amplicon Length** – 50 to 150 bases for optimum PCR efficiency.
   - **Optimal Primer Length** – 20 bases. Do not overlap primer and probe sequences.
   - **Tm** – 58 °C to 60 °C (**Optimal Tm** – 59 °C).
   - **% GC** – 30% to 80%.
   - **3′ end** – Make sure the last five nucleotides at the 3′ end contain no more than two G + C residues.

   Avoid the following motifs:

   - **Repeating oligonucleotides** – Avoid runs of identical nucleotides. If repeats are present, there must be fewer than four consecutive G residues.

   For secondary structure design considerations, see *Primer Express Software Version 3.0 Online Help*.

To design the Reverse Primer:

1. In the sequence tab, select a sequence (at least 25 bases) to the right of the probe. The sequence should be as close to the probe without overlapping it.

2. Select **Edit > Copy Complement**.

IMPORTANT! The Primer Probe Test Tool eliminates non-ATCG bases. Before copying a sequence, change any non-ATCG bases, or select a different region of the sequence.

3. On the Primer Probe Test Tool dialog box, paste (**Ctrl+V**) the sequence into the Rev Primer field. The Primer Probe Test Tool displays the Tm, %GC, and the oligonucleotide length to the right of the Fwd Primer field.

4. If the Tm is not between 58 °C to 60 °C, highlight a section of the sequence to view the corresponding Tm, %GC, and oligonucleotide length. Once the highlighted region results in the desired Tm, click on **Trim** to delete the non-highlighted bases. Be sure to keep the above guidelines in mind.

Note that you can further customize your primer and probe set by editing the default parameter values found under the Parameters tab. For more information on editing parameters, see *Primer Express 3.0 Software Online Help*.

Saving Primer and Probe Sequences

Copy and paste the primer and probe sequences into a text document, then save for future reference.

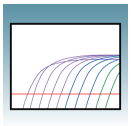
Notes

Ordering Primers and Probes

To order your selected primer/probe set, refer to Chapter 4, "Ordering Primers and Probes."

IMPORTANT! Before running your samples, Applied Biosystems recommends that you run control samples to verify the performance of the selected primers and probes.

Notes



Automatically Designing Primers for SYBR® Green Dye Assays

Note: This procedure generates primers and TaqMan probes. However, only the primers need to be ordered for SYBR® Green Dye assays. If desired, you can save the probe sequence for future use in TaqMan assays.

Creating a Quantification Document

To create a new quantification document:

1. Select **File** > **New**. The New dialog box opens.
2. In the **Type** list, select **TaqMan® MGB Quantification** or **TaqMan® Quantification**.
3. Click **OK**.

The document window opens to the Sequence tab.

Loading a DNA Sequence File

A sample sequence *NM_002217*, is located in the sample sequences folder within the Primer Express folder. You can use this sample file to experiment with the software and design your primers.

To load a sequence file:

1. Select **Tools** > **Add DNA File** ( ). Note you can also copy and paste or type your sequence file in the Sequence tab.
2. At the Add DNA File dialog box, navigate to and select the desired file. For information on the various file formats supported, see *Primer Express Software Version 3.0 Online Help*.
3. Click **Add**. Primer Express software loads the nucleotide sequence from the file and displays the sense strand in the Sequence tab (see "Figure 5. Sequence tab" on page 20). The sequence serves as the starting point for primer design.

Notes

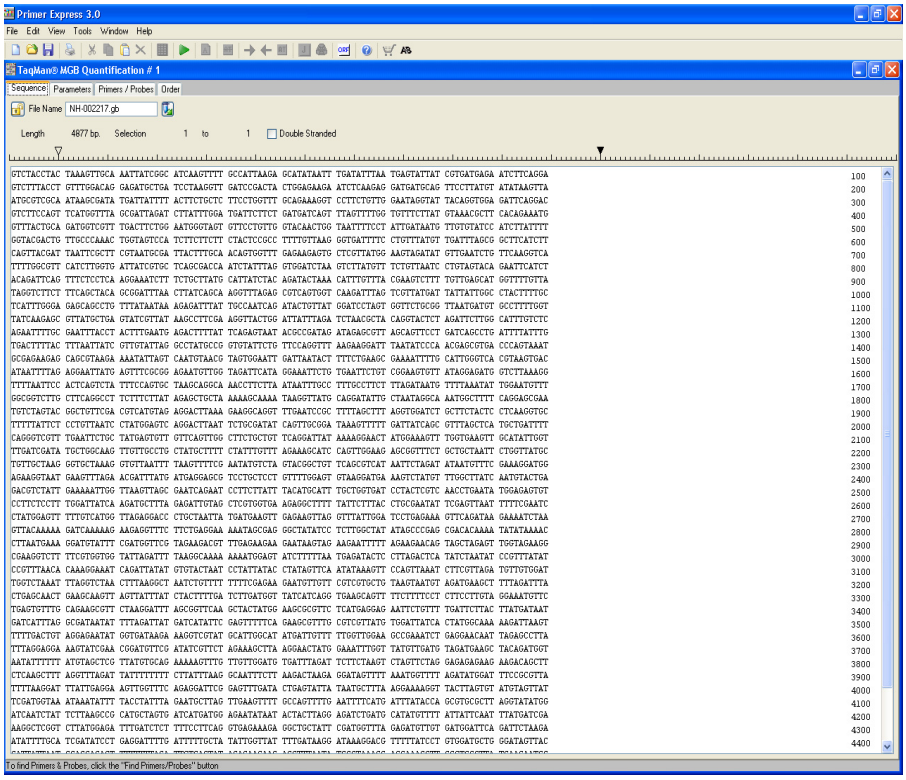


Figure 5. Sequence tab

Note: If you select the Double-Stranded checkbox in the Sequence tab, both sense and reverse strands will be displayed. However, primers are designed using the sense strand sequence only.

Finding Primers

To find primers:

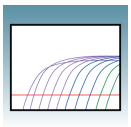
Select **Tools > Find Primers/Probes** ( ). Primer Express software performs its calculations based on default parameter values.

The status bar, located at the bottom of the window, displays information about the progress of the calculations as the software searches for primer/probe sets. If primers are found, go to "Viewing Results" on page 21.

If primers were not found:

If the software does not find primers using default parameters, a pop-up will appear stating that no acceptable primer pairs were found and that you can see the Interim Results window. For more information on Interim Results, see *Primer Express Software Version 3.0 Online Help*.

Notes



At this point, you can:

- Manually design primers as described in "Manually Designing Primers for SYBR® Green Dye Assays" on page 23.
- Design using the complementary sequence. For more information, see *Primer Express Software Version 3.0 Online Help*.

Viewing Results

Primer Express software automatically displays the Primers/Probes tab, if it finds primers and probes. The Primers/Probes tab displays the Candidate Primers & Probes table that contains information about the candidate primers, probes, and amplicons (see "Figure 6. Primers/Probes tab displaying candidate primers and probes"). The forward primer sequences are displayed using the left-to-right 5′-to-3′ convention, and reverse primer sequence using the right-to-left 5′-to-3′ convention.

