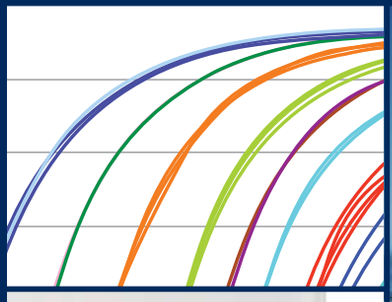
Applied Biosystems

Applied Biosystems
Real-Time PCR Systems



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Contents

Preface

How to Use This Guide

Purpose of This Guide

This guide provides information about the reagents you can use on the Applied Biosystems Real-Time PCR Systems, including:

- An introduction to TaqMan® and SYBR® Green reagents
- Descriptions and design guidelines for:
  - Quantitation experiments
  - Genotyping experiments
  - Presence/absence experiments

Audience

This guide is intended for laboratory staff and principal investigators who perform experiments using Applied Biosystems Real-Time PCR Systems.

Assumptions

This guide assumes that you:

- Are familiar with the Microsoft Windows® XP operating system.
- Are familiar with the Internet and Internet browsers.
- Know how to handle DNA and/or RNA samples and prepare them for PCR.
- Understand data storage, file transfer, and copying and pasting.
- Have networking experience, if you plan to integrate the Real-Time PCR System into your existing laboratory data flow.

Text Conventions

This guide uses the following conventions:

- **Bold** text 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 symbol (▸) separates successive commands you select from a drop-down or shortcut menu. For example:
  Select **File ▸ Open**.

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 reagent kit use, or safe use of a chemical.

Examples of the user attention words appear below:

Note: The Calibrate function is also available in the Control Console.

IMPORTANT! To verify your client connection, you need a valid user ID.

How to Obtain More Information

Related Documentation

The documents listed in this section are available from Applied Biosystems. To obtain this and additional documentation, see "How to Obtain Support" on page xii.

Documents Related to Genotyping Experiments

Document	PN
Allelic Discrimination Pre-Developed TaqMan® Assay Reagents Quick Reference Card	4312212
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Allelic Discrimination Getting Started Guide	4347822
Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Genotyping Experiments	4387784
Applied Biosystems 7900HT Fast Real-Time PCR System Allelic Discrimination Getting Started Guide	4364015
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Genotyping Experiments	4376786
Custom TaqMan® Genomic Assays Protocol	4367671
Custom TaqMan® SNP Genotyping Assays Protocol	4334431
Ordering TaqMan® SNP Genotyping Assays Quick Reference Card	4374204
Performing a Custom TaqMan® SNP Genotyping Assay for 96-Well Plates Quick Reference Card	4371394
Performing a TaqMan® Drug Metabolism Genotyping Assay for 96-Well Plates Quick Reference Card	4367636
Pre-Developed TaqMan® Assay Reagents Allelic Discrimination Protocol	4312214
TaqMan® Drug Metabolism Genotyping Assays Protocol	4362038
TaqMan® SNP Genotyping Assays Protocol	4332856

Documents Related to Presence/Absence Experiments

Document	PN
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System *Plus/Minus Getting Started Guide*	4347821
Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Presence/Absence Experiments	4387785
Applied Biosystems 7900HT Fast Real-Time PCR System Plus-Minus Getting Started Guide	4364017
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Presence/Absence Experiments	4376787
DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol	4343586
NucPrep® Chemistry: Isolation of Genomic DNA from Animal and Plant Tissue Protocol	4333959
PrepMan® Ultra Sample Preparation Reagent Protocol	4318925

Documents Related to Relative Standard Curve and Comparative C_T Experiments

Document	PN
Amplification Efficiency of TaqMan® Gene Expression Assays Application Note	127AP05
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide	4347824
Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments	4387783
Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide	4364016
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments	4376785
Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol	4375575
Custom TaqMan® Gene Expression Assays Protocol	4334429
Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR	4371095
Primer Express® Software Version 3.0 Getting Started Guide	4362460
TaqMan® Gene Expression Assays Protocol	4333458
User Bulletin #2: Relative Quantitation of Gene Expression	4303859

Documents Related to Standard Curve Experiments

Document	PN
Amplification Efficiency of TaqMan® Gene Expression Assays Application Note	127AP05
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide	4347825
Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Standard Curve Experiments	4387779
Applied Biosystems 7900HT Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide	4364014
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments	4376784
Custom TaqMan® Gene Expression Assays Protocol	4334429
Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR	4371095
Primer Express® Software Version 3.0 Getting Started Guide	4362460
TaqMan® Gene Expression Assays Protocol	4333458
User Bulletin #2: Relative Quantitation of Gene Expression	4303859

Documents Related to the Reagent Guide

Document	PN
Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol	4375575
Custom TaqMan® Gene Expression Assays Protocol	4334429
Custom TaqMan® Genomic Assays Protocol: Submission Guidelines	4367671
Custom TaqMan® SNP Genotyping Assays Protocol	4334431
Fast SYBR® Green Master Mix Protocol	4385372
Power SYBR® Green PCR Master Mix and RT-PCR Protocol	4367218
Pre-Developed TaqMan® Assay Reagents Allelic Discrimination Protocol	4312214
Primer Express® Software Version 3.0 Getting Started Guide	4362460
SYBR® Green PCR and RT-PCR Reagents Protocol	4304965
SYBR® Green PCR Master Mix and RT-PCR Reagents Protocol	4310251
TaqMan® Drug Metabolism Genotyping Assays Protocol	4362038
TaqMan® Exogenous Internal Positive Control Reagents Protocol	4308335
TaqMan® Fast Universal PCR Master Mix (2X) Protocol	4351891
TaqMan® Gene Expression Assays Protocol	4333458
TaqMan® Gene Expression Master Mix Protocol	4371135
TaqMan® Genotyping Master Mix Protocol	4371131
TaqMan® SNP Genotyping Assays Protocol	4332856
TaqMan® Universal PCR Master Mix Protocol	4304449
User Bulletin #2: Relative Quantitation of Gene Expression	4303859
Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies Application Note	127AP08

Note: For more documentation, see “How to Obtain Support” on page xii.

Accessing the Software Help

The software Help for each Real-Time PCR System describes how to use each feature of the user interface. Access the Help from within the software by doing one of the following:

- Press **F1**.
- Click in the toolbar.
- Select **Help** ▸ *<software name>*, where *<software name>* is the name of the instrument software

To find topics of interest in the Help:

- Review the table of contents.
- Search for a specific topic.
- Search an alphabetized index.

Send Us Your Comments

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

techpubs@appliedbiosystems.com

IMPORTANT! The e-mail address above is only for submitting comments and suggestions relating to documentation. To order documents, download PDF files, or for help with a technical question, go to **http://www.appliedbiosystems.com**, then click the link for **Support**. (See "How to Obtain Support" on page Front Matter-xii).

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:

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

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Introduction 1

This chapter covers:

About Applied Biosystems Real-Time PCR Systems

Applied Biosystems Real-Time PCR Systems use fluorescence-based polymerase chain reaction (PCR) reagents to provide:

- Quantitative detection of target nucleic acid sequences (targets) using real-time analysis.
- Qualitative detection of targets using post-PCR (endpoint) analysis.
- Qualitative analysis of the PCR product (achieved by melt curve analysis that occurs post-PCR).

About Data Collection

Applied Biosystems Real-Time PCR Systems collect raw fluorescence data at different points during a PCR, depending on the type of run that the instruments perform:

Run Type		Data Collection Point
Real-time runs	Standard curve	The instrument collects data following each extension step of the PCR.
	Relative standard curve	
	Comparative C_T ($\Delta\Delta C_T$)	
Post-PCR (endpoint) runs	Genotyping	The instrument collects data: • Before the PCR. (For presence/absence experiments, data collection before the PCR is optional, but recommended.) • (Optional) During the PCR. The instrument can collect data during the run (real-time); collecting data during the run can be helpful for troubleshooting. • After the PCR.
	Presence/absence	

Regardless of the run type, a data collection point or *read* on the instruments consists of three phases:

1. **Excitation** – The instrument illuminates all wells of the reaction plate within the instrument, exciting the fluorophores in each reaction.

2. **Emission** – The instrument optics collect the residual fluorescence emitted from the wells of the reaction plate. The resulting image collected by the device consists only of light that corresponds to the range of emission wavelengths.

3. **Collection** – The instrument assembles a digital representation of the residual fluorescence collected over a fixed time interval. The Real-Time PCR System software stores the raw fluorescence image for analysis.

After a run, the system software uses calibration data (spatial, dye, and background) to determine the location and intensity of the fluorescence signals in each read, the dye associated with each fluorescence signal, and the significance of the signal.

For More Information

For more information on Applied Biosystems Real-Time PCR Systems, refer to the manuals or guides specific to your instrument.

Preparing for Experiments

General Workflow

Before performing experiments on a Real-Time PCR System, you select the:

1. Experiment type (page 1-4).
2. Reagent type (page 1-5).
3. Assay type (page 1-6).

Information in This Guide

This chapter provides general information on the experiment types, reagent types, and assay types you can use with the Applied Biosystems Real-Time PCR Systems.

Subsequent chapters provide specific information:

Chapter	Description
Chapter 2, Reagent Overview	• Describes and compares the TaqMan® and SYBR® Green reagents. • Provides information on minimizing DNA contamination.
Chapter 3, Quantitation Experiments	• Explains how the experiment type works. • Provides a specific workflow for the experiment type. • Provides design guidelines for each assay type.
Chapter 4, Genotyping Experiments	
Chapter 5, Presence/Absence Experiments	

Select the Experiment Type

In this guide, the term *experiment* refers to the entire process of performing a run using an Applied Biosystems Real-Time PCR System, including setup, run, and analysis. You can perform the following types of experiments on the Real-Time PCR Systems:

- Quantitation, including:
  - Standard curve
  - Relative standard curve
  - Comparative C_T ($\Delta\Delta C_T$)
- Genotyping
- Presence/absence

You can also perform melt curve analysis on the Real-Time PCR Systems. For more information, access the Help from within the Real-Time PCR System software by clicking in the toolbar, pressing **F1**, or selecting **Help** ▸ <*software name*> (where <*software name*> is the name of the instrument software).

Endpoint vs. Real-Time Experiments

The three experiment types can be categorized as real-time or endpoint experiments, as described below.

Category	Properties	Experiment Type
Real-time	• The instrument monitors the progress of the PCR as it occurs.‡ • Data are collected throughout the PCR process. • Reactions are characterized by the point in time during cycling when amplification of a target is first detected.§	Quantitation
Endpoint	• Data are collected at the end of the PCR process. • Reactions are characterized by the quantity of the target accumulated at the end of PCR.§ • The datapoint is the normalized intensity of the reporter dye, or R_n. **Note:** Some endpoint experiments also include pre-PCR datapoints. If so, the system calculates the delta R_n (ΔR_n) value according to the following formula: $\Delta Rn = Rn$ (post-PCR read) – Rn (pre-PCR read) where Rn = normalized reporter	Genotyping Presence/absence

‡ Kwok and Higuchi, 1989.

§ Saiki *et al*., 1985.

Select the Reagent Type

You can use the following reagent types (chemistries) on Applied Biosystems Real-Time PCR Systems:

- TaqMan® reagents
- SYBR® Green reagents
- Other fluorescence-based reagents

TaqMan Reagents

TaqMan reagents include Applied Biosystems TaqMan® assays (preformulated mixes that contain probe and primer sets) and Applied Biosystems TaqMan® master mixes. You can use TaqMan reagents for:

- Quantitation experiments, including:
  - Standard curve
  - Relative standard curve
  - Comparative C_T ($\Delta\Delta C_T$)
- Genotyping experiments
- Presence/absence experiments

SYBR Green Reagents

SYBR Green reagents include primers and master mixes that contain SYBR® Green I dye. You can use SYBR Green reagents for the following quantitation experiments:

- Standard curve
- Relative standard curve
- Comparative C_T ($\Delta\Delta C_T$)

Note: You cannot perform multiplex PCR using SYBR Green reagents. For more information, see "Select Singleplex or Multiplex PCR" on page 3-10.

Other Reagents

You can use other fluorescence-based reagents on Applied Biosystems Real-Time PCR Systems. If you are using other reagents, ensure that the spectra of the dye(s) you select are appropriate, as follows:

- For filter-based instruments, consider the available filters on your instrument.
- For laser-based instruments, consider the spectral range of data collection.

For information on your instrument's filters or spectral range, see your instrument's installation and/or maintenance guide.

Select the Assay Type

The following assay types are available from Applied Biosystems for quantitation, presence/absence, and genotyping experiments:

Experiment Type	Assay Type	See...
Quantitation‡	• Inventoried/Made to Order • Custom • User-Designed	Below
Presence/absence		
Genotyping	• Pre-Designed/Validated • Custom • User-Designed	Page 1-7

‡ Quantitation experiments include standard curve, relative standard curve, and comparative C_T experiments.

