



For Research Use Only. Not for use in diagnostic procedures.



Life Technologies Corporation | 200 Oyster Point Blvd | South San Francisco, California 94080 USA For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

The information in this guide is subject to change without notice.

DISCLAIMER: TO THE EXTENT ALLOWED BY LAW, THERMO FISHER SCIENTIFIC INC. AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Revision history: Pub. No. 4362460

Revision	Date	Description
D	22 March 2022	The computer operating system was updated to Windows [™] 10.
С	11 May 2015	Baseline for this revision history.

NOTICE TO PURCHASER: DISCLAIMER OF LICENSE: Purchase of this software product alone does not imply any license under any process, instrument or other apparatus, system, composition, reagent or kit rights under patent claims owned or otherwise controlled by Thermo Fisher Scientific, either expressly, or by estoppel.

TRADEMARKS: All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. Microsoft and Windows are trademarks of Microsoft Corporation. Intel and Pentium are trademarks of Intel Corporation or its subsidiaries. Macintosh is a trademark of Apple Inc.

©2022 Thermo Fisher Scientific Inc. All rights reserved.

Contents

	Preface	,
Chapter 1	Before You Begin 1 Introduction 2 Installing the Software 2 Basics 7	
Chapter 2	Designing Primers and Probes for Quantification Assays 9 Overview 10 Automatically Designing Primers and Probes 11 Manually Designing Primers and Probes 15 Automatically Designing Primers for SYBR® Green Dye Assays 21 Manually Designing Primers for SYBR® Green Dye Assays 25	5
Chapter 3	Designing Primers and Probes for Allelic Discrimination 29 Assays 29 Overview 30 Automatically Designing Primers and Probes 31 Manually Designing Primers and Probes 37)
Chapter 4	Ordering Primers and Probes 43 Overview 44 Ordering Primers and Probes 44	; +
	Index 47	,





Preface

How to Use This Guide

Purpose of This Guide	The <i>Primer Express® Software Version 3.0 Getting Started Guide</i> 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[®] 10 operating systems 						
Text Conventions	 Bold indicates user action. For example: Type 0, then press Enter for each of the remaining fields. <i>Italic</i> text indicates new or important words and is also used for emphasis. For example: Before analyzing, <i>always</i> 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 this document. 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. 						
	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.

How to Obtain Support

For the latest services and support information for all locations, go to **http://www.thermofisher.com/support**.

At the Support page, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities.
- Order 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







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 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.



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 TAMRATM) 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 monitor800 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 RAM10 GB EIDE hard drive
Network Adaptors	• 10/100 NIC with RWV (internal)	
Printer	Any PC-compatible printer.	
Operating System	• Windows [®] 10 Professional, Service Pack 1 or later	• Windows [®] 10 Professional, Service Pack 1 or later

Operating Systems Not Supported

- Microsoft[®] Windows[®] NT, 2000, and XP
- Macintosh[®]



Installing the Software

Installing Primer Express Software Version 3.0 **Note:** We recommend 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 6.

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

4. Set the Windows system locale language:

IMPORTANT! To ensure that you can view all results details in the Primer Express software, set the Windows system locale language to **English (United States)** as described in this step.



a. Launch the Windows Start menu, then select **Control Panel > Region and Language** to launch the Region and Language dialog.

Note: The path to the Windows Region and Language menu may differ depending on your Windows version and configuration.

b. In the Formats tab, confirm that Format is set to English (United States).

Format: English (United State	es) 🔹			
Date and time form	iats			
Short date:	M/d/yyyy			
Long date:	dddd, MMMM dd, yyyy			
Short time:	h:mm tt 👻			
Long time:	h:mm:ss tt 🔹			
First day of week:	Sunday 👻			
What does the nota	ation mean?			
Examples				
Short date:	3/30/2015			
Long date:	Monday, March 30, 2015			
Short time:	9:04 AM			
Long time:	9:04:44 AM			
	Additional settings			
Consultant to have all				

c. In the Administrative tab, select Change system locale. In the drop-down list, select English (United States), then click OK, OK.

Formats Location Keyboards and Languages Administrative	
Welcome screen and new user accounts View and copy your international settings to the welcome screen, system accounts and new user accounts.	
Copy settings Tell me more about these accounts	
Language for non-Unicode programs	
This setting (system local) controls the language used when displaying text in programs that do not support Unicode.	
Current language for non-Unicode programs:	
English (United States)	
🛞 Change system locale	🦻 Region and Language Settings
What is system locale?	Select which language (system locale) to use when displaying text in program that do not support Unicode. This setting affects all user accounts on the computer.
	Current system locale:
	English (United States)

Notes.

1



To Uninstall
Primer Express
Software
Version 3.0To uninstall Primer Express Software:
1. On the taskbar, select Start > Control Panel. The Control Panel window opens.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 3.0.3. 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.In Primer Express software version 2.0, information about your oligonucleotide designs

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.



Basics

Starting and	To start Primer Express software for the first time:							
Express Software	 On your desktop, select Start > Programs > Applied Biosystems > Primer Express > Primer Express 3.0. After you start Primer Express 3.0 for 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 <i>Primer Express Software Version 3.0 Online Help</i> for more information on these Primer Express software functions:							
	 Annotating Sequences Exporting Printing 							
	 Using the Batch Process Tool 							

Notes

1



Chapter 1 Before You Begin Basics





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



2

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	To create a new quantification document:					
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 ($\boxed{\mathbb{I}}$). 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 12). The sequence serves as the starting point for primer and probe design.