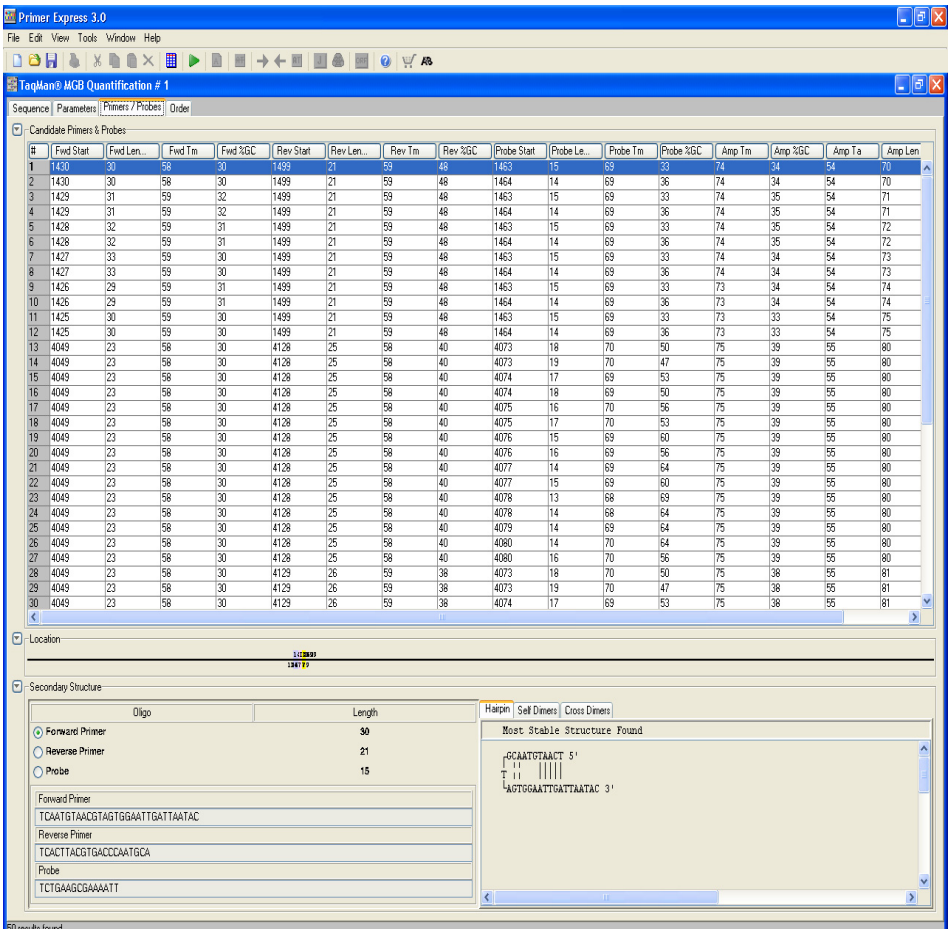


Figure 6. Primers/Probes tab displaying candidate primers and probes

Notes

Evaluating the candidate primer and probe sets:

The Location section of the Primers/Probes tab illustrates the location of the primers and probes within the sequence. The number above the line is the starting base; the number below the line is the ending base. Note that you can also see the corresponding location of a selected candidate Primer/Probe set in the Sequence tab.

In the Sequence tab, the probe will be highlighted in pink, the forward primer in blue, and the reverse primer in yellow. These default color designations can be changed by clicking **Tools > Options**. If you place your cursor over any of these annotations, a tool tip will appear showing the name of the annotation (Probe, Forward Primer, Reverse Primers) start and end locations, Tm and %GC.

As a general guideline, select the primer/probe sets with a low Penalty score and a low amplicon length (if the Penalty score and Amplicon Length fields are not displayed, scroll to the right in the table). However, all primer/probe sets generated using default parameters meet primer and probe guidelines. For more information regarding Penalty scores, see *Primer Express Software Version 3.0 Online Help*.

Note: After the software finds primers and probes, the sequence box is locked. To edit the sequence, click to unlock.

Saving the Document

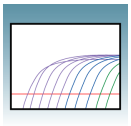
Select **File > Save As** to save the document for future use.

Ordering Primers

To order your selected primers, refer to Chapter 4, "Ordering Primers and Probes."

IMPORTANT! Before running your samples, Applied Biosystems recommends that you run control samples to verify the performance of the selected primers and probes.

Notes



Manually Designing Primers for SYBR® Green Dye Assays

You may choose to manually design primers and probes for a various reasons:

- Automated primer/probe design did not find primers.
- To design primers according to your own specifications.

Creating a Quantification Document

Create a Quantification document and load a sequence file as you would for automatic primer/probe set design. See "Creating a Quantification Document" on page 9.

Manually Designing the Primers

To design the Forward Primer:

1. In the Sequence tab, select a putative forward primer sequence region containing at least 25 bases.
2. Copy (**Ctrl+C**) the sequence.

IMPORTANT! The Primer Probe Test Tool eliminates non-ATCG bases. Before copying a sequence, change any non-ATCG bases, or select a different region of the sequence.

3. Select **Tools > Primer Probe Test Tool**. The Primer Probe Test Tool dialog box appears (see "Figure 7. Primer Probe Test Tool dialog box" on page 24).

Notes

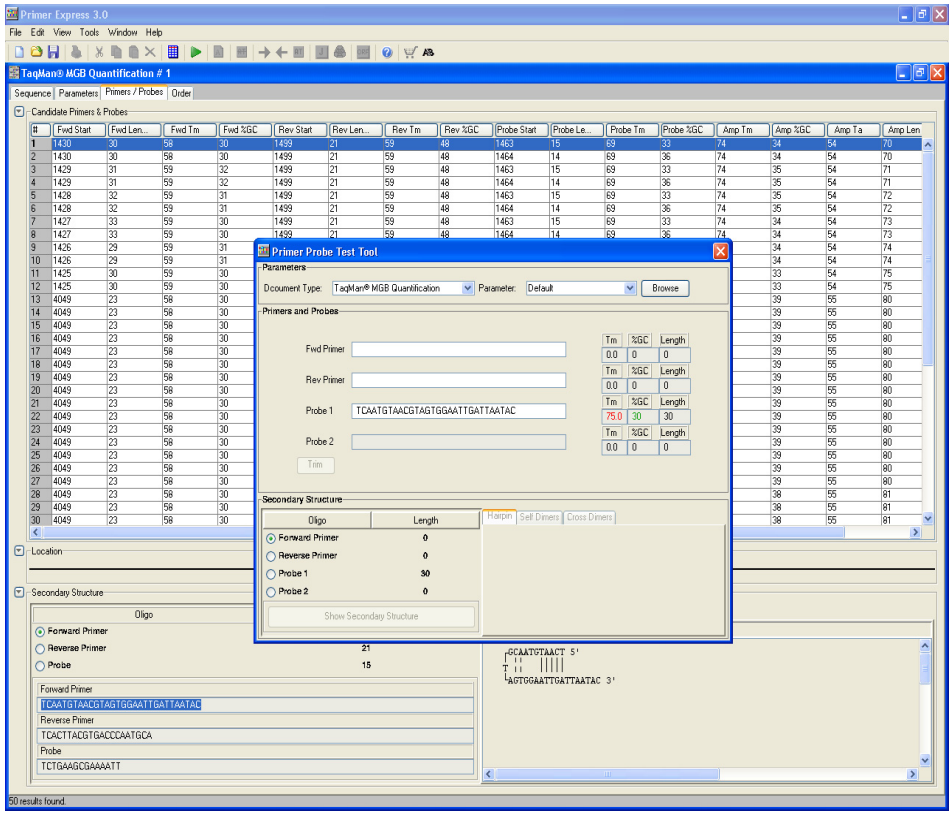


Figure 7. Primer Probe Test Tool dialog box

4. From the **Document Type** drop down menu, select the desired document type. Verify that the Parameter field is set to **Default**. For more information about changing parameters, see *Primer Express Software Version 3.0 Online Help.*