Quantitation and Presence/ Absence Experiments

For quantitation or presence/absence experiments, Applied Biosystems has the following assays:

Assay Type	Assay
Inventoried/Made to Order	• **TaqMan® Gene Expression Assays, Inventoried** – Predesigned FAM™ dye-labeled TaqMan® MGB (minor groove binder) probe and primer sets that can be purchased off-the-shelf. The assay mix is available in a single, preformulated 20× tube. • **TaqMan® Gene Expression Assays, Made to Order** – Predesigned FAM dye-labeled TaqMan MGB probe and primer sets that are manufactured at the time of order. The assay mix is available in a single, preformulated 20× tube.
Custom	**Custom TaqMan® Gene Expression Assays** – FAM dye-labeled TaqMan MGB probe and primer sets that are designed, synthesized, and formulated by the Custom TaqMan® Genomic Assays service based on sequence information that you submit. The assay mix is available in a single, preformulated 20× or 60× tube.

Assay Type	Assay
User-Designed	If you want to design your own assays (primers and probes) for quantitation or presence/absence experiments, Applied Biosystems recommends that you: • Use the Primer Express® Software with TaqMan or SYBR Green reagents. For more information, refer to the *Primer Express® Software Version 3.0 Getting Started Guide*. • Follow the Applied Biosystems Assay Design Guidelines to achieve optimal results. The Assay Design Guidelines are provided in Appendix C. **Note:** Chapter 3 provides guidelines for designing your own assays (see "User-Designed Assays" on page 3-23). Applied Biosystems also has application notes and tutorials that can help you design your own assays; go to: **http://www.appliedbiosystems.com/support/apptech** Select **Real-Time PCR: Applications & Reagents**, then select the appropriate documentation listed under **Primer and Probe Design**.

Genotyping Experiments

For genotyping experiments, Applied Biosystems has the following assays:

Assay Type	Assay
Pre-Designed/Validated	• **TaqMan® SNP Genotyping Assays** – Predesigned FAM™ dye- and VIC® dye-labeled TaqMan® MGB (minor groove binder) probes and primer sets that are available as TaqMan® Pre-Designed SNP Genotyping Assays. The TaqMan Pre-Designed SNP Genotyping Assays are manufactured at the time of order (Made to Order). The assay mix is available in a single, preformulated 20× tube. • **TaqMan® Drug Metabolism Genotyping Assays** – Predesigned FAM dye- and VIC dye-labeled TaqMan MGB probes and primer sets that can be purchased off the shelf (Inventoried). The assay mix is available in a single, preformulated 20× tube. • **Pre-Developed TaqMan® Assay Reagents for Allelic Discrimination (TaqMan® PDARs for AD)** – Predesigned FAM dye- and VIC dye-labeled TaqMan MGB probes and primer sets that can be purchased off-the-shelf (Inventoried). The assay mix is available in a single, preformulated 10× tube.
Custom	**Custom TaqMan® SNP Genotyping Assays** – FAM dye- and VIC dye-labeled TaqMan MGB probes and primer sets that are designed, synthesized, and formulated by the Custom TaqMan® Genomic Assays service based on sequence information that you submit. The assay mix is available in a single, preformulated 40× or 80× tube.
User-Designed	If you want to design your own assays (primers and probes) for genotyping experiments, refer to the *Primer Express® Software Version 3.0 Getting Started Guide*.

Reagent Overview 2

This chapter covers:

Overview

Applied Biosystems offers two reagent types (chemistries) that can be used to detect PCR products on the Applied Biosystems Real-Time PCR Systems:

- TaqMan® reagents (below)
- SYBR® Green reagents (page 2-4)

TaqMan® Reagents

Experiment Types

TaqMan® reagents include Applied Biosystems TaqMan® assays (preformulated mixes that contain probe and primer sets) and Applied Biosystems TaqMan® master mixes. The assays are specific to the target of interest. The master mixes contain the remaining components needed for the PCR reaction. You can use TaqMan reagents for:

- Quantitation experiments, including:
  - Standard curve
  - Relative standard curve
  - Comparative C_T ($\Delta\Delta C_T$)
- Genotyping experiments
- Presence/absence experiments

Development of TaqMan Reagents

Initially, intercalator dyes were used to measure real-time PCR products. The primary disadvantage of this method is that it detects accumulation of both specific and nonspecific PCR products.

Real-time systems for PCR were improved by the introduction of fluorogenic-labeled probes that use the 5′ nuclease activity of Taq DNA polymerase. The availability of these fluorogenic probes enabled the development of a real-time method for detecting only specific amplification products.

How the TaqMan Reagents Work

TaqMan reagents use a fluorogenic probe to detect a specific PCR product as it accumulates during the PCR. Here is how it works:

Step 1 – An oligonucleotide probe is constructed with a fluorescent reporter dye bound to the 5′ end and a quencher on the 3′ end.

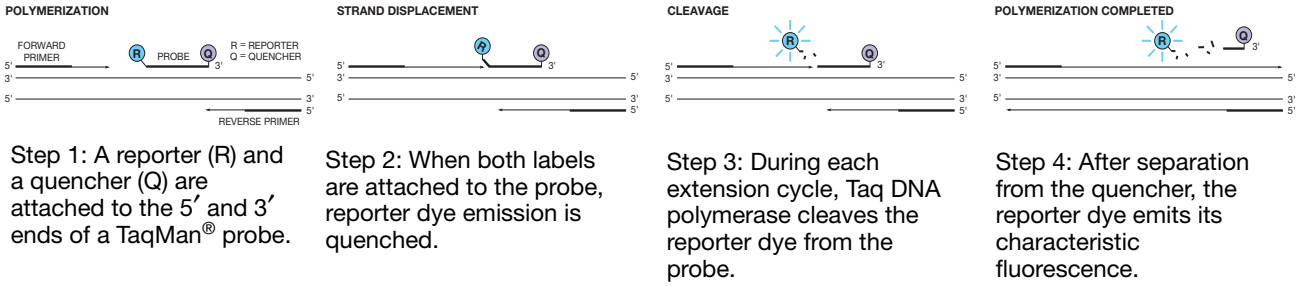
Step 2 – While the probe is intact, the proximity of the quencher to the reporter dye greatly reduces the fluorescence emitted by the reporter dye.

Step 3 – If the target is present, the probe anneals between primer sites and is cleaved by the 5′ nuclease activity of Taq DNA polymerase during extension. Cleavage of the probe:

- Separates the reporter dye from the quencher, increasing the reporter dye fluorescence signal.
- Removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process.

Step 4 – More reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the quantity of amplicon produced. The higher the starting copy number of the nucleic acid target, the earlier a significant increase in fluorescence is observed.

Figure 2-1 illustrates this process.



Step 1: A reporter (R) and a quencher (Q) are attached to the 5′ and 3′ ends of a TaqMan® probe.

Step 2: When both labels are attached to the probe, reporter dye emission is quenched.

Step 3: During each extension cycle, Taq DNA polymerase cleaves the reporter dye from the probe.

Step 4: After separation from the quencher, the reporter dye emits its characteristic fluorescence.

Figure 2-1 How the TaqMan reagents work

Types of TaqMan® Probes

Applied Biosystems offers two types of TaqMan® probes:

- TaqMan® MGB (minor groove-binder) probes with nonfluorescent quencher (NFQ)
- TaqMan® TAMRA™ probes with TAMRA™ dye as quencher

Applied Biosystems offers the following TaqMan probes for use on the Real-Time PCR Systems:

Probe	Assays	5′ Label Dye	3′ Label Dye	MGB?
TaqMan® MGB probe	TaqMan® Gene Expression Assays	FAM™ dye	NFQ	Yes
	Custom TaqMan® Gene Expression Assays	FAM™ dye		
	TaqMan® SNP Genotyping Assays	FAM™ and VIC® dyes		
	Custom TaqMan® SNP Genotyping Assays	FAM™ and VIC® dyes		
	TaqMan® Endogenous Control Assays	FAM™ and VIC® dyes		
Custom TaqMan® MGB probes	User-designed assays	FAM™, TET™, NED™, or VIC® dye		
TaqMan® TAMRA™ probes	TaqMan® Endogenous Control Assays	VIC® dye	TAMRA™ dye	No
Custom TaqMan® TAMRA™ probes	User-designed assays	FAM™, TET™, or VIC® dye		

TaqMan MGB Probes Recommended

Applied Biosystems recommends the general use of TaqMan MGB probes, especially when conventional TaqMan probes exceed 30 nucleotides. The TaqMan MGB probes contain:

- **A fluorescent reporter dye at the 5′ end –** Generates a signal when cleaved by the 5′ nuclease activity of Taq DNA polymerase.
- **A nonfluorescent quencher (NFQ) at the 3′ end –** Allows Real-Time PCR Systems to measure the reporter dye contributions more precisely than probes with TAMRA dye because the quencher does not fluoresce.
- **A minor groove binder (MGB) at the 3′ end –** Increases the melting temperature (Tm) of probes without increasing probe length (Afonina *et al.*, 1997; Kutyavin *et al.*, 1997), thereby allowing the design of shorter probes. Consequently, the TaqMan MGB probes exhibit greater differences in Tm values between matched and mismatched probes; greater differences in Tm values provide for more accurate genotyping.

SYBR® Green Reagents

Experiment Types

SYBR® Green reagents include primers and master mixes that contain SYBR® Green I dye. You can use SYBR Green reagents for the following quantitation experiments:

- Standard curve
- Relative standard curve
- Comparative C_T ($\Delta\Delta C_T$)

Note: You cannot perform multiplex PCR using SYBR Green reagents. For more information, see "Select Singleplex or Multiplex PCR" on page 3-10.

Development of SYBR Green Reagents

Small molecules that bind to double-stranded DNA can be divided into two classes: those that intercalate DNA and those that bind the minor groove of DNA. Higuchi (Higuchi *et al.*, 1992) used the intercalator ethidium bromide for real-time detection of PCR. Hoechst 33258 is an example of a minor groove-binding dye whose fluorescence increases when bound to double-stranded DNA (Higuchi *et al.*, 1993).

Regardless of the binding method, there are at least two requirements for a DNA binding dye for real-time detection of PCR products:

- Increased fluorescence when bound to double-stranded DNA
- No inhibition of PCR

Applied Biosystems has developed conditions that permit the use of the SYBR® Green I dye in PCR without PCR inhibition and with increased sensitivity of detection compared with ethidium bromide.

How the SYBR Green Reagents Work

The SYBR Green reagents use the SYBR Green I dye to detect PCR products by binding to double-stranded DNA formed during the PCR. Here is how it works:

Step 1 – When added to a sample, SYBR Green I dye immediately binds to all double-stranded DNA.

Step 2 – During the PCR, a DNA polymerase amplifies the target, which creates the PCR product, or "amplicon." The double-stranded DNA is denatured to single-stranded molecules and the SYBR Green I dye is released.

Step 3 – The primers anneal to the single-stranded DNA and the DNA polymerase amplifies the target, creating more double-stranded DNA. As the PCR progresses, more amplicon is created.

Step 4 – The SYBR Green I dye then binds to each new copy of double-stranded DNA that is generated during each PCR cycle. Because the SYBR Green I dye binds to all double-stranded DNA, the result is an increase in fluorescence proportional to the quantity of double-stranded PCR product produced.

Figures 2-2 illustrates this process.

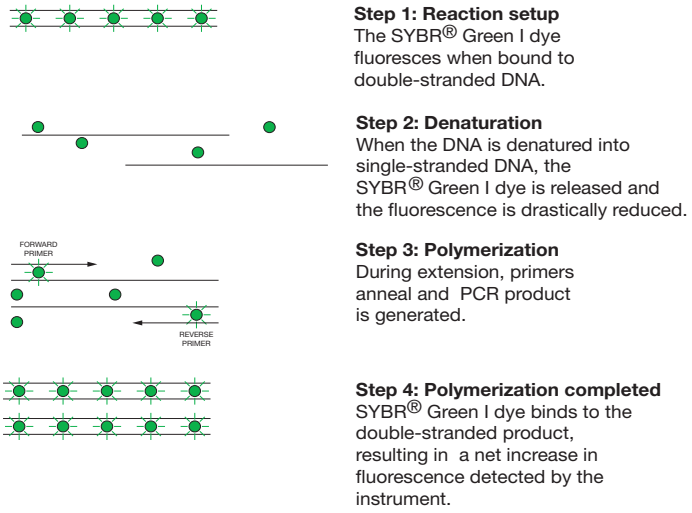


Figure 2-2 How the SYBR Green reagents work

Selecting the Appropriate Reagent Type

The TaqMan and SYBR Green reagents can be used according to the experiment types indicated below.