s dd Vew Todi Wridow Help C	
TorAncha KK GRUpunification # 1 counce Parameteri Primers / Probes Order Fie Name NH-002217.gb Longth 4877 bp. Selection 1 to 1 Double Standed TorAncha TaAADTTorCA AATTATCOGE ATCAATTAT GEATAATAT TORATATTAT TORATATTAT COTGATORGA ATCTTLAGGA TOTACCTA TAAADTTOrCA AATTATCOGE ATCCAATCA TECHAAGAA ATCTTCAAGAA ATCTTCAAGGA TOTACCTAT TORATTORCAA GEATCACTCA TOCTLAGGGA ACCTTCAAGGA ATCTTCAAGGA ATCTTCAAGGA	
Indivation Alex Qualification of a second se	
ozence: Parameter Pinnes / Pickes Dider Pie Name NH-002277.gb Length 4877 bp. Selecton 1 to 1 □ Double Stranded TCTACCTAC TAAASTICAC ANTATACOGC ATCAACTT GOCATTAAGT CATATATT TOAGTATTAT COTGATORAGA ATCTTCAGGA TCTACCTAC TAAASTICAC GAACTACTCA TOCTAAGOT GATCCACTA COCACTATCCACGA ATCTTCAACGA ATCTTCAGGA TCTACCTAC TATAGACGA GAACTACTCA TOCTAAGOT GATCCACTA COCACTATCCACGA ATCTTCAACGA TTCCTATATT	
If Fix Name NH-02227.gb Image: NH-02227.gb Length 4877 bp. Selection 1 to TCTACCTAC TABAGETICGA AATTATCOGGE ACCAATTATE CONTATATATA TOAGTATTAT CONTATTAGE AATCTTCAGGE TCTACCTAC TATAGETICGA CATTATCOGGE ACCAATTAGETIGA GCATATAATT TOATATTATA TOAGTATTAT CONTATTGAGE ACCTTCAGGE TCTACCTAC TATAGETIGA CATTATCOGGE ACCAATTACTGCAGTATATT CONTATTTAT CONTATTATA TOAGTATTAT	
Length 4877 bp. Selection 1 to 1 □ Double Stearded	
▼ Тетастае таллятиска алититское апсалатит восатилаля салиталит токитатита токоталитат сотолетовка алетискова тетасетае таллятиска салитаская салиталит стебалака алетисала балектельска истетскова	
ТЕТИСТ ПЛЕНИТИТЕТИТЕТИТЕТИТЕТИТЕТИТЕТИТЕТИТЕТИТЕТ	minimi
TCTACCTAC TAAAGTTOCA AATTATCOGC ATCAAGTTIT GCCATTAAGA GCATATAATT TGATATTTAA TGAGTATTAT CGTGATGAGA ATCTTCAGGA TCTTTACCT GTTTGGACAG GAGATGCTGA TCCTAAGGTT GATCCGACTA CTGGAGAAGA ATCTCAAGAG GATGATGCAG TTCCTTATGT ATATAAGTTA	
TETTTALET GITTGGALAG GAGARGERGA TEETAAGGET GATELGALTA ETGGAGAAGA ATETLAAGAG GATGATGEAG TILETTAIGT ATATAAGTTA	100
	200
Incenteela Aladochara Iomilanii Aciinele Iileine iileine elabaaalei eliiteine daaradela iakadelee aantakede	300
ICTICIALE ILAIGETTA GUARTAGAT CTATTEGA TAATTETTE GATATLAGT TAGTTEGG TETTETTAT GTAAACGETT LALAGAAATG	400
TITACIOLA GALGALICITA CALLICIA AL ALGALIA CONTRATA AL ALGALIA CALLICIA ALGALIA CONTRATA ALGALIA CALLICIA CALLICIA CALLICIA ALGALIA CALLICIA	500
GIACGACIG INGCICARACIDETAGUICA TETETICTE CIACICOLO E TITETIARA GUIGATITE CIGITATIS DATIRACO CONCALCENTI	600
ACTIVICATI NATIGUTI UTIMATUGA TALATITUGA LAGATGATI CAGAGAGATI UTUGTIATOR ANTIACATA UTUGTI UTUGTIATO	700
THEOLOGI CALLIGUES ATALGES ILAGGALLA ALLATIAS SEGALTAA SETTARETI LESTAAL CHEMANAL GAATLALT	800
LAGATITAG TITETUETA AGGAAATETI TETGETTARG LAITATUTAG AGATACTAGA UATITUTTA UGAAGTUTIT TETTGAGAT GETTITETTA	900
AUGITITUT TILIGUTIKA GUGGATTIAA UTATLAGA UGGTTIAGA UGTTIAGA UTATLAGTIAG TILIGAT TATLATTAGU TALTTITUG	1000
LATITUGUA GAOLAGULIG TITATAATAA AGAGATTAT TULLAATLAG ATALIGITAT GGATLITAGI GGITLIGUG TIAATGATGI GULTITUGI	1100
ATLAGEGE STATESTA SATUSTA ASSULTTESA ASSTALLES ATATTASA THAALSTA LAGIALTET ASATUTTES LAGIALTES	1200
GAATITIDE GAATITAELT AETITGAATG AGAETITAT TEAGAGTAAT AUGEEGATAG ATAGAGEGIT AGEAGTTEET GATEAGEGIG ATTTATTG	1300
SALTITIAL TITAATIATU GITUTATAG GULTATGUG GIGIATITU TITUAGGITI AAGAAGGATI TAATATULLA AUGAGUGIGA ULLAGIAGAAT	140
CCACAGAG CAGCENARGA AAATATTAGT CAATSTAACE TAGTGGAATT GATTAATACT TTTCTGAACE GAAAATTTTG CATTGGGTCA CGTAAGTGAC	1500
FAATTITAG AGGAATTATG AGTTITGGOG AGAATTITGG TAGATTITATA GGAAATTITG TGAATTITGT CGGAAGTGTT ATAGGAGATG GTUTTAAAGG	1600
FITAATICE AETCAGICE TAAGCAGGCE AACCITETTE ATAATTIGEE TITGECTUUT TIGGATATG TITTAAATAT TGGAATGITT	1700
GEGETETTE ETTEAGGEET TETTTETTAT AGAGETGETA AAAAGEAAAA TAAGETTATE CAGGATATTE ETAATAGGEA AATGEETTIT CAGGAGEGAA	1800
STCTAGTAC GECTETTUGA CETCATETAG AGGACITAAA GAAGGCAGGET TEGAATCUGC TETTAGUTT AGEIGGATUT GETTETACTU CICAAGGEGC	1900
ITTTATTCT CCTGTTAATC CTATGCAGTC AGGACTTAAT TCTGCGATAT CAGTGCGGA TAAGGTTTTT GATTATCAGC GTTTAGCTCA TGCTGATTTT	2000
AGGETCETT TGAATTETEC TATGAGTGIT GTTCAGTTEG ETTETECTET TCAGGATATT AAAAGGAALT ATGGAAAGTT TGETGAAGTT GCATATTEGT	2100
TEATCEATA TECTEGEAAG THETESCETE CTATECTITI CTATITETITI AGAAAGCATC CAGITEGAAG AGCGGTITET GETETAATT CTEGTTATEC	2200
GTIGCTAAG GGIGCTAAAG GIGTTAATIT TAAGTITITCG AATATGITCTA GTACGGCTGT TCAGCGTCAT AATITCTAGAT ATAATGITITC GAAAGGAIGG	2300
GAAGGTAAT GAAGTITAGA ACGAITTATG ATGAGGAGCG TCCTGCTCCT GITTTGGAGT GTAAGGATGA AAGTCTATGT TTGGCTTATC AATGTACTGA	2400
ACGTCTATT GAAAAATTGG TTAAGTTAGC GAATCAGAAT CCTTCTTATT TACATGCATT TGCTGGTGAT CCTACTCGTC AACCTGAATA TGGAGAGTGT	2500
CTICTCCTT IGGATTATCA AGATECTITA GAGATTETAG CTCETEGIGA AGAGECITIT TATTCTITAC CIECEGAATAT ICEAGITAAT ITTTCEGAATC	2600
TATGGAGTT TITGTCATGG TTAGAGGACC CTGCTAATTA TGATGAAGTT GAGAAGTTAG GTTTATTGGA TCCTGAGAAA GITCAGATAA GAAAATCTAA	2700
TTACAAAAA GATCAAAAAG AAGAGGTTTC TTCTGAGGAA AAATAGCGAG GGCTATATCC TCTTGGCTAT ATAGCCCCGAG CGACACAAAA TATATAAAAAC	2800
ITAATGAAA GGATGTATTI CGATGGITCG TAGAAGACGT TIGAGAAGAA GAATAAGTAG AAGAATITITI AGAAGAACAG TAGCTAGAGT TGGTAGAAGG	290
GAAGGTCTT TTCGTGGTGG TATTAGATTT TAAGGCAAAA AAAATGGAGT ATCTTTTTAA TGAGATACTC CTTAGACTCA TATCTAATAT CCGTTTATAT	300
SGITTAACA CAAAGGAAAT CAGATTATAT GIGTACTAAT CCTATTATAC CTATAGTICA ATATAAAGIT CCAGTTAAAT CITCGITAGA TGITGIGGAT	310
GETCTARAT TTAGETETAR CTTTARGECT RATETETTIT TTITCERGRA GRATETTETE CETCETECTE TRAGTRATET RERIGATERA	320
IGAGCAACT GAAGCAAGTT AGTTATTTAT CTACTTTTGA TCTTGATGGT TATCATCAGG TGAAGCAGTT TTCTTTTCCT CTTCCTTGTA GGAAATGTTC	330
GAGTEVITTE CAGAGGEGTT CTAGGGETTCAA GCTACTATEG AAGCGCGTTE TCATGAGGAS AATTETEVITT TGATTETTAC TTATGATAAT	340
ITCATTING GCGATAATAT TITAGATTAT GATCATATTC GAGTITITCA GAAGCGITTG COTCOTTATG TGGATTATCA CTATGGCAAA AAGATTAAGT	350
ITTGACTGT AGGAGAATAT GGTGATAAGA AAGGTCGTAT GCATTGGCAT ATGAITGITT TTGGTTGGAA GCCGAAATCT GAGGAACAAT TAGAGCCTTA	360
ITAGGAGGA AAGTATCGAA CGGATGITCG ATATCGTTCT AGAAAGCITA AGGAACTATG GAAATITGGT TATGITGATG TAGATGAAGC TACAGATGGT	370
ATATITITI AIGIAGUTUG TTATGIGUAG AAAAAGTTIG TTGTTGGATG TGATTTAGAT TCTTCTAAGT CTAGTTCTAG GAGAGAAGAA AAGACAGCTT	380
ICAAGUTIT AGGTITAGAT TATIFITITI UTATITAAG GCAATTTETT AAGACTAAGA GGATAGTTIT AAATGGTTIT AGATATGGAT TECCGCGTTA	390
TITAAGGAT TIAITGAGGA AGTIGGITIC AGAGGATICG GAGTITGATA CIGAGTATIA TAATGCTITA AGGAAAAGGI TACITAGIGI ATGIAGITAT	400
CGATGGTAA ATAAATATIT TACCTATITA GAATGCITAG TIGAAGTIIT GCCAGTITIG AATITICAIG AITTATACCA GCGIGCGCII AGGIATAIGG	410
ICAATCTAT TETTAAGCCG CATGCTAGTG ATCATGATGG AGAATATAAT ACTACTTAGG AGATCTGATG CATATGTTTT ATTATTCAAT TTATGATCGA	420
AGGETEGGT ETTATGGAGA TITGATETET TITEETTEAG GTGAGAAAGA GGETGETATI EGATGGTITIA GAGATGTET GATGGATTEA GATTETAAGA	4300
TATTTTGCA TCGATATCCT GAGGATTTTG ATTTTTGCTA TATTGGTTAT TTTGATAAGG ATAAAGGACG TTTTTATCCT GTGGATGCTG GGATAGTTAC	440

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 13.