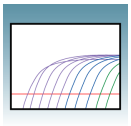
5. Paste (**Ctrl+V**) the annotated sequence in the Fwd Primer field. The software displays the Tm, %GC, and the oligonucleotide length to the right of the Fwd Primer field.

6. If the Tm is not between 58 °C to 60 °C, highlight a section of the sequence to view the corresponding Tm, %GC, and oligonucleotide length as if those highlighted bases were deleted. Once the highlighted region results in the desired Tm, click on **Trim** to delete the highlighted bases.

   Ensure the following guidelines are met (for more information on design guidelines, refer to *Primer Express Software Online Help*):

   - **Amplicon Length** – 50 to 150 bases for optimum PCR efficiency.
   - **Optimal Primer Length** – 20 bases. Do not overlap primer and probe sequences.
   - **Tm** – 58 °C to 60 °C (**Optimal Tm** – 59 °C).
   - **% GC** – 30% to 80%.

Notes



- **3′ end** – Make sure the last five nucleotides at the 3′ end contain no more than two G + C residues.

Avoid the following motifs:

- **Repeating oligonucleotides** – Avoid runs of identical nucleotides. If repeats are present, there must be fewer than four consecutive G residues.

For secondary structure design considerations, see *Primer Express Software Version 3.0 Online Help*.

Note: If you cannot achieve the recommended Tm, you can design using the complementary sequence. For more information, see *Primer Express Software Version 3.0 Online Help*.

To design the Reverse Primer:

1. In the sequence tab, select a putative reverse primer sequence region (containing at least 25 bases).

IMPORTANT! The Primer Probe Test Tool eliminates non-ATCG bases. Before copying a sequence, change any non-ATCG bases, or select a different region of the sequence.

2. Select **Edit > Copy Complement**.
3. On the Primer Probe Test Tool dialog box, paste (**Ctrl+V**) the primer sequence into the Rev Primer field. The Primer Probe Test Tool displays the Tm, %GC, and the oligonucleotide length to the right of the Rev Primer field.
4. If the Tm is not between 58 °C to 60 °C, highlight a section of the sequence to view the corresponding Tm, %GC, and oligonucleotide length. Once the highlighted region results in the desired Tm, click on **Trim** to delete the non-highlighted bases. Be sure to keep the above guidelines in mind.

Note that you can further customize your primer by editing the default parameter values found under the Parameters tab. For more information on editing parameters, see *Primer Express 3.0 Software Online Help*.

Saving Primer Sequences

Copy and paste the primer and probe sequences into a text document, then save for future reference.

Ordering Primers

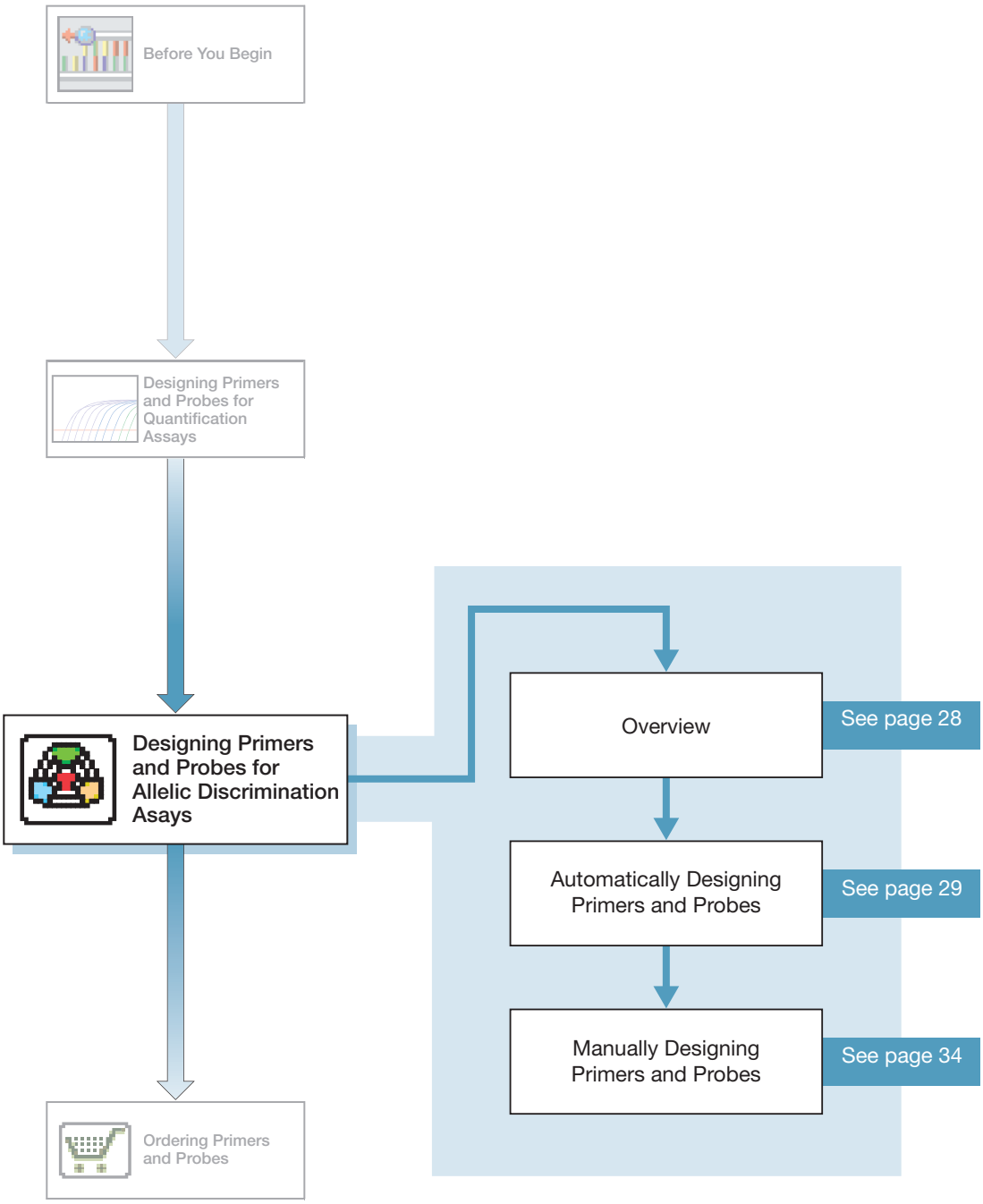
To order your selected primer/probe set, refer to Chapter 4, "Ordering Primers and Probes."