Reagent Type	Experiment Type		
	Quantitation‡ (Chapter 3)	Genotyping (Chapter 4)	Presence/ Absence (Chapter 5)
SYBR® Green reagents	Yes	Not recommended	Not recommended
TaqMan® reagents	Yes	Yes	Yes

‡ Includes standard curve, relative standard curve, and comparative C_T experiments.

Considerations for Quantitation Experiments

Quantitation experiments can be performed with either TaqMan or SYBR Green reagents. Consider the following when choosing between the two reagent types:

Reagent Type	Description	Advantage	Limitation
TaqMan® reagents	TaqMan reagents use a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles.	• Provides increased specificity with the addition of a fluorogenic probe. • Provides multiplex capability. • Includes preformulated assays, optimized to run under universal thermal cycling conditions. • Can be used for either 1- or 2-step RT-PCR.	Requires synthesis of a unique fluorogenic probe.
SYBR® Green reagents	SYBR Green reagents use SYBR® Green I dye, a double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles.	• Is economical (no probe needed). • Allows for melt curve analysis to measure the Tm of all PCR products. • Can be used for either 1- or 2-step RT-PCR.	Binds nonspecifically to all double-stranded DNA sequences. To avoid false-positive signals, check for nonspecific product formation using melt curve or gel analysis.

Minimizing DNA Contaminants

The DNA amplification capability of the PCR process makes special laboratory practices necessary when you perform experiments using TaqMan or SYBR Green reagents. Potential contamination can be introduced by samples with high DNA concentrations, either from the DNA template controls or from PCR carryover.

In addition, due to the nonspecific nature of the SYBR Green I dye, any double-stranded DNA is detected. When using SYBR Green reagents, check for nonspecific product formation by using melt curve or gel analysis. Take care to avoid contamination with target DNA. Gene expression assays that span exon-exon junctions minimize the effect of genomic DNA (gDNA) contaminants.

Using UNG to Minimize Reamplification of Carryover Products

AmpErase® uracil-N-glycosylase (UNG) is a 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-N-glycosylase gene. This gene has been inserted into an *E. coli* host to direct expression of the native form of the enzyme (Kwok and Higuchi, 1989).

UNG acts on single- and double-stranded dU-containing DNA by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA (Longo *et al.*, 1990).

TaqMan Assays

For TaqMan® assays, AmpErase® UNG treatment can prevent the reamplification of carryover PCR products from previous PCR reactions. When dUTP replaces dTTP in PCR amplification, AmpErase UNG treatment can remove up to 200,000 copies of amplicon per 50-µL reaction.

Note: AmpErase UNG (also abbreviated as UDG, for uracil-DNA-glycosylase) is included in some Applied Biosystems TaqMan master mix formulations and can also be purchased individually. When purchasing TaqMan master mixes, check the product information to see if the master mix contains AmpErase UNG.

SYBR Green I Dye Assays

For SYBR Green I dye assays, AmpErase UNG treatment can prevent the reamplification of carryover PCR products from previous PCR reactions. Although Fast SYBR® Green Master Mix, *Power* SYBR® Green PCR Master Mix, and SYBR® Green PCR Master Mix do not contain AmpErase UNG, they contain dUTP and are therefore compatible with AmpErase UNG. If contamination from PCR carryover is suspected, use AmpErase UNG to troubleshoot the problem.

Note: AmpErase UNG can be purchased individually or as part of the SYBR® Green PCR Core Reagents kit.

General PCR Practices

Use the following precautions to minimize sample contamination and PCR product carryover:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification. Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas, dedicated equipment, and supplies for:
  - Sample preparation.
  - PCR setup. Never bring amplified PCR products into the PCR setup area.
  - PCR amplification.
  - Analysis of PCR products.
- Open and close all sample tubes carefully. Avoid splashing or spraying PCR samples.
- Use positive-displacement pipettors or air-displacement pipettors with filter-plugged tips. Change tips after each use.
- Keep reactions and components capped as much as possible.
- Clean lab benches and equipment periodically with 10% bleach solution or 70% ethanol.

Quantitation Experiments 3

This chapter covers:

Section 3.1 About Quantitation Experiments

This section covers:

Overview

What Is a Quantitation Experiment?

A quantitation experiment is a real-time experiment that measures the quantity of a target nucleic acid sequence (target) during each amplification cycle of the polymerase chain reaction (PCR). The target can be DNA, cDNA, or RNA.

Three types of quantitation experiments are discussed in this guide:

- Standard curve (page 3-5)
- Relative standard curve (page 3-5)
- Comparative C_T ($\Delta\Delta C_T$) (page 3-6)

How Quantitation Experiments Work

In real-time quantitation experiments, the reactions are characterized by the point in time during cycling when amplification of a PCR product achieves a fixed level of fluorescence, rather than the final quantity of PCR product accumulated after a fixed number of cycles. An amplification plot graphically displays the fluorescence detected over the number of cycles that were performed.

In the initial cycles of PCR, no significant change in fluorescence signal occurs. This predefined range of PCR cycles is called the *baseline*. First, the Real-Time PCR System software generates a baseline-subtracted amplification plot by calculating a mathematical trend of the normalized fluorescent reporter signal (Rn values corresponding to the baseline cycles). Then, an algorithm searches for the point on the amplification plot at which the baseline-corrected normalized fluorescent reporter signal (delta Rn [ΔRn] value) crosses a set threshold. The cycle at which the ΔRn value crosses the threshold is defined as the C_T.

Workflow

Before performing quantitation experiments on an Applied Biosystems Real-Time PCR System, you need to:

1. Select a quantitation method (page 3-5).
2. Select 1- or 2-step RT-PCR (page 3-8).
3. Select singleplex or multiplex PCR reactions (page 3-10).
4. Select the reagent type (page 3-12).
5. Select the assay type (page 3-12).
6. Review the design guidelines for the assay type you selected (Section 3.2 on page 3-17).

Select a Quantitation Method

About Standard Curve Experiments

The standard curve method is used to determine the absolute target quantity in samples. With the standard curve method, the Real-Time PCR System software measures amplification of the target in samples and in a standard dilution series. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates the absolute quantity of target in the samples.

Components

The following components are required when setting up PCR reactions for standard curve experiments:

- **Sample** – The sample in which the quantity of the target is unknown.
- **Standard** – A sample that contains known standard quantities; used in quantitation experiments to generate standard curves.
- **Standard dilution series** – A set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards.
- **Replicates** – The total number of identical reactions containing identical samples, components, and volumes.
- **Negative Controls** – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

About Relative Standard Curve Experiments

The relative standard curve method is used to determine relative target quantity in samples. With the relative standard curve method, the Real-Time PCR System software measures amplification of the target and of the endogenous control in samples, in a reference sample, and in a standard dilution series. Measurements are normalized using the endogenous control. Data from the standard dilution series are used to generate a standard curve for each of the target gene(s) and the endogenous control. If your Real-Time PCR System software supports the relative standard curve method, the software interpolates target quantity in the samples and in the reference sample. The software determines the relative quantity of target in each sample by comparing target quantity in each sample to target quantity in the reference sample.

Relative standard curve experiments are commonly used to:

- Compare expression levels of a gene in different tissues.
- Compare expression levels of a gene in a treated sample vs. an untreated sample.

Components

The following components are required when setting up PCR reactions for relative standard curve experiments:

- **Sample** – The sample in which the quantity of the target is unknown.
- **Reference sample** – The sample used as the basis for relative quantitation results. For example, in a study of drug effects on gene expression, an untreated control would be an appropriate reference sample. Also called *calibrator*.
- **Standard** – A sample that contains known standard quantities; used in quantitation experiments to generate standard curves.
- **Standard dilution series** – A set of standards containing a range of known quantities or dilutions. The standard dilution series is prepared by serially diluting standards.
- **Endogenous control** – A target or gene that should be expressed at equivalent levels in all samples you are testing. The endogenous control is used to normalize the expression levels of target genes by correcting differences in the starting sample. Housekeeping genes can be used as endogenous controls.
- **Replicates** – The total number of identical reactions containing identical samples, components, and volumes.
- **Negative Controls** – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

About Comparative C_T Experiments

The comparative C_T ($\Delta\Delta C_T$) method is used to determine the relative target quantity in samples. With the comparative C_T method, the Real-Time PCR System software measures amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the endogenous control. The software determines the relative quantity of target in each sample by comparing normalized target quantity in each sample to normalized target quantity in the reference sample.

Comparative C_T experiments are commonly used to:

- Compare expression levels of a gene in different tissues.
- Compare expression levels of a gene in a treated sample vs. an untreated sample.

Components

The following components are required when setting up PCR reactions for comparative C_T experiments:

- **Sample** – The sample in which the quantity of the target is unknown.
- **Reference sample** – The sample used as the basis for relative quantitation results. For example, in a study of drug effects on gene expression, an untreated control would be an appropriate reference sample. Also called *calibrator*.
- **Endogenous control** – A target or gene that should be expressed at equivalent levels in all samples you are testing. The endogenous control is used to normalize the expression levels of target genes by correcting differences in the starting sample. Housekeeping genes can be used as endogenous controls.

- **Replicates** – The total number of identical reactions containing identical samples, components, and volumes.
- **Negative Controls** – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

Comparison of Quantitation Methods

Consider the following when choosing between standard curve, relative standard curve, and comparative C_T experiments:

Experiment Type	Description	Advantage	Limitation
Standard curve	Uses a standard curve to determine the absolute quantity of a target in a sample. Typically used for quantifying viral load.	Allows comparisons against known standard quantities.	Because a standard curve must be constructed for each target, standard curve experiments require more reagents and more space in the reaction plate.
Relative standard curve	Uses a standard curve to determine the change in expression of a target in a sample relative to the same target in a reference sample. Best option when your target assay and your endogenous control assay do not have equivalent efficiencies.	Requires the least amount of validation because the PCR efficiencies of the target and endogenous control do not need to be equivalent.	Because a standard curve must be constructed for each target, relative standard curve experiments require more reagents and more space in the reaction plate.
Comparative C_T ($\Delta\Delta C_T$)	Uses arithmetic formulas to determine the change in expression of a target in a sample relative to the same target in a reference sample. Best option for high-throughput measurements of relative gene expression of many genes in many samples.	• Relative levels of target in samples can be determined without the use of a standard curve, provided that the PCR efficiencies of the target and endogenous control are approximately equal. • Reduced reagent usage. • More space is available in the reaction plate.	Before you use the comparative C_T method, Applied Biosystems recommends that you determine that the PCR efficiencies for the target assay and the endogenous control assay are approximately equal. Assays that do not have equivalent efficiencies produce inaccurate results.

For More Information

For more information on quantitation methods, refer to *User Bulletin #2: Relative Quantitation of Gene Expression* and *Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR*.

Select 1-Step or 2-Step RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) is used to quantify RNA. RT-PCR can be performed as a 1-step or 2-step procedure.

About 1-Step RT-PCR

In 1-step RT-PCR, you perform reverse transcription and the PCR in a single buffer system (Figure 3-1). The reaction proceeds without the addition of reagents between the RT and PCR steps. 1-step RT-PCR offers the convenience of a single-tube preparation for RT and PCR amplification. However, the carryover prevention enzyme, AmpErase® uracil-N-glycosylase (UNG), cannot be used with 1-step RT-PCR. In 1-step RT-PCR, the presence of UNG would destroy the cDNA as it is being made. For information about UNG, see "Using UNG to Minimize Reamplification of Carryover Products" on page 2-7.