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*.



At this point, you can:

- Manually design primers and probes as described in "Manually Designing Primers and Probes" on page 15.
- Design using the complementary sequence. For more information, see *Primer Express Software Version 3.0 Online Help.*

Viewing Results IMPORTANT! To ensure that you can view all results details, set the Windows system locale language to English (United States) as described in step 4 on page 4.

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.

🗓 Pi	rime	er Express 3	3.0														
File	Edit	View Tool	s Window H	Help													
	0		X 🖿 🗎 >	< 🔳 🕨		← III		0 🛒 A	8								
П	мре	an® MGB Q	uantificatio	n#1													T P 🛛
Seg	uenc	e Parameter:	Primers / Pro	bes Order													
	Can	didate Primers	& Prohes														
	#	Fwd Start	FwdLen	Ewd Tm	Fwd %GC	Bey Start	Beylen	Bev Tm	Rev %GC	Probe Start	Probelle	Probe Tro	Probe %GC	Amn Tm	Amn %GC	Amp Ta	Amplen
	1	1430	30	58	30	1499	21	59	48	1463	15	69	33	74	34	54	70 🔨
	2	1430	30	58	30	1499	21	59	48	1464	14	69	36	74	34	54	70
	3	1429	31	59	32	1499	21	59	48	1463	15	69	33	74	35	54	71
	4	1429	31	59	32	1499	21	59	48	1464	14	69	36	74	35	54	
	о 6	1428	32	59	31	1499	21	59	48	1463	10	69	33	74	35	54	72
	7	1427	33	59	30	1499	21	59	48	1463	15	69	33	74	34	54	73
	8	1427	33	59	30	1499	21	59	48	1464	14	69	36	74	34	54	73
	9	1426	29	59	31	1499	21	59	48	1463	15	69	33	73	34	54	74
	10	1426	29	59	31	1499	21	59	48	1464	14	69	36	73	34	54	74
	11	1425	30	59	30	1499	21	59	48	1463	15	69	33	73	33	54	75
	12	4049	23	58	30	4128	25	58	40	4073	18	70	50	75	39	55	80
	14	4049	23	58	30	4128	25	58	40	4073	19	70	47	75	39	55	80
	15	4049	23	58	30	4128	25	58	40	4074	17	69	53	75	39	55	80
	16	4049	23	58	30	4128	25	58	40	4074	18	69	50	75	39	55	80
	17	4049	23	58	30	4128	25	58	40	4075	16	70	56	75	39	55	80
	18	4049	23	56	30	4128	25	58	40	40/5	1/	/0	53 CN	75	39	55	8U =
	20	4043	23	58	30	4128	25	58	40	4076	15	69	56	75	39	55	80
	21	4049	23	58	30	4128	25	58	40	4077	14	69	64	75	39	55	80
	22	4049	23	58	30	4128	25	58	40	4077	15	69	60	75	39	55	80
	23	4049	23	58	30	4128	25	58	40	4078	13	68	69	75	39	55	80
	24	4049	23	58	30	4128	25	58	40	4078	14	68	64	75	39	55	80
	20	4049	23	56	30	4128	25	56	40	40/9	14	59	64	75	39	50	80
	27	4045	23	58	30	4128	25	58	40	4080	16	70	56	75	39	55	80
	28	4049	23	58	30	4129	26	59	38	4073	18	70	50	75	38	55	81
	29	4049	23	58	30	4129	26	59	38	4073	19	70	47	75	38	55	81
	30	4049	23	58	30	4129	26	59	38	4074	17	69	53	75	38	55	81 🞽
-																	<u> </u>
	Loc	ation															
	8					1347 79											
	Sec	ondary Structu	ire														
	F		06				long	5k		Hairpin Self Di	mers Cross Di	ners					
	Olgo Lengn						[Next Stable Structure Found									
		Development Dela							-			are round					
	0	Heverse Prin	ner				21			GCAATGT	AACT 5'						
	0	Probe					15			Ţ							E .
	F	orward Primer								AGTGGAA	TIGATTAATA	. 3'					
	T	CAATGTAACO	GTAGTGGAAT	TGATTAATAC													
	B	leverse Primer															
	TCACTTACGTGACCCAATGCA																
	P	robe															
	T	CTGAAGCGA	AAATT														~
										<		Ш			_	_	>
50 res	ubal	found		_			_	_				_	_	_	_	_	

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

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 **(a)** 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, we recommend that you run control samples to verify the performance of the selected primers and probes.



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 11.

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 16).



🌆 Prir	ner Express 3	8.0														- 8)
File E	lit View Tools	s Window He	əlp													
		X 🖬 🗎 🗙	🔳 🍉		→ ← 🗉 🗾		🛛 😧 🛒 AS	5								
📓 Taq	Man® MGB Q	uantification	#1													- 6 🛛
Seque	nce Parameters	Primers / Prot	es Order													
0-0	andidate Primers	& Probes														
Œ	Fwd Start	Fwd Len	Fwd Tm	Fwd %GC	Rev Start	Rev Len.		Rev %GC	Probe Start	Probe Le	Probe Tm	Probe %GC	Amp Tm	Amp %GC	Amp Ta	Amp Len
1	1430	30	58	30	1499	21	59	48	1463	15	69	33	74	34	54	70 🔨
23	1429	31	59	30	1499	21	59	48	1463	15	69	33	74	39	54	71
4	1429	31	59	32	1499	21	59	48	1464	14	69	36	74	35	54	71
5	1428	32	59	31	1499	21	59	48	1463	15	69	33	74	35	54	72
6	1428	32	59	31	1499	21	59	48	1464	14	69	36	74	35	54	72
1	1427	33	59	30	1499	21	59	48	1463	10	69	33	74	34	54	73
9	1426	29	59	31	Drimor Droh	Tost T	nol.		. ITVT		199			34	54	74
10	1426	29	59	31	Primer Probe	rest fi	000							34	54	74
1	1425	30	59	30	admicicia									33	54	75
1	1425	30	59	30	Dcoument Type:	TaqMan(MGB Quantificati	on 🔽 P	Parameter: Defa	ault	~	Browse		33	54	/5
14	4045	23	58	30	Primers and Prob	BS							_	39	55	80
18	4049	23	58	30										39	55	80
16	4049	23	58	30							Tm %GC	Length		39	55	80
1	4049	23	58	30	Fwd Pr	imer					0.0 0	0		39	55	80
18	4049	23	58	30							Tm %GC	Length		39	55	80
2	4045	23	58	30	Hev Pr	imer					0.0 0	0		39	55	80
2	4049	23	58	30	Duba	TO	ANTOTANCOTAC	TCCAATICAT	TAATAC	_	Tm %GC	Length		39	55	80
2	4049	23	58	30	Probe		AATGTAALGTAG	TGGAATTGAT	TAATAL		75.0 30	30		39	55	80
2	4049	23	58	30	Duba	. –				_	Tm %GC	Length		39	55	80
24	4049	23	59	30	Probe a	<u>د</u>				_	0.0 0	0		39	55	80
28	4049	23	58	30	Trim									39	55	80
23	4049	23	58	30										39	55	80
28	4049	23	58	30	-Secondary Struct	ure							_	38	55	81
23	4049	23	58	30	0600		Lana	the 1	Hairpin Self Di					38	55	81
4	4043	2.5	130	30	O Comment Dates		Leng	un					1	130	50	
	cation				Forward Prim	er	0									
00					O Reverse Prime	er	0									
					O Probe 1		30									
	condary Structu	re			O Probe 2		0									
F		Oliao				now Seco	odam Structure									
	Forward Prin	ner					nosty on dotato									
	Beverse Prim	her.				,	1		0.0111000			_				~
G	Probe					1	5		T		0.000					=
	Forward Primer								<i>AGTGGAA</i>	TTGATTAATA	C 3'					-
	TCAATGTAACG	GTAGTGGAATT	GATTAATAC													
	Reverse Primer															
	TEACTTACGTO	GACCEAATGEA				_										
	Probe															_
	TCTGAAGCGA	AAATT														× *
Ŀ									N					_	_	2
E0	1 1			_		_										

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)
- $\mathbf{Tm} 68 \ ^{\circ}\mathbf{C}$ to 70 $^{\circ}\mathbf{C}$.
- % 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.