IMPORTANT! Before running your samples, Applied Biosystems recommends that you run control samples to verify the performance of the selected primers and probes.

Notes

Notes

Designing Primers and Probes for Allelic Discrimination Assays



Notes

Overview

About This Chapter

This chapter provides information on using Primer Express® software to automatically design primers and probes for allelic discrimination assays using default parameters. It also includes information on how to manually design primers and probes to obtain customized results.

Workflow

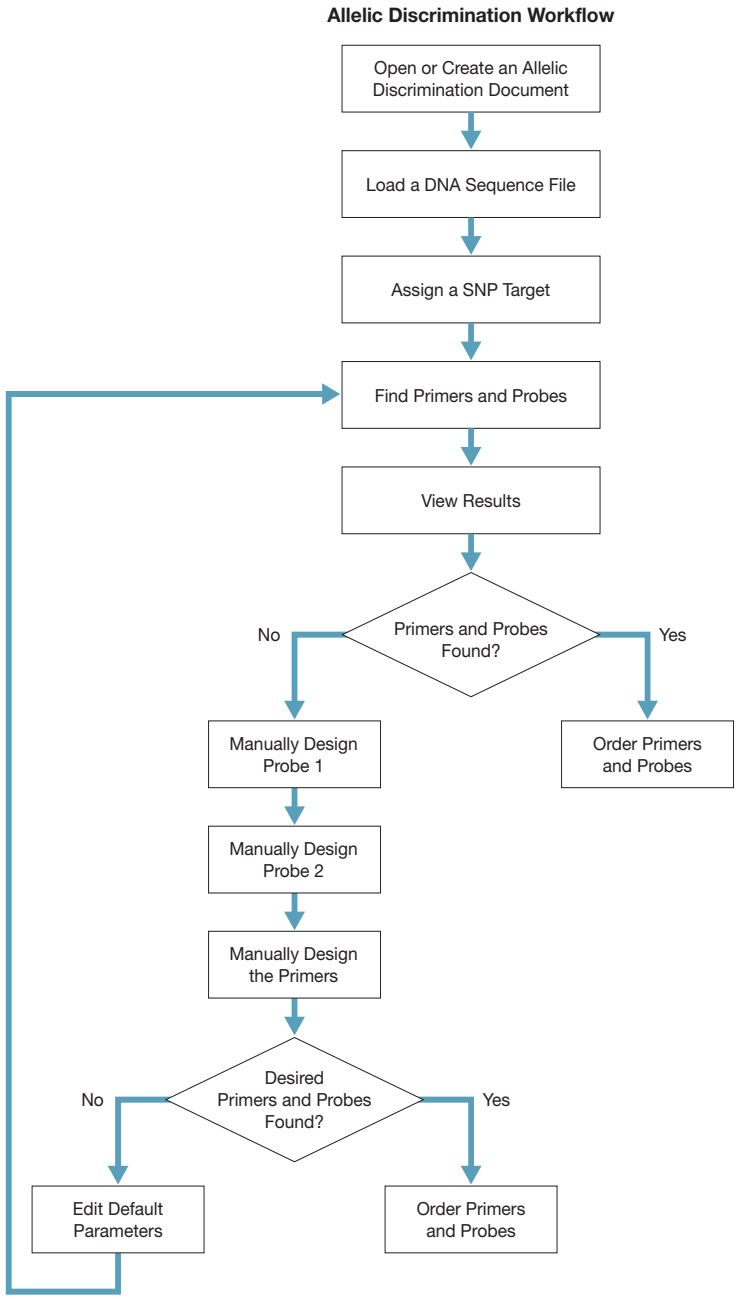


Figure 8. Allelic Discrimination workflow

Notes

Automatically Designing Primers and Probes

Creating an Allelic Discrimination Document

To create a new allelic discrimination document:

1. Select **File** > **New** to open the New dialog box.
2. In the **Type** list, select **TaqMan® MGB Allelic Discrimination** or **TaqMan® Allelic Discrimination**. For best results, use TaqMan MGB probes. MGB probes are shorter than conventional probes and are more specific to the target sequence.
3. Click **OK**.

Loading a DNA Sequence File

A sample sequence file, *AY228765.txt*, is located in the sample sequences folder within the Primer Express folder. You can use this sample file to experiment with the software and design your primers and probes. Note that one forward primer, one reverse primer, and two probes are designed. The two probes, one for each of the SNP sites, will not be identical. However, the two probes must be designed using the same strand (sense strand).

To load a sequence file:

1. Select **Tools** > **Add DNA File** ( ). Note you can also copy and paste or type your sequence file in the Sequence tab.
2. At the Add DNA File dialog box, navigate to and select the desired file. For information on the various file formats supported, see *Primer Express Software Version 3.0 Online Help*.
3. Click **Add**. Primer Express software loads the nucleotide sequence from the file and displays the sense strand in the Sequence tab (see "Figure 9. Sequence tab" on page 30). The sequence serves as the starting point for primer and probe design.

Notes

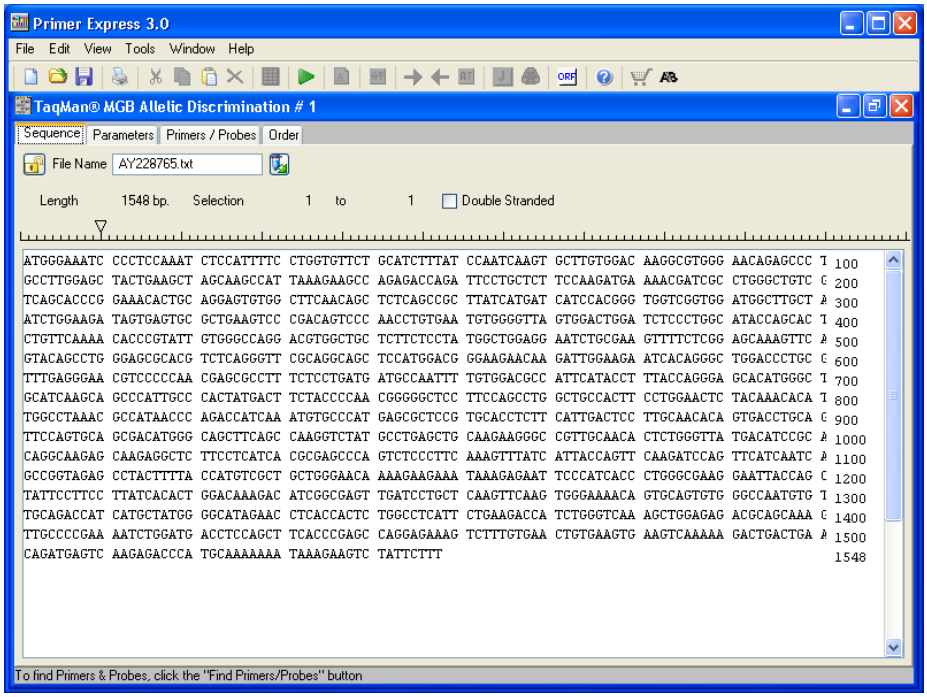


Figure 9. Sequence tab

Note: If you select the Double-Stranded checkbox in the Sequence tab, both sense and anti-sense strands will be displayed. However, primers and probes are designed using the sense strand sequence only.