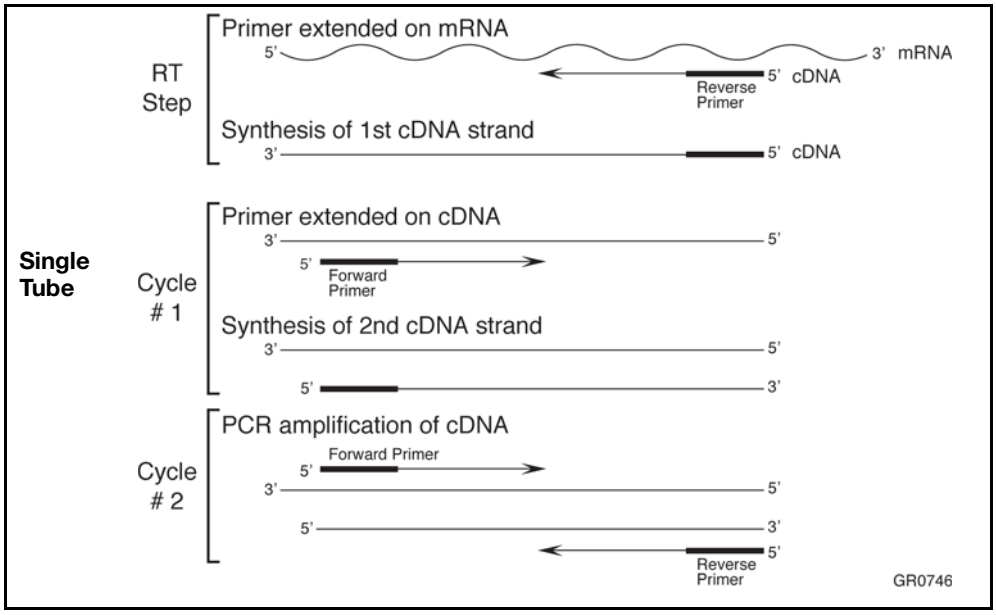


Figure 3-1 1-step RT-PCR

About 2-Step RT-PCR

In 2-step RT-PCR, you perform two separate reactions: one for RT and one for PCR (Figure 3-2). 2-step RT-PCR is useful when detecting multiple transcripts from a single cDNA reaction, or when storing a portion of the cDNA for later use. When you perform PCR using dUTP as one of the bases, you can use AmpErase® UNG enzyme to prevent carryover contamination. For information about UNG, see "Using UNG to Minimize Reamplification of Carryover Products" on page 2-7.

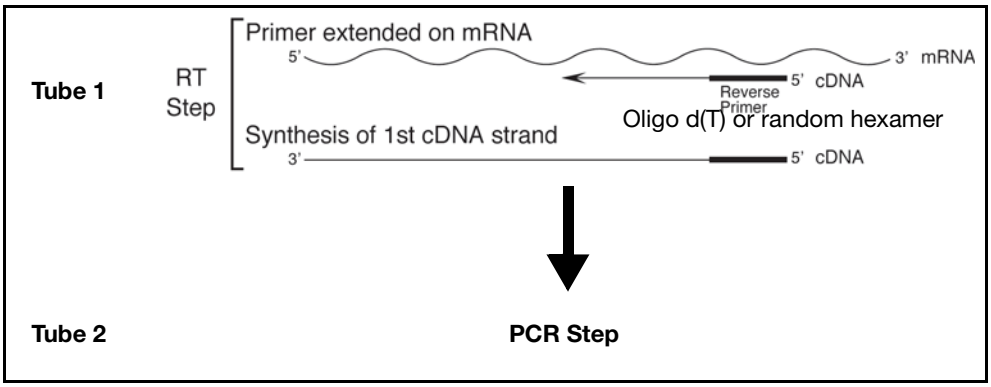


Figure 3-2 2-step RT-PCR

Primers Used for cDNA Synthesis

For 1-step RT-PCR, sequence-specific reverse primers can be used for cDNA synthesis.

For 2-step RT-PCR, the following primers can be used for cDNA synthesis:

- Oligo $d(T)_{16}$
- Random primers
- Sequence-specific reverse primers

The choice of primers for reverse transcription is best made after experimentally evaluating all three priming systems. For short RNA sequences containing no hairpin loops, any of the three priming systems works equally well. For longer RNA transcripts or sequences containing hairpin loops, consider the following guidelines:

Primers	Selection Guidelines
Oligo $d(T)_{16}$	• Use to reverse transcribe only eukaryotic mRNAs and retroviruses with poly-A tails • Avoid long mRNA transcripts or amplicons greater than 1.5 kilobases upstream from the poly-A site
Random primers	• Try first for use with long transcripts or transcripts containing hairpin loops • Use to transcribe all RNA (rRNA, mRNA, and tRNA)
Sequence-specific reverse primers	• Use to reverse transcribe RNA-containing complementary sequences only • Use in 1-step RT-PCR

Comparison of RT-PCR Methods

Method	Primers for cDNA Synthesis	Attributes
1-step RT-PCR	Sequence-specific reverse primer	• Requires single reaction mix • AmpErase® UNG cannot be used
2-step RT-PCR	Random hexamers	• cDNA can be stored for later use • AmpErase® UNG can be used • Requires two reaction mixes
	Oligo $d(T)_{16}$	
	Sequence-specific primers	

Select Singleplex or Multiplex PCR

You can perform a PCR reaction using either:

- **Singleplex PCR** – A single primer/probe set is present in the reaction tube or well. Only one target or endogenous control can be amplified per reaction.

  or

- **Multiplex PCR** – Two or more primer/probe sets are present in the reaction tube or well. Each set amplifies a specific target or endogenous control. Typically, a probe labeled with FAM™ dye detects the target, and a probe labeled with VIC® dye detects the endogenous control.

  IMPORTANT! SYBR® Green reagents cannot be used for multiplex PCR.

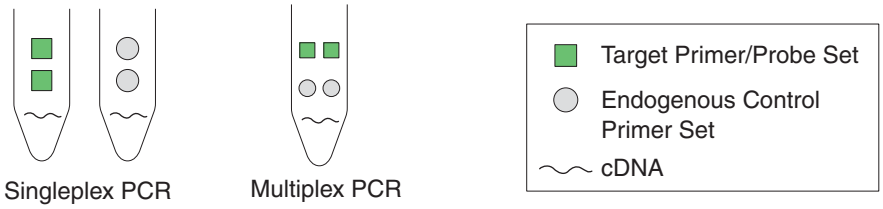


Figure 3-3 Singleplex vs. multiplex PCR

About Multiplex PCR

To perform multiplex PCR you must:

- Ensure that the endogenous control you have selected is more abundant (lower C_T value) than all the targets that you are trying to quantify under all conditions.
- Run the endogenous control assay as a primer-limited assay. The endogenous control assay (for the more abundant template) in each reaction must be primer-limited to avoid competitive PCR that may alter the C_T of the less abundant template.

Primer Limiting in Multiplex PCR

To generate an accurate multiplex assay, the amplification of one target gene must not compromise the amplification of the other target gene. Otherwise, the amplification of a highly abundant expressed gene can prevent the less abundantly expressed gene from amplifying efficiently.

If the less abundantly expressed gene does not amplify efficiently, your experiment may produce inaccurate results or, in severe cases, detection of the less abundantly expressed gene may be inhibited completely. You can avoid this situation by limiting the concentrations of the primers used to amplify the more abundantly expressed gene, thereby causing the amplification to plateau early, not long after the C_T has been established. Primer limitation should not change the C_T value. However, a primer-limited assay may be more susceptible to fluctuations in reaction conditions than the non-limited-primer target assay that it is normalizing. For more information, see Appendix B, “Primer Limiting in Multiplex PCR.”

Singleplex vs. Multiplex PCR

Primer limiting in multiplex PCR becomes increasingly more complex as the number of targets you quantify increases. When you analyze multiple numbers of targets, it may be more effective to use singleplex PCR.

In multiplex PCR, you must find a suitable endogenous control that is also more abundantly expressed than all of your target genes. You must also run all of your target and endogenous control assays in both the singleplex and multiplex formats, then compare C_T values from both formats to determine if there are any effects from the multiplexing on your C_T values. If you are studying more than two or three targets, the advantages of multiplexing diminish as the work of optimization becomes more complex and requires more reagents and time.

Consider the following when choosing between multiplex and singleplex PCR:

PCR	Description	Advantage	Limitation
Singleplex	A reaction in which a single target or endogenous control is amplified in the reaction tube or well.	• Primer optimization is not required for TaqMan® assays. • Flexibility to use TaqMan® or SYBR® Green reagents.	Requires sample for both the target and the endogenous control.
Multiplex	A reaction in which more than one target or endogenous control is amplified in the reaction tube or well.	Reduces both the running costs and the dependence on accurate pipetting when splitting a sample into two separate tubes. **Note:** This advantage diminishes if you are studying more than two or three targets.	• The endogenous control assay must be run as a primer-limited assay. • The expression of the endogenous control is greater than all the targets. • Requires validation and optimization. • May not be as effective as singleplex PCR when analyzing multiple numbers of targets. • You cannot use SYBR® Green reagents.

Select the Reagent Type

TaqMan vs. SYBR Green Reagents

You can perform quantitation experiments on the Applied Biosystems Real-Time PCR Systems with:

- TaqMan® reagents
- SYBR® Green reagents

For information on choosing between TaqMan and SYBR Green reagents, see "Considerations for Quantitation Experiments" on page 2-6.

Select the Assay Type

You can select the following assay types for quantitation experiments:

- Inventoried/Made to Order (page 3-13)
- Custom (page 3-14)
- User-designed (page 3-15)

Inventoried/Made to Order Assays

The table below lists the products available for the Applied Biosystems Inventoried/Made to Order assay type. The assays are specific to the target of interest. The master mixes contain the remaining components needed for the PCR reaction.

Note: For guidelines on designing your experiments with Inventoried/Made to Order assays, see page 3-18.

Product		Attributes
Assay	TaqMan® Gene Expression Assays	• Predesigned, gene-specific primer/probe sets for human, mouse, rat, *Arabidopsis*, *Drosophila*, *C. elegans*, *C. familiares* (dog), and *M. mulatta* (Rhesus) genes. • Probe is a FAM™ dye-labeled TaqMan® MGB probe. • Provided in a convenient, single 20× tube. • Available as Inventoried or Made to Order assays.
	TaqMan® Endogenous Control Assays **Note:** FAM™ dye-labeled TaqMan® Endogenous Control Assays are available as TaqMan® Gene Expression Assays.	• Optimized, preformulated, ready-to-use endogenous control assays. • Cost-effective, gene-specific primer/probe sets for human, mouse, and rat species, and 18S rRNA. (18S rRNA detects any eukaryotic species.) • Choice of: – FAM™ dye-labeled TaqMan® MGB probes – VIC® dye-labeled TaqMan® MGB probes – VIC® dye-labeled TaqMan® TAMRA™ probes **Note:** The VIC® dye-labeled probes are primer limited.
Master mixes	TaqMan® Gene Expression Master Mix	Designed for precise quantitation by real-time PCR for routine and challenging experiments: • Sensitive detection down to 1 copy of target. • Multiplex PCR for co-amplifying two targets in a single reaction. • Specificity for differentiation between gene family members. • Validated with TaqMan® Gene Expression Assays. • Uses one reagent for all assays to simplify assay preparation.
	TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG)	• Provides optimal performance for TaqMan® assays that use cDNA or DNA as a template. • Contains components that ensure excellent assay performance. • Uses one reagent for all assays to simplify assay preparation.
	TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG	• Provides the same attributes listed above for TaqMan® 2× Universal PCR Master Mix. • Allows you to run quantitation experiments in <40 min. **IMPORTANT!** TaqMan® Fast Universal PCR Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.

Custom Assays

The table below lists the products available for the Applied Biosystems Custom assay type. The assays are specific to the target of interest. The master mixes contain the remaining components needed for the PCR reaction.

Note: For guidelines on designing your experiments with Custom assays, see page 3-18.

Product		Attributes
Assay	Custom TaqMan® Gene Expression Assays	• Any species or organism. • Target of your choice. • Probe is a FAM™ dye-labeled TaqMan® MGB probe. • Provided in a convenient, single 20× tube.
Master mix	TaqMan® Gene Expression Master Mix	Designed for precise quantitation by real-time PCR for routine and challenging experiments: • Sensitive detection down to 1 copy of target. • Multiplex PCR for co-amplifying two targets in a single reaction. • Specificity for differentiation between gene family members. • Validated with TaqMan® Gene Expression Assays. • Uses one reagent for all assays to simplify assay preparation.
	TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG)	• Provides optimal performance for TaqMan® assays that use cDNA or DNA as a template. • Contains components that ensure excellent assay performance. • Uses one reagent for all assays to simplify assay preparation.
	TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG	• Provides the same attributes listed above for TaqMan® 2× Universal PCR Master Mix. • Allows you to run quantitation experiments in <40 min. **IMPORTANT!** TaqMan® Fast Universal PCR Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.

User-Designed Assays

The table below lists the products available from Applied Biosystems for designing your own assays. The primers and probes are specific to the target of interest. The master mixes contain the remaining components needed for the PCR reaction.

Note: For guidelines on designing your experiments with user-designed assays, see page 3-23.