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 18.
- 8. To automatically find primers after manually designing the probe, select Edit > Annotate > Probe (I). 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 18.

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 18.



Viewing Results IMPORTANT! To ensure that you can view all results details, set the Windows system locale language to **English (United States)** as described in step 4 on page 4.

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 **a** 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

Primer Express Software 3.0 Getting Started Guide



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.



Ordering Primers and Probes

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

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



2

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	To create a new quantification document:
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 <i>NM_002217</i> , 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:
	 Select Tools > Add DNA File (
	2. At the Add DNA File dialog box, navigate to and select the desired file. For information on the various file formats supported, see <i>Primer Express Software Version 3.0 Online Help</i> .
	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 22). The sequence serves as the starting point for primer design.



🖬 Primer Express 3.0		7 🗙
File Edit View Tools Window Help		
🕅 TaoMan® MGB Oventification # 1		
SQUATE: Parameters Primers / Primers / Primers		_
Length 4877 bp. Selection 1 to 1 Double Stranded		
ΥΥ		
CHITRACTAC AMETRICA METATORIC ATCALETTE SCALTARS CONTAINT TRAVETTAL TRADUTTA CONTAINS ATTENDED	100	
CICUTENCIE STUDIERAS GARATETIS TUCIARATE SUCCESSION AUTOMASIA ATTIVASIS ATTIVASIS ATTIVASIS AUTOMAS	200	-
ATSCISTICAL ATALONGATA TRATTATTIT ACTITITICTICTI TICTIGITTI GUARAAAGGI COTTITITITI GAATAGGIAT TACAGGIGGA GATTIAGGAC	200	
CHURCH TRANSPORTER ACCAUTEAGE CTRATTERGE TRATTETTER REALIZED TRACTICE TRACTICE CONSIGNED ACCAUTE	500	
CITEMATICA CATEGORIET TRACTITIES ANTIGONAE CONCENTES CRAFALING TANTITIES TRACTARE TENEDATIC AVETANTE	400	
STRAGATE TECHNAL TECHNETCA TETETTET CLATECOCC TETENTIAG GENALTET CLATECOLE AND	500	
CARTRACING TANTIFICATION CONTINUES INCOMENTATION ADDRESS TO ADDRESS AD	600	
THITESCH ANTION INTO THE TRACE TRACE AND ANTION A COMMAND A	700	
Information and a second management of the second of the second s	800	
ALAMATICAY ITELECTICA AVANANCIT FUTUTINO CATANICAL OPENATORY CANOLITY ITE COAMICTIT TOTINAOCAL WITHIGHTA	900	
INVOLUTI I LANGIALA GUNATIAN CIAILANA ANTIANA GUNANNI ANTIAN COLLANDI INI INI DOCUMANTIAN	1000	
TARILIGUNA GARCARCETO TITATARIAR ANNATTATI DECARTAN ATALIATIATI DITATO DELL'ANDI DETETETO DI DECETTI DEI DECARTANA ANNATTATI DECARTANA ANNATTATI DI DECARTANA ANNATTATI DECARTANA ANNATTATI DI DECARTANA ANNA DI	1100	
INTERNARAGE GITATELEGA GIALDITAT ANGULTUGA MEGITATEGA ITATIANA LITAK ANGULA CAGINETE AGAITETEG ANTITUGU	1200	
AGAINING GANTIACT ACTIGATE ACAINT TAGAGAAN ACCOUNT ALLOCANA ALLO	1300	
Indeliniae interinite sitesianae curatoles pistantite intervente adaptorin nariatella elevatoria entre constructoria adaptoria elevatoria	1400	
GEDAGAGAG CACCETAAGA AAATATTAGT CAATGTAACG TAGTGGAATT GATTAATACT TITCTGAAGC GAAAATTITG CATTGGGTCA CUTAAGTGAC	1500	
ATAATITTAG AGGAATTATG AGTITCGCGG AGAATGITGG TAGATTCATA GGAAATTCTG TGAATTCTGT CGGAAGTGTT ATAGGAGATG GTCTTAAAGG	1600	
TITTAATTCC ACTCAGTCTA TITCCAGTGC TAAGCAGGCA AACCTTCTTA ATAATTTGCC TITGCCTTCT TTAGATAATG TITTAAATAT TGGAATGTTT	1700	
GEGEGETETTE ETTEAGECET TETTTETTAT AGAGETEETA AAAAGEAAAA TAAGETTATE CAGGATATTE CTAATAGEEA AATGEETTTE CAGGAGEGAA	1800	
TETCTAGTAC GECTETTCEA CETCATETAG AGEACITAAA GAAGGCAGET TTEAATCCCC TITTAGCTIT AGETGGATET GCTTCTACTC CTCAAGGTGC	1900	
TITITATEE CEETAATE CAAGAGE AGGACTTAAT EETGEGATAA CAGTEGEGGA TAAAGETTITE GATTATEAGE GITTAGEEGA TAGEGATITE	2000	
CAGGGTCGTT TGAATTCTSC TATGAGTGTT GTCAGTTGG CTTCTGCTGT TCAGGAATTAT AAAAGGAACT ATGGAAAGTT TGGTGAAGTT GCATATTGGT	2100	
ITGATEGATA TECTOGCAAG ITGITECCTE CTATECTITI CTATITETTI AGAAAGCATE CAGITEGAAG AGEGETITET ECTECTAATI ETEGTIATEC	2200	
TETTECTAAG GETECTAAAG GEGETAATTT TAAGTTTTCE AATATGTCTA GTACGGCTGT TCAGCGCTCAT AATTCTAGAT ATAATGTTTC GAAAGGATGG	2300	
AGAAGGTAAT GAAGTTTAGA ACGATTTATG ATGAGGAGGG TCCTGCTCCT GTTTTGGAGT GTAAGGATGA AAGTCTATGT TTGGCTTATC AATGTACTGA	2400	
GACGTETATT GAAAAAATTGG TTAAGTTAGE GAATCAGAAT CETTETTATT TACATGEATT TGETGGTGAT CETAETCGTC AACETGAATA TGGAGAGTGT	2500	
CCTTCTCCTT TGGATTATCA AGATGCTTTA GAGATTGTAG CTCGTGGTGA AGAGGCTTTT TATTCTTTAC CTGCGAATAT TCGAGTTAAT TTTTCGAATC	2600	
CTATGGAGTT TTTGTCATGG TTAGAGGACC CTCCTAATTA TGATGAAGTT GAGAAGTTAG GTTTATTGGA TCCTGAGAAA GTTCAGATAA GAAAATCTAA	2700	
GTTACAAAAA GATCAAAAAA GATCAAAAAAG AAGAGGTTTE TTCTGAGGAA AAATAGCGAG GGCTATATCC TCTTGGCTAT ATAGCCCGAG CGACACAAAA TATATAAAAC	2800	
CTTAATGAAA GGATGTATTT CGATGGTTCG TAGAAGACGT TTGAGAAGAA GAATAAGTAG AAGAATTTTT AGAAGAACAG TAGCTAGAAT TGGTAGAAGG	2900	
CGAAGGTCTT TTCGTGGTGG TATTAGATTT TAAGGCAAAA AAAATGGAGT ATCTTITTAA TGAGATACTC CTTAGACTCA TATCTAATAT CCGTTTATAT	3000	
CCGTTTAACA CAAAGGAAAT CAGATTATAT GTGTACTAAT CCTATTATAC CTATAGTTCA ATATAAAGTT CCAGTTAAAT CTTCGTTAGA TGTTGTGGAT	3100	
TGGTCTAAAT TTAGGTCTAA CTTTAGGGCT AATCTGTTTT TTTTCGAGAA GAATGTTGTT CGTCGTGCTG TAAGTAATGT AGATGAAGCT TTTAGATTTA	3200	
CTGAGCAACT GAAGCAAGTT AGTTATTTAT CTACTTTTGA TCTTGATGGT TATCATCAGG TGAAGCAGTT TTCTTTTCCT CTTCCTTGTA GGAAATGTTC	3300	
TEASTSTITE CASAASCETT CTAASGATTT ASCEGETTCAA SCTACTATES AASCEGETTC TCATEASGAS AATTCTETTT TEATTCTAC TTATEATAAT	3400	
GATCATITIAG GCGATAATAT TITAGATTAT GATCATATIC GAGTITITCA GAAGCGITIG COTCOTIATG TOGATTATCA CTATOGCAAA AAGATTAAGT	3500	
TTTTGACTGT AGGAGAATAT GGTGATAAGA AAGGTCGTAT GCATTGGCAT ATGATTGTTT TTGGTTGGAA GCCGAAATCT GAGGAACAAT TAGAGCCTTA	3600	
TTTAGGAGGA AAGTATUGAA CEGATGTTUG ATATUGTTUT AGAAAGCTTA AGGAACTATG GAAATTTUGT TATGTTGATG TAGATGAAGU TACAGATGGT	3700	
AATATITITA AUGTAGETEG TATEGTEGAS AAAAAGTITE TIGTIGGATE GEATITAGAT CUTCURAGE CAGGITETAG GAGAGAGAAS AAGACAGETT	3800	
CTCAAGCTTT AGGTTTAGAT TATTITITT CTTATTTAAG GCAATTTCTT AAGACTAAGA GGATAGTTTT AAATGGTTTT AGATATGGAT TTCCCCGTTA	3900	
TTTTAAGGAT TTAITGAGGA AGTTGGTTTC GCAGGATTCG GAGTTTGATA CTGAGTATTA TAATGCTTTA AGGAAAAGGT TACTTAGTGT ATGTAGTTAT	4000	
Incratogram atamatatit tacctatita gaatecitag tigaagitit gccagitite aatiticate attitatacca gcgtocgcit aggtatateg	4100	
ATCAATCTAT TCTTAAGCCG CATGCTAGTG ATCATGATGG AGAATATAAT ACTACTITAGG AGATCTGATG CATATGTTTT ATTATTCAAT TTATGATCGA	4200	
AAGGCTCCGT CTTATGGAGA TITGATCTCT TITCCTTCAG GTGAGAAAAG GGCTGCTAIT CGATGGTTTA GAGATGTTTG GATGGATTCA GATTCTAAGA	4300	
ATATTITICA TEGATATECT GAGGATITES ATTITECTA TATEGETAT TITEATAAGG ATAAAGGACG TITETATECT GEGATAGETE GATAGETAC	4400	
CATTENTIALE CONCENCION TERMINETALE MODIFICATE ACLICALCALE ACCOUNTANT CONTAINED ACCALACCENT CONTRACTOR	4400	×
To find Primers & Probes, click the "Find Primers/Probes" button		