Assigning a SNP Target

To assign a SNP target:

1. Highlight the SNP target site.
2. Select **Edit > Annotate > SNP Target** ( ) then select the variant for the SNP site. To determine the variant to select, find the two possible variant bases for your SNP, then click the code between the two bases. In the example sequence provided, the SNP target is located at position 528 as a **G/A** variant, so click **R**, then **OK** (see "Figure 10. Determining variant using SNP Target Tool" on page 31).

Notes

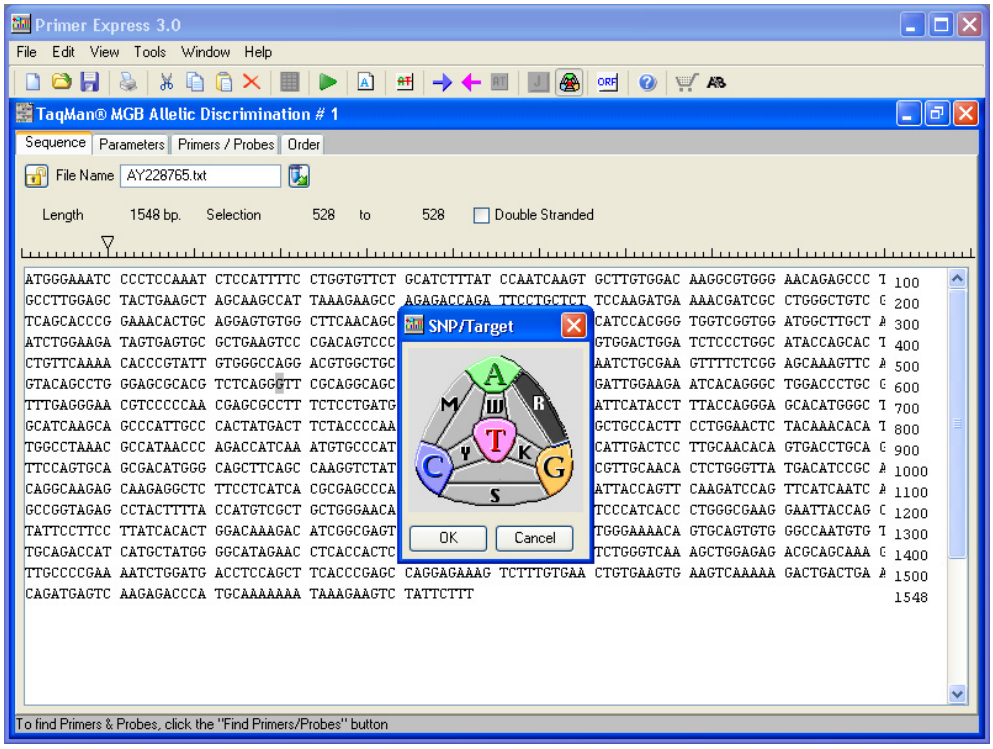


Figure 10. Determining variant using SNP Target Tool

Finding Primers and Probes

To find primers and probes:

Select **Tools > Find Primers/Probes** ( ). Primer Express software performs its calculations based on default parameter values.

The status bar, located at the bottom of the window, displays information about the progress of the calculations as the software searches for primer, probe, and amplicon sets. If primers and probes are found, go to "Viewing Results".

If primers and probes were not found:

If the software does not find primers and probes using default parameters, a pop-up will appear stating that no acceptable primer pairs were found and that you can see the Interim Results window. For more information on Interim Results, see *Primer Express Software Version 3.0 Online Help*.

Viewing Results

Primer Express software automatically displays the Primers/Probes tabs if it finds primers and probes (see "Figure 11. Primer/Probe Tab displaying candidate primers and probes" on page 32). The Primers/Probes tab displays the candidate Primers & Probes table that contains information about forward primers, reverse primers, probes, and amplicons. The forward primer sequences are displayed using the left-to-right 5′-to-3′ convention, and reverse primer sequences are displayed using the right-to-left 5′-to-3′ convention.

Notes

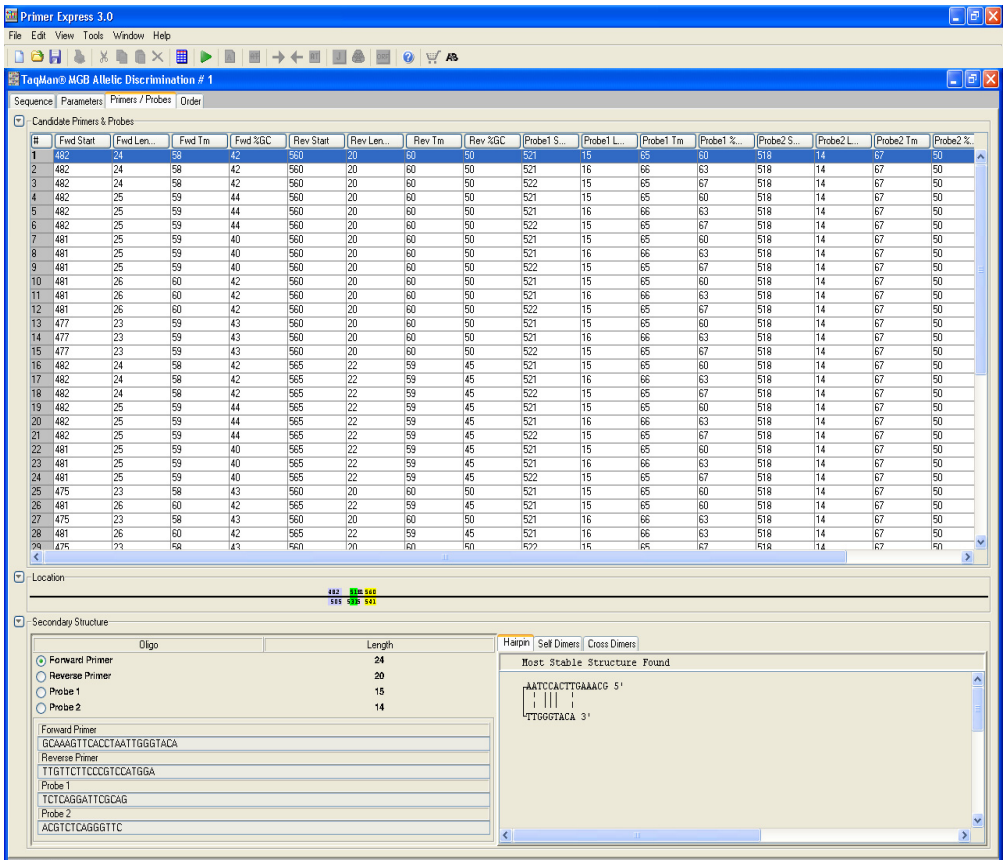


Figure 11. Primer/Probe Tab displaying candidate primers and probes

Evaluating the candidate primer and probe sets:

The Location section illustrates the location of the primers and probes within the line sequence. The number above the line is the starting base; the number below the line is the ending base. Note that you can also see the corresponding location of a selected candidate Primer/Probe set in the Sequence tab.