Product		Attributes
Assay design	Custom TaqMan® MGB probes	• Any species or organism. • Choice of dye labels, quenchers, and synthesis scales. • For use with Applied Biosystems Assay Design Guidelines. **Note:** TaqMan® MGB and TAMRA™ probes cannot be used with SYBR® Green reagents.
	Custom TaqMan® TAMRA™ probes	
	Sequence Detection Primers	
	Primer Express® Software	Software that designs primers and probes for real-time PCR using the Applied Biosystems Assay Design Guidelines.
Master mix	TaqMan® Gene Expression Master Mix	Designed for precise quantitation by real-time PCR for routine and challenging experiments: • Sensitive detection down to 1 copy of target. • Multiplex PCR for co-amplifying two targets in a single reaction. • Specificity for differentiation between gene family members. • Validated with TaqMan® Gene Expression Assays. • Uses one reagent for all assays to simplify assay preparation.
	TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG)	• Provides optimal performance for TaqMan® assays that use cDNA or DNA as a template. • Contains components that ensure excellent assay performance. • Uses one reagent for all assays to simplify assay preparation.
	TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG	• Provides the same attributes listed above for TaqMan® 2× Universal PCR Master Mix. • Allows you to run quantitation experiments in <40 min. **IMPORTANT!** TaqMan® Fast Universal PCR Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.

Product		Attributes
Master mix *(continued)*	*Power* SYBR® Green PCR Master Mix	• Highly sensitive quantitation enables low-copy-number detection (as few as two copies of a target gene). • Detects double-stranded DNA, so specific probes are not required. • Contains highly purified AmpliTaq Gold® DNA Polymerase LD to minimize nonspecific product formation (including primer-dimer). • For use with the Sequence Detection Primers.
	Fast SYBR® Green Master Mix	• Detects double-stranded DNA, so specific probes are not required. • Allows you to run quantitation experiments in <40 min. • Contains AmpliTaq® Fast DNA Polymerase UP to minimize nonspecific product formation (including primer-dimer). • For use with the Sequence Detection Primers. **IMPORTANT!** Fast SYBR® Green Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.
	SYBR® Green PCR Master Mix	• Detects double-stranded DNA, so specific probes are not required. • For standard applications when high sensitivity is not required. • Contains AmpliTaq Gold® DNA Polymerase to minimize nonspecific product formation (including primer-dimer). • For use with the Sequence Detection Primers.

Section 3.2 Design Guidelines

This section covers:

Inventoried/Made to Order and Custom Assays

Workflow

The recommended workflow for performing quantitation experiments with Applied Biosystems Inventoried/Made to Order and Custom assay types is the same. Applied Biosystems recommends that you:

1. Select the assay:
   - TaqMan® Gene Expression Assays (below).
   - Custom TaqMan® Gene Expression Assays (page 3-20).
2. Select the master mix (page 3-21).
3. Design the experiment (page 3-22).

TaqMan® Gene Expression Assays

Product Description

TaqMan® Gene Expression Assays are a comprehensive collection of Inventoried and Made to Order probe and primer sets for performing quantitation experiments on human, mouse, rat, *Arabidopsis*, *Drosophila*, *C. elegans*, *C. familiares* (dog), and *M. mulatta* (Rhesus) genes.

The assays:

- Use TaqMan® reagents to amplify and detect the target in cDNA samples.
- Are designed using an automated design and quality-controlled system. Inventoried assays are manufactured and placed in inventory; Made to Order assays are predesigned and manufactured when ordered.
- Are designed and optimized to work with an Applied Biosystems TaqMan® master mix using universal thermal cycling conditions.
- When possible, amplify target cDNA without amplifying genomic DNA (*m* suffix in assay ID) by designing probes that cross exon-exon junctions.

Product Requirements

All TaqMan Gene Expression Assays require:

- Three components:
  - 1 to 100 ng of cDNA sample (converted from RNA) per well, with all wells in a study having the same amount of cDNA.
  - 20× Gene Expression Assay Mix (specific for each target). Each assay mix consists of two unlabeled PCR primers and a FAM™ dye-labeled TaqMan® MGB (minor groove binder) probe in a preformulated 20× mix. 1× final concentrations are 250 nM for the probe and 900 nM for each primer.
  - TaqMan® Gene Expression Master Mix, TaqMan® Universal PCR Master Mix (with or without AmpErase® UNG), or TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG.
- Only one PCR amplification step during each PCR cycle and a simultaneous real-time reading to obtain results.

Available Assays

TaqMan Gene Expression Assays are available for human, mouse, rat, *Arabidopsis*, *Drosophila*, *C. elegans*, *C. familiares* (dog), and *M. mulatta* (Rhesus) genes. The part numbers are:

- 4331182 for Inventoried assays
- 4351372 for Made to Order assays

The prefix of the assay name indicates the species for which the assay was designed: *Hs* for *Homo sapiens* (human), *Mm* for *Mus musculus* (mouse), *Rn* for *Rattus norvegicus* (rat), *At* for *Arabidopsis thaliana*, *Dm* for *Drosophila melanogaster*, Ce for *C. elegans*, Cf for *C. familiares* (dog), and Rh for *M. mulatta* (Rhesus).

The suffix of the assay name indicates the assay placement, as described in the table below.

Suffix	Description
_m	The assay's probe spans an exon junction; the assay does not detect genomic DNA.
_s	The assay's primers and probes are designed within a single exon; the assay detects genomic DNA.
_g	The assay may detect genomic DNA; the assay's primers and probes may be within a single exon.
_mH	The assay was designed to a transcript belonging to a gene family with high sequence homology. The assay provides between 10 C_T and 15 C_T difference between the target gene and the gene with the closest sequence homology. Therefore, the assay detects the target transcript with 1000- to 30,000-fold greater discrimination (sensitivity) than the closest homologous transcript, if both transcripts are present at the same copy number in a sample.
_sH	
_gH	
_u	The assay's amplicon spans an exon junction, and the probe sits completely in one of the spanned exons.
_ft	The assay is designed to detect fusion transcripts that result from chromosomal translocation. The probe and one primer are on one side of the fusion transcript breakpoint; the second primer is on the other side of the fusion transcript breakpoint. The assay does not detect genomic DNA.
_at	The assay is designed to detect a specific synthetic RNA transcript with a unique sequence that lacks homology to current annotated biological sequences.

TaqMan® Endogenous Control Assays

TaqMan® Endogenous Control Assays are available as:

- Inventoried/Made to Order TaqMan Gene Expression Assays (PN 4331182 and PN 4351372) – Each assay contains a FAM™ dye-labeled TaqMan® MGB probe in a single, preformulated 20× tube.
- Individual control assays for human, mouse, and rat species, and 18S rRNA; 18S rRNA detects any eukaryotic species (various part numbers) – Each assay contains either a FAM™ dye-labeled TaqMan® MGB probe, a VIC® dye-labeled TaqMan® MGB probe, or a VIC® dye-labeled TaqMan® TAMRA™ probe. TaqMan Endogenous Controls with VIC dye labels are primer limited.

For More Information

- For information on the latest available products and specific product uses, go to **http://www.appliedbiosystems.com/**:
  a. In the Home page, under TaqMan® Products, select **TaqMan® Gene Expression Assays**.
  b. On the Gene Expression Assays & Arrays page:
     - Under Individual Assays, select **TaqMan® Gene Expression Assays**. This option links to all TaqMan Gene Expression Assays.

       or
     - Under Individual Control Assays, select **TaqMan® Endogenous Controls**. This option links to the individual TaqMan Endogenous Control Assays (that contain FAM dye-labeled TaqMan MGB probes, VIC dye-labeled TaqMan MGB probes, or VIC dye-labeled TaqMan TAMRA probes).
- For information on Custom TaqMan Endogenous Control Assays, refer to the *Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies Application Note*.
- For information on preparing PCR reactions using the TaqMan Gene Expression Assays, refer to the *TaqMan® Gene Expression Assays Protocol*.

Custom TaqMan® Gene Expression Assays

Product Description

Custom TaqMan® Gene Expression Assays are TaqMan probe and primer sets that are designed, synthesized, and formulated by the Custom TaqMan® Genomic Assays service based on sequence information that you submit. Custom TaqMan Gene Expression Assays allow you to perform quantitation experiments on any gene or splice variant in any organism.

The assays:

- Use TaqMan® reagents to amplify and detect the target in cDNA samples.
- Are developed using proprietary assay-design software.
- Are designed and optimized to work with an Applied Biosystems TaqMan® master mix, using universal thermal cycling conditions.

Product Requirements

All Custom TaqMan Gene Expression Assays require:

- A submission file that includes your target sequence. You create the submission file using free File Builder software, then submit the file to the Custom TaqMan® Genomic Assays service.
- Three components:
  – 1 to 100 ng of cDNA sample (converted from RNA) per well, with all wells in a study having the same amount of cDNA.
  – 20× Gene Expression Assay or 60× Gene Expression Assay (specific for each target). Each assay consists of two target-specific primers and a FAM™ dye-labeled TaqMan MGB probe in a preformulated 20× or 60× mix. 1× final concentrations are 250 nM for the probe and 900 nM for each primer.
  – TaqMan® Gene Expression Master Mix, TaqMan® Universal PCR Master Mix (with or without AmpErase® UNG), or TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG.

- Only one PCR amplification step during each PCR cycle and a simultaneous real-time reading to obtain results.

For More Information

- For information on the latest available products and specific product uses, go to the **http://www.appliedbiosystems.com/**:
  a. In the Home page, under TaqMan® Products, select **TaqMan® Gene Expression Assays**.
  b. In the Gene Expression Assays & Arrays page, under Individual Assays, select **Custom TaqMan® Gene Expression Assays**.
- For information on ordering Custom TaqMan Gene Expression Assays, refer to the *Custom TaqMan® Genomic Assays Protocol: Submission Guidelines*.
- For information on preparing PCR reactions using the Custom TaqMan Gene Expression Assays, refer to the *Custom TaqMan® Gene Expression Assays Protocol*.

Select the Master Mix

Available Master Mixes

For quantitation experiments, Applied Biosystems Inventoried/Made to Order and Custom assay types are designed to work with the following TaqMan® master mixes:

Master Mix	Part Number
TaqMan® Gene Expression Master Mix, 1-Pack (1 × 5 mL), 200 reactions	4369016
TaqMan® Gene Expression Master Mix, 10-Pack (10 × 5 mL), 2000 reactions	4369542
TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG, 250 reactions **IMPORTANT!** TaqMan® Fast Universal PCR Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.	4352042
TaqMan® 2× Universal PCR Master Mix, 200 reactions	4304437
TaqMan® 2× Universal PCR Master Mix, 2000 reactions	4326708
10-Pack, TaqMan® 2× Universal PCR Master Mix	4305719
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 200 reactions	4324018
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 2000 reactions	4326614
10-Pack, TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG	4324020

For More Information

For information on using the TaqMan reagents, refer to:

- *TaqMan® Fast Universal PCR Master Mix (2×) Protocol*
- *TaqMan® Gene Expression Master Mix Protocol*
- *TaqMan® Universal PCR Master Mix Protocol*

Design the Experiment

For information on designing quantitation experiments on the Real-Time PCR Systems, refer to:

System	Document
7300 System	• *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide* • *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide*
7500/7500 Fast System	• *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Standard Curve Experiments* • *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments*
7900HT Fast System	• *Applied Biosystems 7900HT Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide* • *Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide*
StepOne™ and StepOnePlus™ Systems	• *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments* • *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments*

Note: For document part numbers, see "Related Documentation" on page viii. For information on obtaining these documents, see "How to Obtain Support" on page xii.

User-Designed Assays

Workflow

If you are designing your own assays (primers and probes) for quantitation experiments, Applied Biosystems recommends that you follow the workflow for the Applied Biosystems Assay Design Guidelines:

1. Design primers and probes using Primer Express® Software (below).
2. Select the appropriate reagents (page 3-26).
3. Use the recommended thermal cycling conditions (page 3-30).
4. Begin with default primer and probe concentrations. If needed, optimize the primer concentrations (page 3-33) and probe concentrations (page 3-37).

IMPORTANT! These steps provide a rapid and reliable system for assay design and optimization only when used in their entirety. Adopt the system as a whole to achieve the highest level of success. For a more detailed description of Applied Biosystems Assay Design Guidelines, see Appendix C.

Design Primers and Probes Using Primer Express® Software

The Primer Express® Software uses recommended parameters to select primers and probes based on the DNA sequence that you provide.

If you are designing your own assay, follow the summary of the primer and probe design guidelines for quantitation experiments on page 3-25. These guidelines are part of the default parameters set in Primer Express Software v3.0. For a detailed discussion of these guidelines, see "About the Primer and Probe Design Guidelines" on page 3-24.

Note: Although a probe is not required for SYBR® Green I dye detection, it is a good practice to use Primer Express software to select a primer and probe set when you design an assay for SYBR® Green reagents. Although no probe will be used, the primers will meet all required criteria; if you need to convert the assay to TaqMan reagents to obtain higher specificity, you can find the probe immediately in the original Primer Express software document.

Selecting an Amplicon Site for Quantitation Assays

Selecting a good amplicon site ensures amplification of the target mRNA/cDNA without co-amplifying the genomic sequence, pseudogenes, and other related genes. SYBR Green reagents can be useful for screening amplicon sites for gene expression.