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 23.

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 as described in "Manually Designing Primers for SYBR® Green Dye Assays" on page 25.
- Design using the complementary sequence. For more information, see *Primer Express Software Version 3.0 Online Help.*

Viewing Results IMPORTANT! To ensure that you can view all results details, set the Windows system locale language to English (United States) as described in step 4 on page 4.

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.

<u>1</u>	Primer	Express 3	.0														
File	Edit	View Tools	Window H	Help													
				× 🔳 🕨		+ € II		0 🦞 R	8								
	lagMa	n® MGB Qi	Jantificatio	n#1													
Se	quence	Parameters	Primers / Pri	obes Order													
F	Candi	date Primers :	Prohes														
5	(H)	Eucl Chart	Fund Lon	Fund Tro	End %CC	Day Start	Review	Rev Tro	Day %GC	Droho Start	Proballa	Proho Tro	Robe %GC	Amp Tm	Amo %GC	Arro Ta	Amplan
	<u> </u>	1430	31	58	31	1499	21	59	48	1463	15	69	33	74	34	54	70
	2	1430	30	58	30	1499	21	59	48	1464	14	69	36	74	34	54	70
	3	1429	31	59	32	1499	21	59	48	1463	15	69	33	74	35	54	71
	4	1429	31	59	32	1499	21	59	48	1464	14	69	36	74	35	54	71
	5	1428	32	59	31	1499	21	59	48	1463	15	69	33	74	35	54	72
	6	1428	32	59	31	1499	21	59	48	1464	14	69	36	74	35	54	72
	7	1427	33	59	30	1499	21	59	48	1463	15	69	33	74	34	54	73
	8	1427	33	59	30	1499	21	59	48	1464	14	69	36	74	34	54	73
	9	1426	29	59	31	1499	21	59	48	1463	15	69	33	73	34	54	74
	10	1426	29	59	31	1499	21	59	48	1464	14	69	36	73	34	54	/4
	10	1425	30	59	30	1439	21	59	48	1463	10	69	33	73	33	54	75
	12	1420	22	59	30	4129	21	59	40	1909	14	70	50	75	33	55	- 20
	14	4043	23	58	30	4128	25	59	40	4073	19	70	47	75	39	55	- 80
	15	4049	23	58	30	4128	25	58	40	4074	17	69	53	75	39	55	80
	16	4049	23	58	30	4128	25	58	40	4074	18	69	50	75	39	55	80
	17	4049	23	58	30	4128	25	58	40	4075	16	70	56	75	39	55	80
	18	4049	23	58	30	4128	25	58	40	4075	17	70	53	75	39	55	80
	19	4049	23	58	30	4128	25	58	40	4076	15	69	60	75	39	55	80
	20	4049	23	58	30	4128	25	58	40	4076	16	69	56	75	39	55	80
	21	4049	23	58	30	4128	25	58	40	4077	14	69	64	75	39	55	80
	22	4049	23	58	30	4128	25	58	40	4077	15	69	60	75	39	55	80
	23	4049	23	58	30	4128	25	58	40	4078	13	68	69	75	39	55	80
	24	4049	23	58	30	4128	25	58	40	4078	14	68	64	75	39	56	80
	25	4049	23	58	30	4126	25	58	40	4079	14	59	64	/5	39	50	80
	26	4049	23	26	30	4126	20	56	40	4060	14	70	54	70	39	50	80
	27	4045	23	50	20	4120	20	50	90	4060	10	70	50	73	30	30	00
	28	4045	23	58	30	4123	20	59	38	4073	19	70	47	75	30	55	
	30	4040	23	58	30	4129	26	59	38	4074	17	69	53	75	38	55	81
	<	1010	100			11125	120							1.0			>
F	-Locat	ion															
						LALEBARD											
	22					134779											
E	Secondary Structure																
			01	02	í					Hairrin Call Di	man Curre Di						
			Ulig	lo			Leng	n		Tranpin Set Di	Inters Closs Di	ildis					
	Forward Primer 30										able Struct	ure Found					
	O Reverse Primer 21										FAACT 5'						^
	O Probe 15																
										LAGTEGAA	TTGATTAATA	2 3'					
	For	ward Primer															
	TC	AATGTAACG	TAGTGGAAT	TGATTAATAC													
	Reverse Primer																
	TC	ACTTACGTO	ACCCAATGO	A													
	Probe																
TCTG4AGCG4AAATT													~				
										<					_	_	>

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



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 **(a)** 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, we recommend that you run control samples to verify the performance of the selected primers and probes.



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 11.

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 26).