In the sequence tab, the probe 1 will be highlighted in pink, probe 2 will be highlighted in green (if probe 1 and 2 overlap, the overlap region will appear green), the forward primer in blue, and the reverse primer in yellow (see "Figure 12. Probe annotations in Sequence tab" on page 33). These default color designations can be changed by clicking **Tools > Options**. If you place your cursor over any of these annotations, a tool tip will appear showing the name of the annotation (Probe, Forward Primer, Reverse Primers) start and end locations, Tm and GC%.

Notes

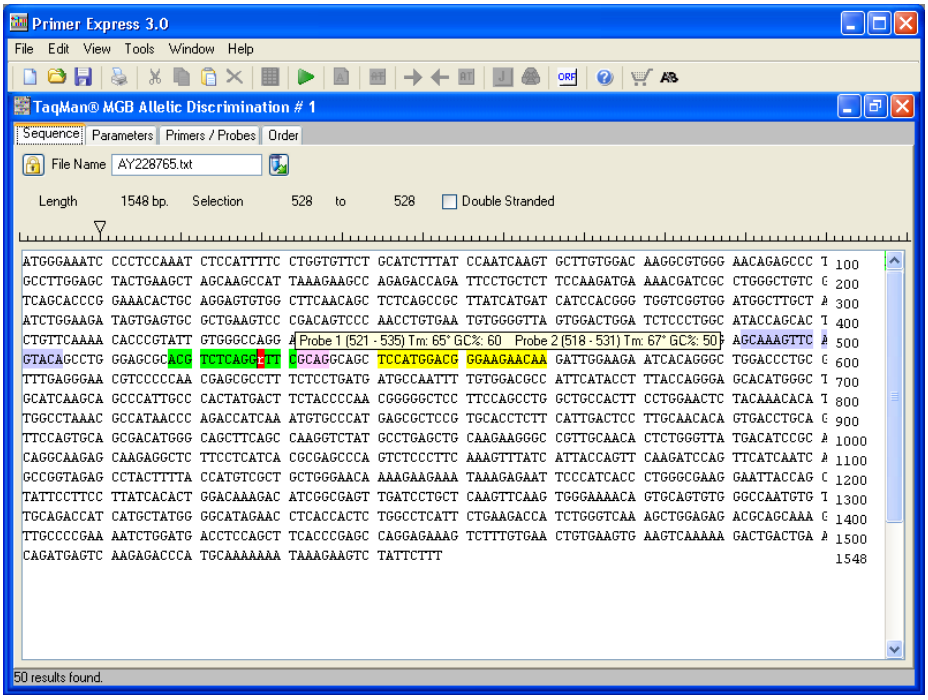


Figure 12. Probe annotations in Sequence tab

Note: After the software finds primers and probes, the sequence box is locked. To edit the sequence, click to unlock.

As a general guideline, select the primer/probe sets with a low Penalty score and a low amplicon length (if the Penalty score and Amplicon Length fields are not displayed, scroll to the right in the table). However, all primer/probe sets generated using default parameters meet primer and probe guidelines. For more information regarding Penalty scores, see *Primer Express Software Version 3.0 Online Help*.

Saving the Document

Before proceeding to other designs, be sure to save the Primer/Probe annotations and results found. Select **File > Save As** to save the document for future use.

Ordering Primers and Probes

To order your selected primers and probes, refer to Chapter 4, "Ordering Primers and Probes."

IMPORTANT! Before running your samples, Applied Biosystems recommends that you run control samples to verify the performance of the selected primers and probes.

Notes

Manually Designing Primers and Probes

You may choose to manually design primers and probes for a various reasons:

- Automated primer/probe design did not find primers or probes.
- To design primers and probes according to your own specifications.

Creating an Allelic Discrimination Document

Create an MGB allelic discrimination document and load a sequence file as you would for automatic primer/probe set design. See "Creating an Allelic Discrimination Document" on page 29.

Manually Designing the Allele 1 Probe

To design the probe for Allele 1:

1. In the Sequence tab, identify the SNP site and the putative probe sequence.
2. Assign the SNP target (see "Assigning a SNP Target" on page 30).
3. Select the sequence for the probe (13 to 25 bases) then select **Edit > Copy with Allele 1**.

IMPORTANT! The Primer Probe Test Tool eliminates non-ATCG bases. Before copying a sequence, change any non-ATCG bases, or select a different region of the sequence.

4. Select **Tools > Primer Probe Test Tool**.
5. From the Document Type drop down menu, select the desired document type. Verify that the Parameter field is set to **Default**. For more information about parameters, see *Primer Express Software Version 3.0 Online Help.*
6. Paste (**Ctrl+V**) the annotated sequence in the Probe 1 field. The software displays the Tm, %GC, and the oligonucleotide length to the right of the Probe 1 field (see "Figure 13. Primer Probe Test Tool dialog box" on page 35). Note that the original Allele 1 variant base appears in lower case on the Primer Probe Test Tool.