Guidelines

- The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA.
- The primer pair should be specific to the target gene to avoid amplification of pseudogenes or other related genes.
- When designing primers, use Primer Express software guidelines.
- If no primers and probes are found in the amplicon site you have chosen, you may need to reexamine the sequence and select one or more target sites for design.

If the gene you are studying does not have introns, it is not possible to design an amplicon that amplifies the mRNA sequence without amplifying the genomic sequence. In this case, run a control of your RNA sample that has not been reverse-transcribed (RT minus controls).

About the Primer and Probe Design Guidelines

Selection of Small Amplicons

An important default parameter in Primer Express software is the selection of amplicons in the 50- to 150-base pair range. Small amplicons are favored because they promote high-efficiency amplification.

In addition, high-efficiency assays enable relative quantitation to be performed using the comparative C_T ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001). This method increases sample throughput by eliminating the need for standard curves.

G/C Content

Whenever possible, select primers and probes in a region with a G/C content of 30 to 80%. Regions with a G/C content >80% may not denature well during thermal cycling, leading to a less efficient reaction. G/C-rich sequences are susceptible to nonspecific interactions that may reduce reaction efficiency and produce nonspecific signal in assays using SYBR Green reagents. Avoid primer and probe sequences containing runs of four or more G bases.

Melting Temperature

When you select primers and probes with the recommended melting temperature (Tm), you can use universal thermal cycling conditions. Applied Biosystems recommends that the probe Tm be 10 °C higher than that of the primers.

5′ End of Probes

Primer Express Software v3.0 does not select probes with a G on the 5′ end. The quenching effect of a G base in this position is present even after probe cleavage. The presence of a G base can result in reduced fluorescence values (ΔRn) that can negatively affect assay performance. G bases in positions close to the 5′ end, but not on it, have not been shown to compromise assay performance.

3′ End of Primers

To reduce the possibility of nonspecific product formation, ensure that the last five bases on the 3′ end of the primers do not contain more than two C and/or G bases. Under certain circumstances, such as a G/C-rich template sequence, you may have to relax this recommendation to keep the amplicon under 150 base pairs in length. In general, avoid primer 3′ ends extremely rich in G and/or C bases.

Summary of Primer and MGB Probe Design Guidelines

<table>
<tr><th>Probe Guidelines</th><th>Primer Guidelines</th></tr>
<tr><td colspan="2">Select the probe first, then design the primers as close as possible to the probe without overlapping the probe (amplicons of 50 to 150 base pairs are strongly recommended).</td></tr>
<tr><td colspan="2">Keep the G/C content in the 30 to 80% range.</td></tr>
<tr><td colspan="2">Avoid runs of an identical nucleotide, especially guanine, where runs of four or more Gs should be avoided.</td></tr>
<tr><td>When using Primer Express® Software, the Tm should be 68 to 70 °C.</td><td>When using Primer Express® Software, the Tm should be 58 to 60 °C.</td></tr>
<tr><td>No G on the 5′ end.</td><td rowspan="2">The five nucleotides at the 3′ end should have no more than two G and/or C bases.</td></tr>
<tr><td>• TaqMan MGB probes should not be shorter than 13 bases
• TaqMan TAMRA probes should not be longer than 40 bases.</td></tr>
</table>

Select the Reagents

Several TaqMan and SYBR Green reagents are available for quantitation experiments. The reagents you use depend on your target:

- DNA or cDNA (below).
- RNA using 1-step RT-PCR (page 3-27).
- RNA using 2-step RT-PCR (page 3-28).

Note: If you use SYBR Green reagents, Applied Biosystems highly recommends the *Power* SYBR Green reagents.

DNA or cDNA Quantitation

Reagent	Kit	Part Number
TaqMan® reagents	TaqMan® Gene Expression Master Mix, 1-Pack (1 × 5 mL), 200 reactions	4369016
	TaqMan® Gene Expression Master Mix, 10-Pack (10 × 5 mL), 2000 reactions	4369542
	TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG, 250 reactions‡	4352042
	TaqMan® 2× Universal PCR Master Mix, 200 reactions	4304437
	TaqMan® 2× Universal PCR Master Mix, 2000 reactions	4326708
	10-Pack, TaqMan® 2× Universal PCR Master Mix	4305719
	TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 200 reactions	4324018
	TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 2000 reactions	4326614
	10-Pack, TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG	4324020
	TaqMan® PCR Core Reagents Kit, 200 reactions	N808-0228

Reagent	Kit	Part Number
SYBR® Green reagents	Fast SYBR® Green Master Mix (1 mL)§	4385610
	Fast SYBR® Green Master Mix (5 mL)	4385612
	Fast SYBR® Green Master Mix (10 × 5 mL)	4385618
	Fast SYBR® Green Master Mix (50 mL)	4385614
	Power SYBR® Green PCR Master Mix (1 mL), 40 reactions	4368577
	Power SYBR® Green PCR Master Mix (5-mL), 200 reactions	4367659
	Power SYBR® Green PCR Master Mix (10 × 5-mL), 2000 reactions	4368708
	Power SYBR® Green PCR Master Mix (50 mL), 2000 reactions	4367660
	SYBR® Green PCR Master Mix (1-mL), 40 reactions	4344463
	SYBR® Green PCR Master Mix (5-mL), 200 reactions	4309155
	SYBR® Green PCR Master Mix (50-mL), 2000 reactions	4334973
	SYBR® Green PCR Core Reagents, 200 reactions	4304886

‡TaqMan® Fast Universal PCR Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.

§Fast SYBR® Green Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.

RNA Quantitation Using 1-Step RT-PCR

Reagent	Kit	Part Number
TaqMan® reagents	TaqMan® One-Step RT-PCR Master Mix Reagents Kit	4309169
	TaqMan® EZ RT-PCR Core Reagents **IMPORTANT!** Use the TaqMan® EZ RT-PCR Core Reagents when a high-temperature RT step is required.	N808-0236
	TaqMan® Gold RT-PCR Reagents without Controls, 200 reactions	N808-0232
	TaqMan® Gold RT-PCR Reagents without Controls, 10-Pack, 2000 reactions	4304133
	TaqMan® Gold RT-PCR Reagents with Controls, 200 reactions	N808-0233
SYBR® Green reagents	*Power* SYBR® Green RT-PCR Reagents Kit	4368711
	SYBR® Green RT-PCR Reagents	4310179

RNA Quantitation Using 2-Step RT-PCR

Reagent	Step	Kit	Part Number
TaqMan® reagents	PCR step only	TaqMan® Gene Expression Master Mix, 1-Pack (1 × 5 mL), 200 reactions	4369016
		TaqMan® Gene Expression Master Mix, 10-Pack (10 × 5 mL), 2000 reactions	4369542
		TaqMan® 2× Universal PCR Master Mix	4304437
		TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG‡	4352042
	RT step only	High-Capacity cDNA Reverse Transcription Kit	4374966
		TaqMan® Reverse Transcription Reagents	N808-0234
	Both RT and PCR steps	TaqMan® Gold RT-PCR Reagents without Controls, 200 reactions	N808-0232
		TaqMan® Gold RT-PCR Reagents without Controls, 10-Pack, 2000 reactions	4304133
		TaqMan® Gold RT-PCR Reagents with Controls, 200 reactions	N808-0233
SYBR® Green reagents	PCR step only	Fast SYBR® Green Master Mix (5 mL)§	4385612
		Power SYBR® Green PCR Master Mix	4367659
		SYBR® Green PCR Master Mix	4309155
	RT step only	High-Capacity cDNA Reverse Transcription Kit	4374966
		TaqMan® Reverse Transcription Reagents	N808-0234
	Both RT and PCR steps	*Power* SYBR® Green RT-PCR Reagents Kit	4368711
		SYBR® Green RT-PCR Reagents	4310179

‡TaqMan® Fast Universal PCR Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.

§Fast SYBR® Green Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.

About DNA Polymerase

A DNA polymerase is included in Applied Biosystems TaqMan and SYBR Green reagents:

Reagent	DNA Polymerase
TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG)	AmpliTaq Gold® DNA Polymerase
TaqMan® Gene Expression Master Mix	AmpliTaq Gold® DNA Polymerase UP (Ultra Pure)‡
TaqMan® Genotyping Master Mix	
TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG	AmpliTaq® Fast DNA Polymerase
SYBR® Green PCR Master Mix	AmpliTaq Gold® DNA Polymerase
Fast SYBR® Green Master Mix	AmpliTaq® Fast DNA Polymerase UP (Ultra Pure)‡
Power SYBR® Green PCR Master Mix	AmpliTaq Gold® DNA Polymerase LD (Low DNA)‡

‡ DNA Polymerase LD and UP have been purified and quality-control tested to verify that <10 copies of bacterial ribosomal RNA gene sequences are present in a 5-Unit aliquot.

AmpliTaq Gold and AmpliTaq Fast DNA polymerases contain a hot-start enzyme. The hot-start enzyme:

- Ensures a robust reaction.
- Can dramatically reduce the amount of nonspecific amplification and primer-dimer formation.
- Simplifies assay setup, which can be performed at room temperature.

About MultiScribe Reverse Transcriptase

MultiScribe™ Reverse Transcriptase is a recombinant Moloney Murine Leukemia Virus (MuLV) Reverse Transcriptase that reverse transcribes RNA into complimentary DNA (cDNA).

Use the Recommended Thermal Cycling Conditions

- DNA or cDNA (below)
- RNA using 1-step RT-PCR (page 3-31)
- RNA using 2-step RT-PCR (page 3-32)

Note: Thermal cycling conditions for Fast reagents differ from thermal cycling conditions for standard reagents.

DNA or cDNA Quantitation

Reagent	Times and Temperatures			
	Initial Steps		PCR (40 Cycles)	
	AmpErase® UNG Activation	Hot-Start DNA Polymerase Activation	Melt	Anneal/Extend
	HOLD	HOLD	CYCLE	
• TaqMan® Gene Expression Master Mix • TaqMan® 2× Universal PCR Master Mix (with AmpErase® UNG) • *Power* SYBR® Green PCR Master Mix	2 min @ 50 °C	10 min @ 95 °C	Use the default thermal cycling conditions for standard reagents, as provided by your Real-Time PCR System software.	
TaqMan® 2× Universal PCR Master Mix (without AmpErase® UNG)	NA	20 sec @ 95 °C	Use the default thermal cycling conditions for standard reagents, as provided by your Real-Time PCR System software.	
SYBR® Green PCR Master Mix	NA	10 min @ 95 °C	Use the default thermal cycling conditions for standard reagents, as provided by your Real-Time PCR System software.	
• TaqMan® Fast Universal PCR Master Mix (25), No AmpErase® UNG • Fast SYBR® Green Master Mix	NA	20 sec @ 95 °C	Use the default thermal cycling conditions for Fast reagents, as provided by your Real-Time PCR System software.	

RNA Quantitation Using 1-Step RT-PCR

Reagent	Times and Temperatures			
	Initial Steps		PCR (40 Cycles)	
	AmpErase® UNG Activation	Hot-Start DNA Polymerase Activation	Melt	Anneal/Extend
	HOLD	HOLD	CYCLE	
• TaqMan® One-Step RT-PCR Master Mix Reagents Kit • *Power* SYBR® Green RT-PCR Reagents Kit	30 min @ 48 °C	10 min @ 95 °C	Use the default thermal cycling conditions for standard reagents, as provided by your Real-Time PCR System software.	

Note: The conditions listed above do not apply to the TaqMan® EZ RT-PCR Kit. See the *TaqMan® EZ RT-PCR Kit Protocol* for the appropriate conditions.

RNA Quantitation Using 2-Step RT-PCR

1. RT Step			
Reagent	**Times and Temperatures**		
	HOLD	**HOLD**	**Prepare for the PCR Step**
High-Capacity cDNA Reverse Transcription Kit	10 min @ 25 °C	120 min @ 37 °C	After reverse-transcribing the RNA into cDNA (RT step), the samples can be stored or used for the subsequent PCR step described below.
IMPORTANT! For most applications and when large amounts of cDNA are required, Applied Biosystems recommends 120 minutes at 37 °C for reverse transcription to achieve optimal conversion.			