2



🚮 P	rimer	Express 3.	.0														- 5	×
File	Edit	View Tools	Window Hel	þ	and I ama I													
		al (4) (3)				→ ← Ⅲ Ⅲ	6 <u></u>	A 12 (0)	8									
1	aqMa	n® MGB Qu	antification i	# 1													_ 6	×
Sec	uence	Parameters	Primers / Probe	ss Order														
	Cand	idate Primers 8	Probes	1	100.000		(V	100.000	200)(2 + 4.00		201 100			-
	<u>п</u>	Fwd Start	Fwd Len	Fwd Im	Fwd %GL	Hev Start	Rev Len.	. Hev Im	Rev %GC	Probe Start	Probe Le	Probe I m	Probe %GC	Amp Im	Amp %GC	Ampla	Amp Len	
	2	1430	30	58	30	1499	21	59	48	1464	14	69	36	74	34	54	70	
	3	1429	31	59	32	1499	21	59	48	1463	15	69	33	74	35	54	71	
	4	1429	31	59	32	1499	21	59	48	1464	14	69	36	74	35	54	71	
	5	1428	32	59	31	1499	21	59	48	1463	15	69	33	74	35	54	72	
	7	1427	33	59	30	1499	21	59	48	1463	15	69	33	74	34	54	73	
	8	1427	33	59	30	1499	21	59	48	1464	14	69	36	74	34	54	73	
	9	1426	29	59	31	💷 Primer Prot	e Test To	ol						X	34	54	74	
	10	1426	29	59	31	Parameters								<u> </u>	34	54	74	
	12	1425	30	59	30	Decument Tune	TaoMan®	MGR Quantifical	ion V P	aramater Def	a di	~	Browen		33	54	75	
	13	4049	23	58	30	b'counterit Type.	Taqualis	Mub Guannica		arameter.	ouk		DIOWSE		39	55	80	
	14	4049	23	58	30	Primers and Prol	890								39	55	80	
	15	4049	23	58	30										39	55	80	
	15	4049	23	58	30	Ewd F	vimer				_	Tm %GC	Length		39	55	80	
	18	4043	23	58	30						1	0.0 0	0		39	55	80	
	19	4049	23	58	30	Rev F	rimer					Tm %GC	Length		39	55	80	
	20	4049	23	58	30						-	0.0 0	0		39	55	80	
	21	4049	23	58	30	Probe	1 TO	AATGTAACGTAG	TGGAATTGAT	TAATAC		Tm %GC	Length		39	55	80	
	22	4049	23	58	30							75.0 30	30		39	55	80	
	24	4049	23	58	30	Probe	2					1m %60	Length		39	55	80	
	25	4049	23	58	30							0.0	0		39	55	80	
	26	4049	23	58	30	Inm									39	55	80	
	27	4049	23	58	30										39	55	80	
	28	4049	23	58	30	Secondary Struc	ture								38	55	81	
	30	4049	23	58	30	Oligo		Len	gth 1	Hairpin Self D	imers Cross Di	mers			38	55	81	
	<				1	Forward Prin	ner	0									>	
	Local	tion				O Beverse Prin	107	0										-
	-					O Deeba 1												-
						OPIQUE			(1
	Seco	ndary Structur	9			O Probe 2		0										1
			Oligo				Show Secor	ndary Structure										-
	0 F	orward Prim	er														1770	
	OF	Reverse Prime	16				2	1		GCAATG	FAACT 5'						<u>^</u>	
	OF	Probe					11	5		TIL	TTGATTAATA	C 31						
	For	rward Primer								AUTODA	ALLOAT LAALA							
	TC	AATGTAACG	TAGTGGAATTG	ATTAATAC														
	Re	verse Primer																
	TC	ACTTACGTG	ACCCAATGCA															
	Pro	obe																
	TC	TGAAGCGAA	AATT							(>	
	r—								<u>L</u>									4
50	N 6																	_

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%.



• 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 PrimerCopy 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, we recommend that you run control samples to verify the performance of the selected primers and probes.







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







Automatically Designing Primers and Probes

... ...

. ...

Creating an Allelic	To create a new allelic discrimination document:
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, <i>AY228765.txt</i> , 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:
	 Select Tools > Add DNA File (Select Tools > Add DNA File (Select Tools > Add DNA File (Select Tools > Add DNA File (
	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 32). The sequence serves as the starting point for primer and probe design.



🔤 Primer Express 3.0	
File Edit View Tools Window Help	
📱 TaqMan® MGB Allelic Discrimination # 1	7 🗙
Sequence Parameters Primers / Probes Order	
🔐 File Name 🛛 AY228765.txt 🛛 🔀	
Length 1548 bp. Selection 1 to 1 🗌 Double Stranded	
γγ	1
ATEGRANATE CONTRACTOR TO CARTETE CONTRACTOR CONTRACTOR CONTRACTOR AND CONTRACTOR AND CONTRACTOR	
RECTEGREE TACTEGREE TACTEGREET TAAGAAGEE AGAGAGEA TECHTEGREET TECHAGAGAGA AAACGATCEE CEGGGETET (200	
TCAGCACCCG GAACACTEC AGGACTERE CITCAGCAGE TCTCAGCGGE TTATCATEAT CATCCACGGG TEGETEGET & 200	
ATCTGGAAGA TAGTGAGTGC GCTGAAGTCC CAACCTGTGAA TGTGGGGTTA GTGGACTGGA TCTCCCTGGC ATACCAGCAC 1 300	
CTETTCAAAA CACCCETATT STEEGECCASE ACETEECTEC TCTTCTCCTA TEECTEGAAE AATCTECEAA STTTTCTCEE AECAAAETTC # 500	
GTACAGECTE GEAGEGEACE TETEAGEGET EGEAGEGEAEE TECATEGAEE GEAAGAACAA GATTEGAAGA ATCAEGEGE TEGAECETEE E	
TITGAGGGAA CGTCCCCCAA CGAGCGCCTT TCTCCTGATG ATGCCAATTT TGTGGACGCC ATTCATACCT TTACCAGGGA GCACATGGGC 1 700	
GCATCAAGCA GCCCATTGCC CACTATGACT TCTACCCCCAA CGGGGGGCTCC TTCCAGCCTG GCTGCCACTT CCTGGAACTC TACAAACACA T 800	=
TEGECTAAAC ECCATAACCE AGACCATCAA ATETECCCCAT GAGESCTCCE TECACCTCTT CATTEACTCE TTECAACACA ETEACCTECA E 900	
TTCCAGTGCA GCGACATGGG CAGCTTCAGC CAAGGTCTAT GCCTGAGCTG CAAGAAGGGC CGTTGCAACA CTCTGGGTTA TGACATCCGC # 1000	
CAGGCAAGAG CAAGAGGCTC TTCCTCATCA CGCGAGCCCA GTCTCCCTTC AAAGTTTATC ATTACCAGTT CAAGATCCAG TTCATCAATC # 1100	
GCCGGTAGAG CCTACTTTTA CCATGTCGCT GCTGGGAACA AAAGAAGAAA TAAAGAGAAT TCCCATCACC CTGGGCGAAG GAATTACCAG C 1200	
TATTCCTTCC TTATCACACT GGACAAAGAC ATCGGCGAGT TGATCCTGCT CAAGTTCAAG TGGGAAAACA GTGCAGTGTG GGCCAATGTG 1 1300	
TGCAGACCAT CATGCTATGG GGCATAGAAC CTCACCACTC TGGCCTCATT CTGAAGACCA TCTGGGTCAA AGCTGGAGAG ACGCAGCAAA © 1400	
TTGCCCCCGAA AATCTGGATG ACCTCCAGCT TCACCCGAGC CAGGAGAAAG TCTTTGTGAA CTGTGAAGTG AAGTCAAAAA GACTGACTGA # 1500	
CAGATGAGTC AAGAGACCCA TGCAAAAAAA TAAAGAAGTC TATTCTTT 1548	
	Y
To find Primers & Probes, click the "Find Primers/Probes" button	

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 To assign a SNP target: 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 33).