Notes

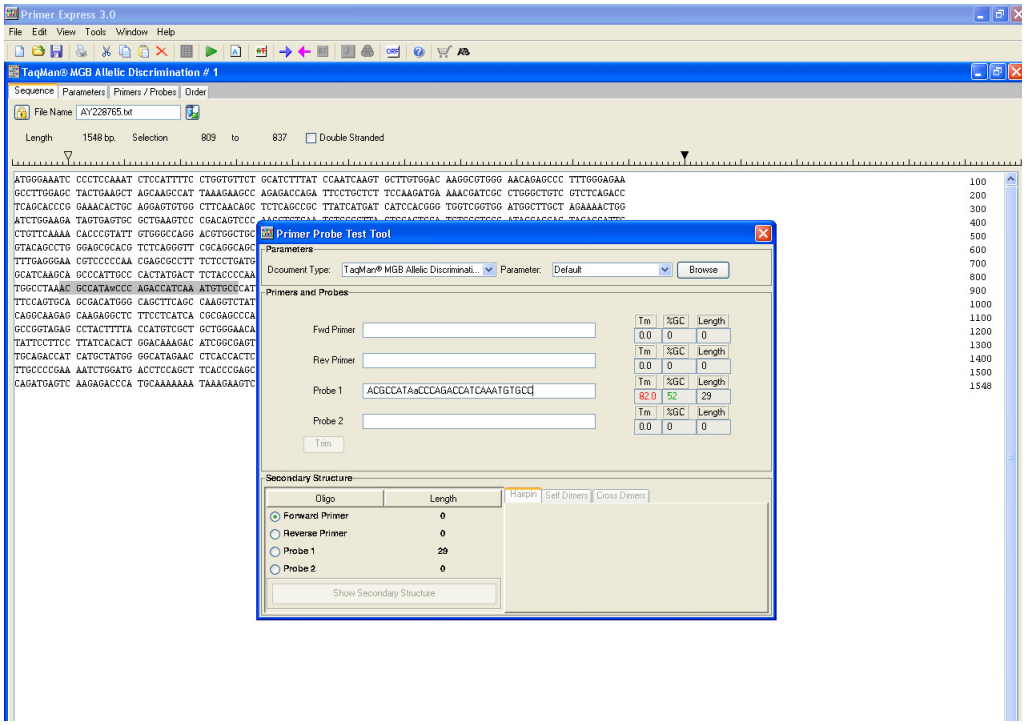


Figure 13. Primer Probe Test Tool dialog box

7. If the Tm is not between 65 °C to 67 °C, highlight a section of the sequence to view the corresponding Tm, %GC, and oligonucleotide length. Once the highlighted region results in the desired Tm, click on **Trim** to delete the non-highlighted bases.

   Ensure the following guidelines are met (for more information on design guidelines, refer to *Primer Express Software Version 3.0 Online Help*):

   - **Amplicon Length** – 50 to 150 bases for optimum PCR efficiency.
   - **Probe Length** – 13 to 25 bases (13 to 30 bases if using conventional TaqMan probes).
   - **Tm** – 65 °C to 67 °C.
   - **% GC** – 30% to 80%.
   - **5′ end** – Cannot be a G residue. A G residue adjacent to the reporter dye will quench the reporter fluorescence somewhat, even after cleavage.
   - **Tm difference between probes** – Not greater than 1 °C
   - **SNP site** – Locate in the middle third of sequence or toward 3′ end but not in the last two bases of 3′ end (see "Figure 14. SNP site in an MGB probe" on page 36).

Notes

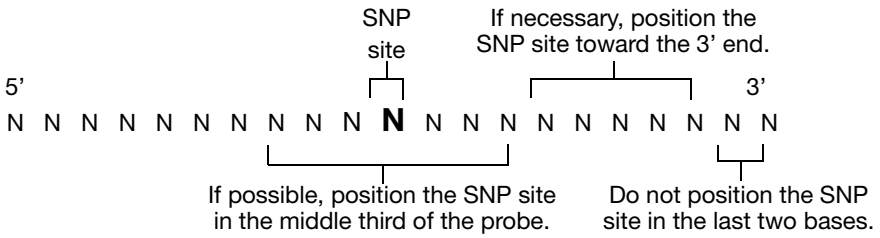


Figure 14. SNP site in an MGB probe

Avoid the following motifs:

- **Repeating oligonucleotides**– Avoid runs of identical nucleotides. If repeats are present, there must be fewer than four consecutive G residues.
- **G residues on the 3′ end** – Avoid 5′-...GGG-MGB-3′ or 5′-...GGAG-MGB-3′
- **Consecutive A residues** – Avoid six consecutive A residues anywhere in the probe.
- **CC dinucleotides** – Avoid two or more CC dinucleotides in the middle of the probe, which can sometimes reduce signal.
- **FAM™-dye labeled probes** – If ordering FAM™-dye labeled probes, avoid a G in the second position on the 5′ end.

For secondary structure design considerations, see *Primer Express Software Version 3.0 Online Help*.

Note: If you cannot achieve the recommended Tm, or probe allele 1 is no longer within the guidelines, you can design using the complementary sequence. For more information, see *Primer Express Software Version 3.0 Online Help*.

Manually Designing the Allele 2 Probe

To design the probe for Allele 2:

Note: Keep the Allele 1 and Allele 2 probe Tms within one degree of each other.

1. In the Sequence tab, select the sequence for the probe (13 to 25 bases and includes the SNP site) then select **Edit > Copy with Allele 2**.

IMPORTANT! The Primer Probe Test Tool eliminates non-ATCG bases. Before copying a sequence, change any non-ATCG bases, or select a different region of the sequence.

2. Select **Tools > Primer Probe Test Tool**.

Notes

3. Paste (**Ctrl+V**) the sequence into the Probe 2 field. The Primer Probe Test Tool displays the Tm, %GC, and sequence length to the right of the field. Note that the original Allele 2 variant base will appear in lower case on the Primer Probe Test Tool.

4. If the Tm is not between 65 °C to 67 °C, highlight a section of the sequence to view the corresponding Tm, %GC, and oligonucleotide length of the highlighted region. Once the highlighted region results in the desired Tm, click on **Trim** to delete the non-highlighted bases. Keep in mind the general design guidelines previously listed on page 35.

Manually Designing the Primers

To design the Forward Primer:

1. Select a sequence (at least 25 bases) to the left of the probe. The sequence should be as close to the probe as possible without overlapping it.

2. Copy (**Ctrl+C**) the sequence.

IMPORTANT! The Primer Probe Test Tool eliminates non-ATCG bases. Before copying a sequence, change any non-ATCG bases, or select a different region of the sequence.

3. On the Primer Probe Test Tool dialog box, paste (**Ctrl+V**) the sequence into the Fwd Primer field. The Primer Probe Test Tool displays the Tm, %GC, and the oligonucleotide length to the right of the Fwd Primer field.

4. If the Tm is not between 58 °C to 60 °C, highlight a section of the sequence to view the corresponding Tm, %GC, and oligonucleotide length as if those highlighted bases were deleted. Once the highlighted region results in the desired Tm, click on **Trim** to delete the non-highlighted bases.

   Ensure the following guidelines are met (for more information on design guidelines, refer to *Primer Express Software Version 3.0 Online Help*):

   - **Amplicon Length** – 50 to 150 bases for optimum PCR efficiency.
   - **Optimal Primer Length** – 20 bases. Do not overlap primer and probe sequences.
   - **Tm** – 58 °C to 60 °C (**Optimal Tm** – 59 °C).
   - **% GC** – 30% to 80%.
   - **3′ end** – Make sure the last five nucleotides at the 3′ end contain no more than two G + C residues.

   Avoid the following motifs:

   - **Repeating oligonucleotides** – Avoid runs of identical nucleotides. If repeats are present, there must be fewer than four consecutive G residues.

   For secondary structure design considerations, see *Primer Express Software Version 3.0 Online Help*.

Notes

To design the Reverse Primer:

1. In the sequence tab, select a sequence (at least 25 bases) to the right of the probe.The sequence should be as close to the probe without overlapping it.

2. Select **Edit > Copy Complement**.

IMPORTANT! The Primer Probe Test Tool eliminates non-ATCG bases. Before copying a sequence, change any non-ATCG bases, or select a different region of the sequence.

3. On the Primer Probe Test Tool dialog box, paste (**Ctrl+V**) the sequence into the Rev Primer field. The Primer Probe Test Tool displays the Tm, %GC, and the oligonucleotide length to the right of the Fwd Primer field.