2. PCR Step				
Reagent	**Times and Temperatures**			
	Initial Steps		**PCR (40 Cycles)**	
	AmpErase® UNG Activation	**Hot-Start DNA Polymerase Activation**	**Melt**	**Anneal/Extend**
	HOLD	**HOLD**	**CYCLE**	
• TaqMan® Gene Expression Master Mix • TaqMan® 2X Universal PCR Master Mix (with AmpErase® UNG) • *Power* SYBR® Green PCR Master Mix	2 min @ 50 °C	10 min @ 95 °C	Use the default thermal cycling conditions for standard reagents, as provided by your Real-Time PCR System software.	
TaqMan® 2X Universal PCR Master Mix (without AmpErase® UNG)	NA	20 sec @ 95 °C	Use the default thermal cycling conditions for standard reagents, as provided by your Real-Time PCR System software.	
SYBR® Green PCR Master Mix	NA	10 min @ 95 °C	Use the default thermal cycling conditions for standard reagents, as provided by your Real-Time PCR System software.	
• TaqMan® Fast Universal PCR Master Mix (2X), No AmpErase® UNG • Fast SYBR® Green Master Mix	NA	20 sec @ 95 °C	Use the default thermal cycling conditions for Fast reagents, as provided by your Real-Time PCR System software.	

Optimize Primer Concentrations

By independently varying forward and reverse primer concentrations in optimization experiments, you can identify the concentrations that provide optimal assay performance.

When you use TaqMan Gene Expression Master Mix or TaqMan 2× Universal PCR Master Mix, Applied Biosystems recommends the primer concentrations listed in "Default Primer Concentrations" below. Detailed discussions follow for the:

- Primer optimization matrix (below)
- TaqMan reagents (below)
- SYBR Green reagents (page 3-34)

Default Primer Concentrations

The recommended starting primer concentrations listed in the table below are for DNA and cDNA quantitation assays.

Reagent	Starting Primer Concentrations (nM)	
	Forward Primer	Reverse Primer
TaqMan® reagents	900	900
SYBR® Green reagents	200	200

Primer Optimization Matrix

A primer optimization matrix allows you to determine the minimum primer concentration that yields the minimum C_T value and maximum ΔRn value.

When using SYBR Green reagents, optimal primer concentration allows you to compensate for nonspecific primer binding and primer-dimer formation. The lower the concentration of primers, the less chance there is for nonspecific primer amplification and primer-dimer formation.

TaqMan Reagents

For quantitation assays using TaqMan reagents, Applied Biosystems recommends the following guidelines for each primer and probe:

TaqMan Reagent Concentrations (nM)		
Forward Primer	Reverse Primer	Probe
900	900	250

Because there is a specific probe in the reaction, nonspecific amplification and primer-dimer formation will not be detected and therefore are not concerns. To obtain a fluorescence signal with TaqMan reagents, you must have primer binding and extension and probe binding and cleavage.

If desired, you can achieve optimal performance by selecting the primer concentrations that provide the lowest C_T value and the highest ΔRn value for a fixed quantity of target template.

Note: Although C_T values are the parameter by which quantitative values are assigned in real-time quantitation assays, ΔRn values can also be important when you try to obtain maximum sensitivity and reproducibility.

SYBR Green Reagents

Optimizing primer concentrations is slightly more complex for quantitation assays using SYBR Green reagents. You must include negative controls for SYBR Green reagents. The primer concentrations you select should provide a low C_T and high ΔRn when run against the target template, but should not produce nonspecific amplification or primer-dimer formation within negative controls. Melt curves or gel analysis can be extremely useful when you select optimal primer concentrations for quantitation assays using SYBR Green reagents.

The results of a typical SYBR Green reagent primer optimization matrix experiment are shown in Figure 3-4 on page 3-35:

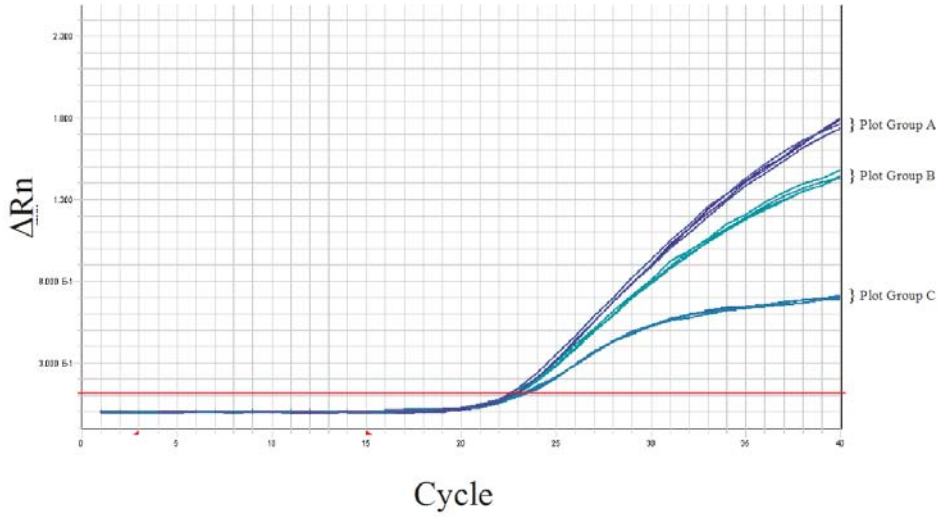
- Figure 3-4a shows the amplification plots for all primer concentration combinations in linear view.
- Figure 3-4b shows the same data in log view format.

Overall, Plot Group A provides optimal performance. The reactions have a final concentration of 300 nM for the forward and reverse primers. This concentration produces the lowest C_T value and the highest ΔRn value. When compared to Plot Group A:

- Plot Group B (where the reactions have a final concentration of 150 nM for both primers) produces a decreased ΔRn value and a slightly higher C_T value.
- Plot Group C (where the reactions have a final concentration of 50 nM for both primers) produces the lowest ΔRn value and the highest C_T value.

You must consider the data from both the primer optimization amplification plots as well as the melt curve analysis and negative control reactions to find the best primer concentration: one that gives a low C_T value, a high ΔRn value, no primer-dimer formation, and no nonspecific amplification.

a) Linear view



b) Log view

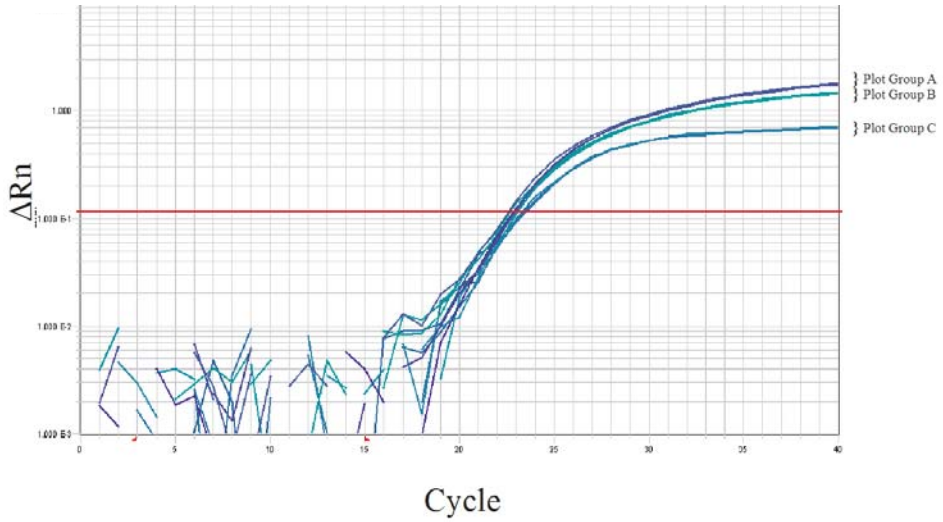


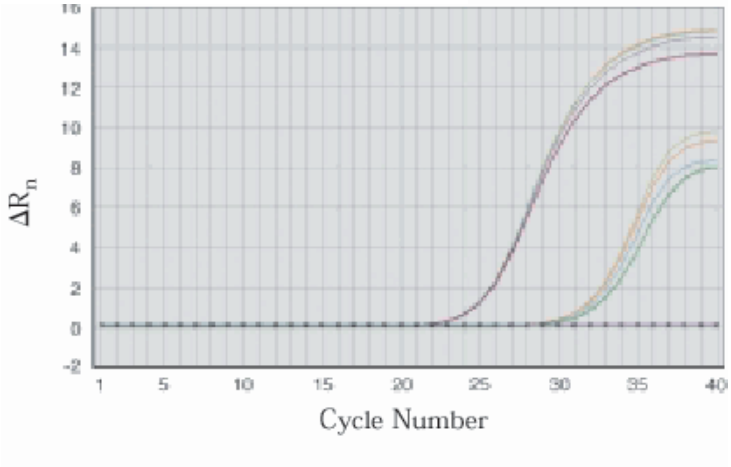
Figure 3-4 SYBR Green reagents primer optimization experimental results showing amplification plots (linear and log views) of primer combinations
Plot group key:
A: Combinations that contain at least 300 nM of forward and reverse primer
B: Combinations that contain at least 150 nM of forward and reverse primer
C: Combinations that contain at least 50 nM of forward and reverse primer

Figure 3-5 below shows the results from a non-optimized reaction using SYBR Green reagents:

- Figure 3-5a shows amplification of the negative control wells, which indicates that significant nonspecific amplification or primer-dimer formation is occurring.
- Figure 3-5b shows that the melting temperature of the product generated in the absence of template is lower than the melting temperature of the specific product generated with template, indicating that significant nonspecific amplification or primer-dimer formation is occurring.

The results shown in Figure 3-5 are typical of primer-dimer formation. These results indicate that lower primer concentrations may provide better results. Additionally, you can redesign another set of primers to the target of interest.

a) Amplification plot, linear view



b) Melt curve analysis

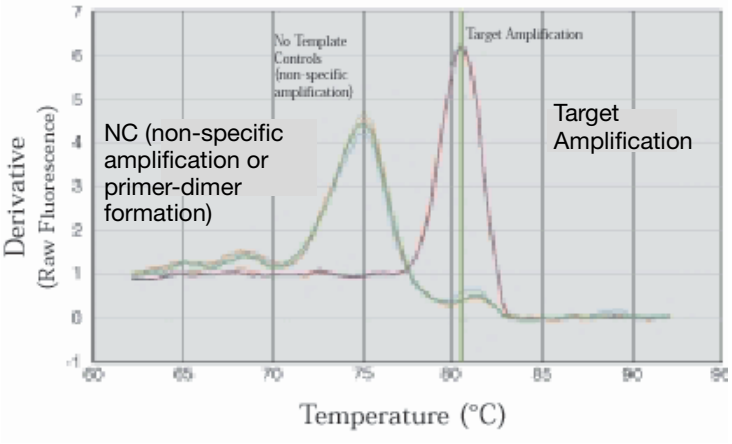


Figure 3-5 Amplification data and melt curve analysis using SYBR Green reagents:
(a) Amplification plot (linear view) demonstrating suspected nonspecific amplification in negative control (NC) wells
(b) Melt curve analysis confirming that product in NC wells has a melting temperature that is different from that of the specific product

Optimize the Probe Concentration

For detection by TaqMan® probes, the recommended probe concentration of 250 nM ensures excellent assay performance. You can optimize the probe concentration, however, decreasing the probe concentration could cause an increase in the C_T value.

Note: No probe is required for SYBR Green I dye detection.

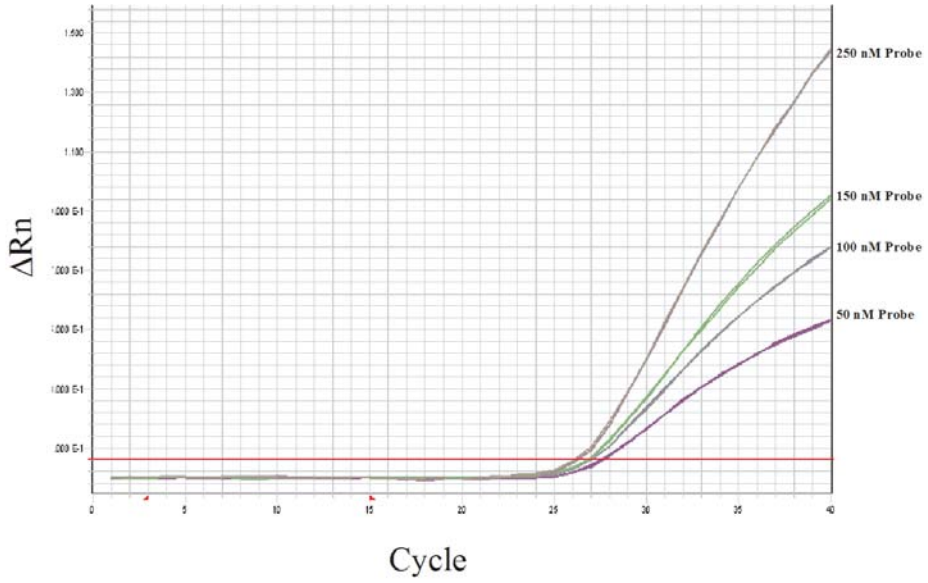
Recommended Probe Concentrations

The recommended probe concentrations for DNA and cDNA quantitation assays using TaqMan reagents is 250 nM. Figure 3-6 shows the results of a probe optimization experiment in which the probe concentration is varied from 50 to 250 nM:

- Figure 3-6a shows an increase in ΔRn as the probe concentration is increased.
- Figure 3-6b shows that the C_T value changes with sufficient probe concentrations.