file Edit View	Tools Win	dow Help									
🗅 😂 📙	& X D				m → ←	at 🗾 🛞		ea `!!			
👹 TaqMan® A	AGB Allelic I	iscriminatio	n#1					والمتحرفين وحافرا	ويستيت والتراجع		7
Sequence Pa	rameters Prime	ers / Probes Ord	ler								
🔐 File Name	AY228765.txt										
Length	1548 bp.	Selection	528 t	0	528 🔲	Double Stranded	ł				
				Lu	muntun		untinn				
ATGGGAAATC	CCCTCCAAAT	CTCCATTTTC	CTGGTG	FTCT	GCATCTTTAT	CCAATCAAGT	GCTTGTGGA	C AAGGCGTGGG	AACAGAGCCC	7 100	^
GCCTTGGAGC	TACTGAAGCT	AGCAAGCCAT	TAAAGA	AGCC	AGAGACCAGA	TTCCTGCTCT	TCCAAGATG	A AAACGATCGC	CTGGGCTGTC	C 200	
TCAGCACCCG	GAAACACTGC	AGGAGTGTGG	CTTCAA	CAGC	🛅 SNP/Tar	get 🔣	CATCCACGG	G TEGTCEGTEG	ATGGCTTGCT	A 300	
ATCTGGAAGA	TAGTGAGTGC	GCTGAAGTCC	CGACAG	гссс			GTGGACTGG	A TCTCCCTGGC	ATACCAGCAC	T 400	
CTGTTCAAAA	CACCCGTATT	GTGGGCCAGG	ACGTGG	CTGC			AATCTGCGA	A GTTTTCTCGG	AGCAAAGTTC	A 500	
GTACAGCCTG	GGAGCGCACG	TCTCAGGGTT	CGCAGG	CAGC		3	GATTGGAAG	A ATCACAGGGC	TGGACCCTGC	6 600	
TTTGAGGGAA	CGTCCCCCAA	CGAGCGCCTT	TCTCCT	GATG	M/ /i	J R	ATTCATACC:	F TTACCAGGGA	GCACATGGGC	T 700	
GCATCAAGCA	GCCCATTGCC	CACTATGACT	TCTACC	CAA	18		GCTGCCACT	CCTGGAACTC	TACAAACACA	1 800	
TGGCCTAAAC	GCCATAACCC	AGACCATCAA	ATGTGC	CCAT			CATTGACTC	TTGCAACACA	GTGACCTGCA	6 900	
TTCCAGTGCA	GCGACATGGG	CAGCTTCAGC	CAAGGT	TAT	C	\sim	CGTTGCAAC	A CTCTGGGTTA	TGACATCCGC	A 1000	
CAGGCAAGAG	CAAGAGGCTC	TTCCTCATCA	CGCGAG	CCCA			ATTACCAGT	CAAGATCCAG	TTCATCAATC	A 1100	
GCCGGTAGAG	CCTACTTTTA	CCATGTCGCT	GCTGGG.	AACA		<u> </u>	TCCCATCAC	C CTGGGCGAAG	GAATTACCAG	£ 1200	
TATTCCTTCC	TTATCACACT	GGACAAAGAC	ATCGGC	GAGT			TGGGAAAAC	A GTGCAGTGTG	GGCCAATGTG	I 1300	
TECAGACCAT	CATGCTATGG	GGCATAGAAC	CTCACC	ACTO		Lancel	TETEGETCA	AGCTGGAGAG	ACGCAGCAAA	F 1400	
TTGCCCCGAA	AATCTGGATG	ACCTCCAGCT	TCACCCI	GAGC	CAGGAGAAAG	TETTEGAA	CTGTGAAGTI	AAGTCAAAAA	GACTGACTGA	A 1500	
CAGATGAGTC	AAGAGACCCA	TGCAAAAAAA	TAAAGA	AGTC	TATTCTT					- 1500	
										1040	

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

IMPORTANT! To ensure that you can view all results details, set the Windows system locale language to **English (United States)** as described in step 4 on page 4.

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 34). 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.

💷 Primei	r Express 3	.0														_ @
File Edit	View Tools	Window He	alp I IIII I IIII IIII	Th set -			0 w/ .									
Tacks		olio Diserimi	ination # 1					•								
Sequence	Parameters	Primers / Prob	es Order													
Cand	fidate Primers 8	Probes														
#	Fwd Start	Fwd Len	Fwd Tm	Fwd %GC	Rev Start	Rev Len	Rev Tm	Rev %GC	Probe1 S	Probe1 L	Probe1 Tm	Probe1 %	Probe2 S	Probe2 L	Probe2 Tm	Probe2 %.
1	482	24	58	42	560	20	60	50	521	15	65	60	518	14	67	50 🔨
2	482	24	58	42	560	20	60	50	521	16	66	63	518	14	67	50
3	482	24	58	42	560	20	60	50	522	15	65	6/	518	14	6/	50
4	482	20	59	44	560	20	60	50	521	10	60	60	518	14	67	50
0 C	402	20	50	44	560	20	60	50	522	15	65	63	510	14	67	50
7	402	20	50	44	560	20	60	50	521	15	65	60	510	14	67	50
r	401	25	59	40	560	20	60	50	521	16	60	60	E10	14	67	50
9	401	25	59	40	560	20	60	50	522	15	65	67	518	14	67	50 -
10	481	26	60	42	560	20	60	50	521	15	65	60	518	14	67	50
11	481	26	60	42	560	20	60	50	521	16	66	63	518	14	67	50
12	481	26	60	42	560	20	60	50	522	15	65	67	518	14	67	50
13	477	23	59	43	560	20	60	50	521	15	65	60	518	14	67	50
14	477	23	59	43	560	20	60	50	521	16	66	63	518	14	67	50
15	477	23	59	43	560	20	60	50	522	15	65	67	518	14	67	50
16	482	24	58	42	565	22	59	45	521	15	65	60	518	14	67	50
17	482	24	58	42	565	22	59	45	521	16	66	63	518	14	67	50
18	482	24	58	42	565	22	59	45	522	15	65	67	518	14	67	50
19	482	25	59	44	565	22	59	45	521	15	65	60	518	14	67	50
20	482	25	59	44	565	22	59	45	521	16	66	63	518	14	67	50
21	482	25	59	44	565	22	59	45	522	15	65	67	518	14	67	50
22	481	25	59	40	565	22	59	45	521	15	65	60	518	14	67	50
23	481	25	59	40	565	22	59	45	521	16	66	63	518	14	67	50
24	481	25	59	40	565	22	59	45	522	15	65	67	518	14	67	50
25	475	23	58	43	560	20	60	50	521	15	65	60	518	14	67	50
26	481	26	60	42	565	22	59	45	521	15	65	60	518	14	67	50
27	475	23	58	43	560	20	60	50	521	16	66	63	518	14	67	50
28	481	26	60	42	565	22	59	45	521	16	66	63	518	14	67	50
29	475	23	58	43	560	20	คา	50	522	15	85	67	518	14	67	50
C-Loca	tion				103											
	442 528 560 505 5335 543															
Seco	Secondary Structure															
	•	01				Locath		H	inin Call Dias							
	Constant Print	Uligo				Length			anpart Sea Dame	sis Closs Dillier	2					
0	Forward Prim	er				24			Host Stab	le Structur	e Found					
0	Heverse Prim-	er				20			ANTOCNOT	TGAAACG FI						^
0	O Probe 1 15									1						
0	Probe 2					14			I III	1						
En	award Primer								TIOUUTAC.	n 0'						
G	CAAAGTTCAC	CTAATTOGGT	606													
Re	werse Primer	en an ruuun			_											
TI	GITCHICOC	GTCCATGGA														
Pn	obe 1															
TO	TCAGGATTO	GCAG														
Pn	obe 2															
A	GTCTCAGGG	TTC														×
								<								>

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 35). 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%.