4. If the Tm is not between 58 °C to 60 °C, highlight a section of the sequence to view the corresponding Tm, %GC, and oligonucleotide length. Once the highlighted region results in the desired Tm, click on **Trim** to delete the non-highlighted bases. Be sure to keep the above guidelines in mind.

Note that you can further customize your primer and probe set by editing the default parameter values found under the Parameters tab. For more information on editing parameters, see *Primer Express 3.0 Software Online Help*.

Saving Primer and Probe Sequences

Copy and paste the primer and probe sequences into a text document, then save for future reference.

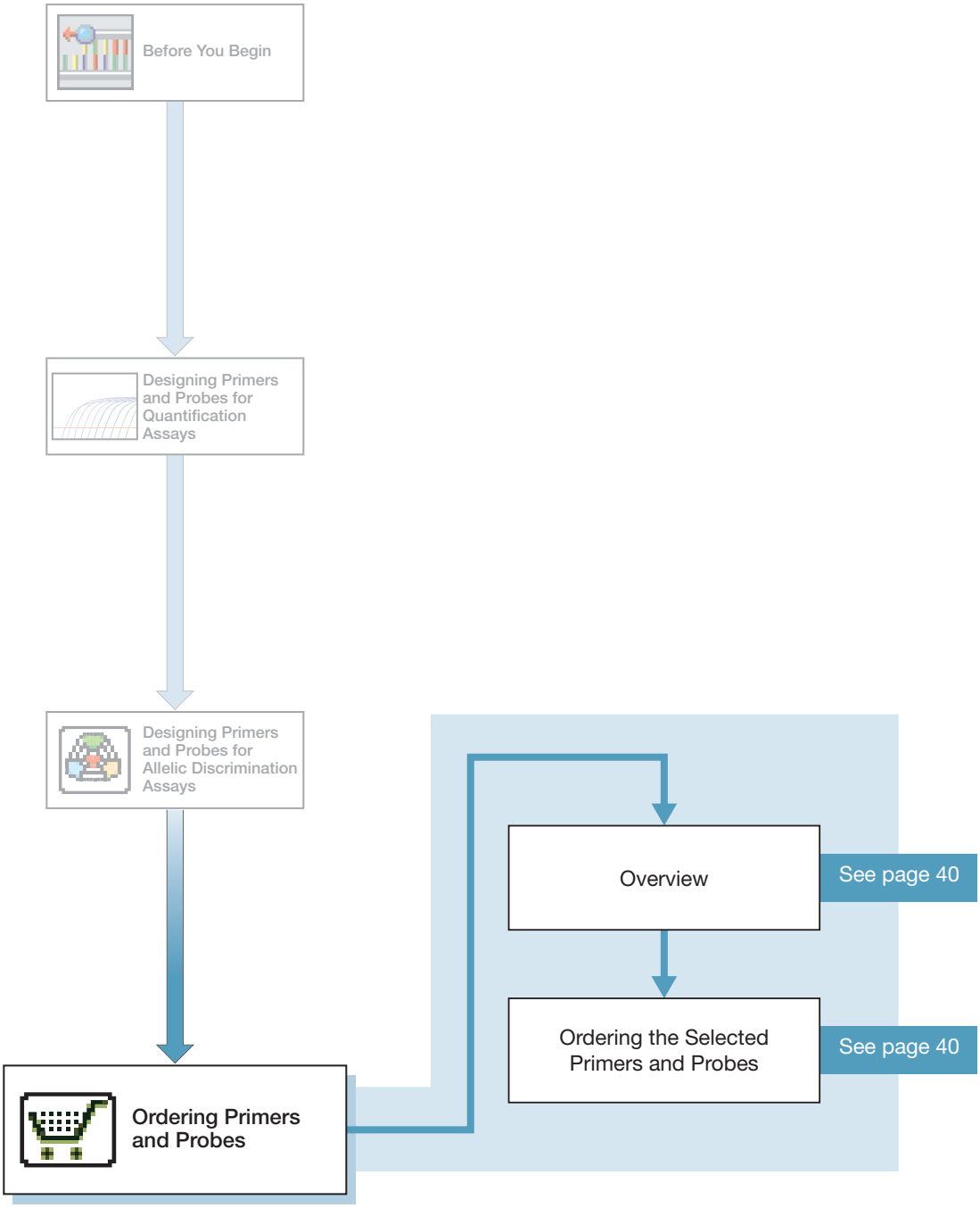
Ordering Primers and Probes

To order primers and probes, see Chapter 4, “Ordering Primers and Probes.”

IMPORTANT! Before running your samples, Applied Biosystems recommends that you run control samples to verify the performance of the selected primers and probes.

Notes

Ordering Primers and Probes



Notes

Overview

About This Chapter

This chapter provides information on how to order your selected primer and probes.

Ordering Primers and Probes

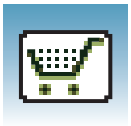
After the Primer Express® Software generates the table of candidate primers and probes, you can order those that best suit your needs.

1. In the Primer/Probe tab, select the primer and probe set you want to order.
2. Click on the **Order** tab.
3. Click on the toolbar to go the Applied Biosystems online store.
4. Log into the AB Store if you have an account, register if you are a new user.

Ordering Primers

1. Below the ABI PRISM® Primers/Probes heading, under the TaqMan Primers and Probes heading, click **Sequence Detection Primers**.
2. In the Product Information tab, select the check box next to the volume of primers to order.
3. Below the primer option you selected, click **Customize**.
4. Follow the instructions on the web page to specify any options.
5. Follow the instructions on the web page to enter or copy your sequence text.
6. Type the name for the Forward Primer, press **Enter**, then copy and paste your primer sequence from either the Order tab or the Primer Probe Test Tool (in the Primer Express software).
7. Type the name for the Reverse Primer, press **Enter**, then copy and paste your primer sequence from either the Order tab or the Primer Probe Test Tool (in the Primer Express software).
8. Click **Continue**.
9. Review your order, then click **Add to Basket**. If this completes your order (SYBR® Green Dye assays), click **Proceed to Checkout** and follow the instructions on the web page to complete your order. Otherwise, click **Continue Shopping** to add Green Dye or probes to your order.

Notes



Ordering Probes

1. Above the Sequence Detection Primers heading, click the **TaqMan® Primers & Probes** link.
2. Below the ABI PRISM® Primers/Probes heading, click the **TaqMan® Primers & Probes** link to expand the list.
3. Select **TaqMan® MGB Probes** or **TaqMan® TAMRA™ dye Probes** (if ordering conventional probes).
4. In the Product Information tab, select the check box next to the volume of probes to order.
5. Below the probe option you selected, click **Customize**.
   a. Follow the instructions on the web page to enter or copy your sequence text. If this probe is for allelic discrimination, be sure to specify the appropriate dyes.
   b. To order additional probes, follow the steps above. Otherwise, review your order, then click **Add to Basket**.
   c. Click **Proceed to Checkout**, then follow the instructions on the web page to complete your order.

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