Primer Express 3.0	
File Edit View Tools Window Help	
🗱 TaqMan® MGB Allelic Discrimination # 1	
Sequence Parameters Primers / Probes Order	
🔒 File Name AY228765.txt	
Length 1548 bp. Selection 528 to 528 🗌 Double Stranded	
	ш
ATGEGAAATC CCCTCCAAAT CTCCATTTTC CTGETGTTCT GCATCTTTAT CCAATCAAET GCTTGTGGAC AAGGCGTGGG AACAGAGCCC 1 100	~
GCCTTGGAGC TACTGAAGCT AGCAAGCCAT TAAAGAAGCC AGAGACCAGA TTCCTGCTCT TCCAAGATGA AAACGATCGC CTGGGCTGTC C 200	
TCAGCACCCG GAAACACTGC AGGAGTGTGG CTTCAACAGC TCTCAGCCGC TTATCATGAT CATCCACGGG TGGTCGGTGG ATGGCTTGCT # 300	
ATCTEGAAGA TAGTGAGTGC GCTGAAGTCC CGACAGTCCC AACCTGTGAA TGTGGGGTTA GTGGACTGGA TCTCCCTGGC ATACCAGCAC 1 400	
CTGTTCAAAA CACCCGTATT GTGGGCCAGG (Probe 1 (521 - 535) Tm: 65° GC%: 60 Probe 2 (518 - 531) Tm: 67° GC%: 50 AGCAAAGTTC 2 500	
GTACAGCCTG GGAGCGCACG TCTCAGGZIT CGCAGGCAGC TCCATGGACG GGAAGAACAA GATTGGAAGA ATCACAGGGC TGGACCCTGC C 600	
TTTGAGGGAA CGTCCCCCAA CGAGCGCCTT TCTCCTGATG ATGCCAATTT TGTGGACGCC ATTCATACCT TTACCAGGGA GCACATGGGC 1 700	
GCATCAAGCA GCCCATTGCC CACTATGACT TCTACCCCAA CGGGGGCTCC TTCCAGCCTG GCTGCCACTT CCTGGAACTC TACAAACACA 1 800	Ξ.
TGGCCTAAAC GCCATAACCC AGACCATCAA ATGTGCCCAT GAGCGCTCCG TGCACCTCTT CATTGACTCC TTGCAACACA GTGACCTGCA C 900	
TTCCAGTGCA GCGACATGGG CAGCTTCAGC CAAGGTCTAT GCCTGAGCTG CAAGAAGGGC CGTTGCAACA CTCTGGGTTA TGACATCCGC 2 1000	
CAGGCAAGAG CAAGAGGETE TECETEATEA EGEGAGEEEA GETETEETE AAAGTTTATE ATTACEAGTT CAAGATEEAG TECATEAATE # 1100	
GCCGGTAGAG CCTACTTTTA CCATGTCGCT GCTGGGAACA AAAGAAGAAA TAAAGAGAAAT TCCCATCACC CTGGGCGAAG GAATTACCAG C 1200	
TATTCCTTCC TTATCACACT GGACAAAGAC ATCGGCGAGT TGATCCTGCT CAAGTTCAAG TGGGAAAACA GTGCAGTGTG GGCCAATGTG 1 1300	
TGCAGACCAT CATGCTATGG GGCATAGAAC CTCACCACTC TGGCCTCATT CTGAAGACCA TCTGGGTCAA AGCTGGAGAG ACGCAGCAAA C 1400	
TTGCCCCGAA AATCTGGATG ACCTCCAGCT TCACCCGAGC CAGGAGAAAG TCTTTGTGAA CTGTGAAGTG AAGTCAAAAA GACTGACTGA # 1500	
CAGATGAGTC AAGAGACCCA TGCAAAAAAA TAAAGAAGTC TATTCTTT 1548	
	~
50 results found.	

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 **(a)** 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
DocumentBefore 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, we recommend that you run control samples to verify the performance of the selected primers and probes.



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
DocumentCreate 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 31.

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 32).
- **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 38). Note that the original Allele 1 variant base appears in lower case on the Primer Probe Test Tool.



Primer Express 3.0		- a -
File Edit View Tools Window Help		
	≝ → ← ■ ■ @ ∞ @ ₩ &	
TagMan® MGB Allelic Discrimination # 1		
Sequence Parameters Primers / Probes Order		
File Name AY228765 bt		
Length 1548 bp. Selection 809 to	837 Double Stranded	
L		
ATGGGAAATC CCCTCCAAAT CTCCATTTTC CTGGTGTTCT	GCATCTITAT CCAATCAAGT GCTTGTGGAC AAGGCGTGGG AACAGAGCCC TTTGGGAGAA	100
GCCTTGGAGC TACTGAAGCT AGCAAGCCAT TAAAGAAGCC	AGAGACCAGA TTCCTGCTCT TCCAAGATGA AAACGATCGC CTGGGCTGTC GTCTCAGACC	200
TCAGCACCCG GAAACACTGC AGGAGTGTGG CTTCAACAGC	TCTCAGCCGC TTATCATGAT CATCCACGGG TGGTCGGTGG ATGGCTTGCT AGAAAACTGG	300
ATCTGGAAGA TAGTGAGTGC GCTGAAGTCC CGACAGTCCC	ANCONTRAN TOTOCOCTTA CTOCACTOCA TOTOCOTOCA ATACCACCAC TACACCATTO	400
CTGTTCAAAA CACCCGTATT GTGGGCCAGG ACGTGGCTGC	Primer Probe Test Tool	500
GTACAGECTG GGAGEGEALG TETCAGGETT CGCAGGEAGE	- Parameters	600
GCATCAAGCA GCCCATTGCC CACTATGACT TCTACCCCAA	Document Type: TaqMar/® MGB Allelic Discriminati 💙 Parameter: Default 🛛 🖌 Browse	700
TGGCCTAAAC GCCATAWCCC AGACCATCAA ATGTGCCCAT	Drinnen and Deckan	900
TTECAGTECA GEGACATEGE CAGETTEAGE CAAGETETAT	Printers and Probes	1000
CAGGCAAGAG CAAGAGGCTC TTCCTCATCA CGCGAGCCCA	Tm 28C Length	1100
GCCGGTAGAG CCTACTTTTA CCATGTCGCT GCTGGGAACA	Fwd Primer	1200
TATTCCTTCC TTATCACACT GGACAAAGAC ATCGGCGAGT	Tm 200 Length	1300
TGCAGACCAT CATGCTATGG GGCATAGAAC CTCACCACTC	Rev Primer 00 0 0	1400
CACATCACTC ANCACACCCA TECANANAA TAAACAACTC	Tm 26C Length	1500
CROATOROIC ARONOACCCA IOCRAARAA IARAOAROIC	Probe 1 ACGCCATAaCCCAGACCATCAAATGTGCC 820 52 29	1548
	Tm 200 Length	
	Probe 2	
	Tim	
	Papanalar / Prustup	
	Secondary Structure	
	Oligo Length Set Differe Closs Differe	
	Forward Primer 0	
	O Reverse Primer 0	
	O Probe 1 29	
	O Probe 2 0	
	Show Secondary Structure	
	John Jecology Shecke	

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).
- $Tm 65 \degree C$ to 67 $\degree C$.
- % 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 39).





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.
	 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.



- **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 38.

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 $^{\circ}$ C to 60 $^{\circ}$ 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 ProbeCopy and paste the primer and probe sequences into a text document, then save for
future reference.Sequences

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

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

Notes.

3



<u></u>	Ordering Primers and Probes
Image: Arrow of the experimentation o	
Image: Arrowski state in the end of	Overview Overview Ordering the Selected Primers and Probes See page 44

4



Overview

About 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 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.



- 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.

Notes

4



Chapter 4 Ordering Primers and Probes Ordering Primers and Probes

Index

Α

add DNA file 11, 21, 31 allele 2, 37, 39, 40 allelic discrimination creating the document 31 allelic discrimination assay defined 2 allelic discrimination assays assigning a SNP target 32 design guidelines 38 finding primers and probes 33 manually designing the allele 1 probe 37 manually designing the allele 2 probe 39 saving primer and probe sequences 41 saving the document 35 amplicon length 14, 16, 18, 19, 24, 26, 35, 38, 40 Annotating Sequences. See Online Help anti-sense strand 2, 3, 12, 32 anti-sense strand defined 2

В

Batch Process Tool. See Online Help

D

document defined 2

Ε

Exporting. See Online Help

F

File format supported. See Online Help

Н

http vi

installing Primer Express Software 4

0

Online Help 7 Ordering TaqMan® TAMRA[™] dye Probes 45 ordering primers 44 ordering probes 45 Overview 10

Ρ

primer defined 3 Primer Express Software Version 2.0 4 Primer Express Software version 2.0 6 primer length 19, 26, 40 Primer Probe Test Tool 15, 16, 17, 18, 19, 25, 26, 27, 37, 38, 39, 40, 41, 44 Printing. See Online Help probe defined 3 probe length 16, 38

Q

quantification assay 3
Quantification Assays
Manually Designing the Probe 15
Primer Design Guidelines 19, 40
quantification assays
saving the document after automatic design 14
viewing results 13
quantification document 11
Quantification Workflow 10

R

rapid assay design guidelines 2 registration code 7

S

sense strand 11, 12, 21, 22, 31, 32 sense strand defined 3 SNP 2, 31, 32, 37, 38, 39 SNP site 31, 32, 37, 38, 39 SNP site in an MGB probe 39 SNP Target 32 SNP target 32 SNP Target 32 SNP Target Tool 33 Starting and Exiting Primer Express Software 7
Support, contacting vi
SYBR Green Dye Assays Primer Guidelines 26
SYBR Green Dye assays manually designing the forward primer 25 manually designing the primers 25 manually designing the reverse primer 27 saving primer sequences 27 viewing results 23
system requirements 3

Т

TaqMan Probe3TaqMan® MGB Probe defined3TaqMan® MGB Quantification11TaqMan® Quantification11Technical Supportvi

U

Uninstalling Primer Express Software 6