To ensure the best reproducibility, especially when you want to detect low copy numbers of a target, avoid probe-limiting concentrations. Run the assay at a probe concentration of 250 nM. At this concentration, you avoid probe limitation and ensure large ΔRn values. Large ΔRn values indicate a robust assay that is performing at high efficiency, giving high product yield and allowing accurate peak measurement.

a) Linear view



b) Log view

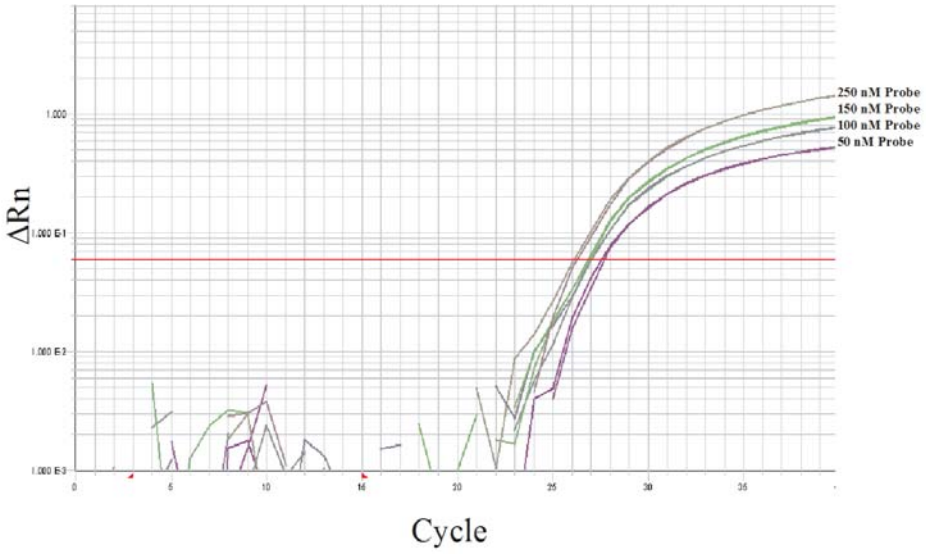


Figure 3-6 Amplification plot (linear and log views) of probe concentration titration from 50 to 250 nM

For More Information

For information on:

- Using the TaqMan reagents, refer to the:
  - *TaqMan® Fast Universal PCR Master Mix (2×) Protocol*
  - *TaqMan® Gene Expression Master Mix Protocol*
  - *TaqMan® Universal PCR Master Mix Protocol*
- Using the SYBR Green reagents, refer to the:
  - *Fast SYBR® Green Master Mix Protocol*
  - *Power SYBR® Green PCR Master Mix and RT-PCR Protocol*
  - *SYBR® Green PCR Master Mix and RT-PCR Reagents Protocol*
  - *SYBR® Green PCR and RT-PCR Reagents Protocol*
- Performing quantitation experiments on the Real-Time PCR Systems, refer to:

System	Document
7300 System	• *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide* • *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide*
7500/7500 Fast System	• *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Standard Curve Experiments* • *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments*
7900HT Fast System	• *Applied Biosystems 7900HT Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide* • *Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide*
StepOne™ and StepOnePlus™ Systems	• *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments* • *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments*

Note: For document part numbers, see "Related Documentation" on page viii. For information on obtaining these documents, see "How to Obtain Support" on page xii.

Genotyping Experiments 4

This chapter covers:

Section 4.1 About Genotyping Experiments

This section covers:

Overview

What Is a Genotyping Experiment?

A genotyping experiment is an endpoint experiment used to determine the genotype of unknown samples. With this experiment type, you can differentiate two alleles of a single nucleotide polymorphism (SNP).

A genotyping experiment determines if unknown samples are:

- Allele 1 homozygotes (samples having only allele 1)
- Allele 2 homozygotes (samples having only allele 2)
- Heterozygotes (samples having both allele 1 and allele 2)

Components

PCR reactions for genotyping experiments include the following components:

- **Sample** – The DNA sample in which the genotype of the target is unknown.
- **(Optional) Replicates** – Identical reactions containing identical components and volumes.
- **Negative Controls** – Samples that contain water or buffer instead of template; also known as no template controls (NTCs). Negative controls should not amplify.
- **(Optional) Positive controls** – Samples that contain known genotypes (homozygotes for allele 1, homozygotes for allele 2, and heterozygotes for alleles 1 and 2).

Instruments

Genotyping experiments require two steps: thermal cycling (PCR amplification) followed by endpoint detection of the resulting fluorescence signals. You can perform the thermal cycling step (PCR amplification) on an Applied Biosystems Real-Time PCR System or on a standalone thermal cycler.

If you use a Real-Time PCR System:

- You can analyze the PCR, which is helpful for troubleshooting.
- Perform the endpoint plate read separately.

How Genotyping Experiments Work

In genotyping experiments, the PCR includes a specific fluorescent-dye-labeled probe for each allele of the target SNP. The probes contain different fluorescent reporter dyes to differentiate each allele.

You can use TaqMan® minor groove binder (MGB) probes on the Applied Biosystems Real-Time PCR Systems. Each TaqMan® MGB probe contains:

- A reporter dye at the 5′ end of each probe:
  - VIC® dye is linked to the 5′ end of the allele 1 probe
  - FAM™ dye is linked to the 5′ end of the allele 2 probe
- A minor groove binder (MGB)

  This modification increases the melting temperature (Tm) of probes without increasing probe length (Afonina *et al.*, 1997; Kutyavin *et al.*, 1997), thereby allowing the design of shorter probes. Consequently, the TaqMan MGB probes exhibit greater differences in Tm values between matched and mismatched probes; greater differences in Tm values provide accurate genotyping.

- A nonfluorescent quencher (NFQ) at the 3′ end of the probe

  Because the quencher does not fluoresce, real-time PCR systems can measure reporter dye contributions more accurately.

During PCR, each probe anneals specifically to its complementary sequence between the forward and reverse primer sites. The DNA polymerase can cleave only probes that hybridize to their specific SNP allele (match). Cleavage separates the reporter dye from the quencher dye, increasing fluorescence of the reporter dye. Thus, the fluorescence signals generated during PCR amplification indicate the alleles that are present in the sample.

Mismatches Between Probe and Allele Sequences

A mismatch between a probe and a SNP allele (Figure 4-1) reduces the efficiency of probe hybridization. Furthermore, the DNA polymerase is likely to displace the mismatched probe rather than to cleave it to release reporter dye.

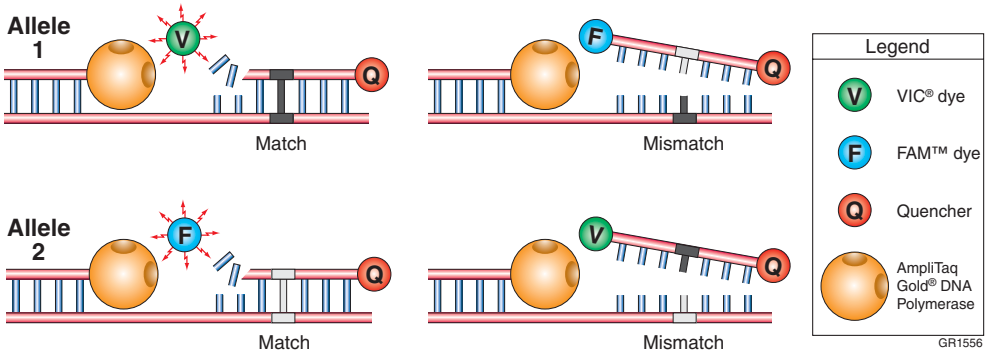


Figure 4-1 Results from matches and mismatches between allele and probe sequences in genotyping experiments

Table 4-1 summarizes the possible results of the genotyping experiment example shown above.

Table 4-1 Genotyping experiment results

A substantial increase in…	Indicates…
VIC® dye fluorescence only	Allele 1 homozygotes.
FAM™ dye fluorescence only	Allele 2 homozygotes.
VIC® dye and FAM™ dye fluorescence	Heterozygotes.

Workflow

Before performing genotyping experiments on a Real-Time PCR System, you need to:

1. Select the assay type (page 4-6).
2. Review the design guidelines for the assay type you selected (Section 4.2 on page 4-9).

Select the Assay Type

You can select the following assay types for genotyping experiments:

- Pre-Designed/Validated (below)
- Custom (page 4-7)

Pre-Designed/ Validated Assays

The table below lists the products available for the Applied Biosystems Pre-Designed/Validated assay type. The assays are specific to the target of interest. The master mixes contain the remaining components needed for the PCR reaction.

Note: For guidelines on designing your experiments with Pre-Designed/Validated assays, see page 4-10.

Note: Genotyping experiments are not supported for Fast or SYBR® Green master mixes and protocols.

Product		Attributes
Assay	TaqMan® SNP Genotyping Assays	• Predesigned assays for high-density, genome-wide marker coverage. • For studying single-nucleotide polymorphisms (SNPs), insertions/deletions (in/dels), and multinucleotide polymorphisms (MNPs). • For screening, association, candidate region, candidate gene, or fine-mapping studies. • Convenient single-tube format.
	TaqMan® Drug Metabolism Genotyping Assays	• Detect polymorphisms in 220 genes that code for various drug metabolism enzymes and drug transporters. • For studying single-nucleotide polymorphisms (SNPs), insertions/deletions (in/dels), and multinucleotide polymorphisms (MNPs).
	Pre-Developed TaqMan® Assay Reagents for Allelic Discrimination	• Genotype purified DNA samples for specific mutations. • Allele 1 and 2 control DNA included to allow each homozygote signal to be generated on each run. • Closed-tube system requires no post-PCR manipulation or gels.
Master mix	TaqMan® Genotyping Master Mix	Optimized for endpoint fluorescence detection in SNP genotyping applications: • Distinct clusters and high call rates for unambiguous allelic discrimination. • Excellent pre- and post-PCR stability for high-throughput setup and analysis. • Validated with TaqMan® SNP Genotyping Assays. • Uses one reagent for all assays to simplify assay preparation.
	TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG)	• Provides optimal performance for TaqMan® assays that use cDNA or DNA as a template. • Contains components that ensure excellent assay performance. • Uses one reagent for all assays to simplify assay preparation.

Custom Assays

The table below lists the products available for the Applied Biosystems Custom assay type. The assays are specific to the target of interest. The master mixes contain the remaining components needed for the PCR reaction.

Note: For guidelines on designing your experiments with Custom assays, see page 4-10.

Note: Genotyping experiments are not supported for Fast or SYBR® Green master mixes and protocols.

Product		Attributes
Assay	Custom TaqMan® SNP Genotyping Assays	• Any possible single-nucleotide polymorphism (SNP) in any organism. • Detect insertions/deletions (in/dels) of up to six bases. • Detect multiple nucleotide polymorphisms (MNPs) of up to six bases. • Convenient single-tube format.
Master mix	TaqMan® Genotyping Master Mix	Optimized for endpoint fluorescence detection in SNP genotyping applications: • Distinct clusters and high call rates for unambiguous allelic discrimination. • Excellent pre- and post-PCR stability for high-throughput setup and analysis. • Validated with TaqMan® SNP Genotyping Assays. • Uses one reagent for all assays to simplify assay preparation.
	TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG)	• Provides optimal performance for TaqMan® assays that use cDNA or DNA as a template. • Contains components that ensure excellent assay performance. • Uses one reagent for all assays to simplify assay preparation.

Section 4.2 Design Guidelines

This section covers:

Pre-Designed/Validated and Custom Assays

Workflow

The recommended workflow for performing genotyping experiments with Applied Biosystems Pre-Designed/Validated and Custom assay types is the same. Applied Biosystems recommends that you:

1. Select the assay:
   - TaqMan® SNP Genotyping Assays (below).
   - TaqMan® Drug Metabolism Genotyping Assays (page 4-11).
   - TaqMan® Pre-Developed Assays Reagents for Allelic Discrimination (page 4-12).
   - Custom TaqMan® SNP Genotyping Assays (page 4-13).
2. Select the master mix (page 4-14).
3. Design the experiment (page 4-15).

TaqMan® SNP Genotyping Assays

Product Description

TaqMan® SNP Genotyping Assays are a comprehensive collection of primer and probe sets for genotyping single nucleotide polymorphisms (SNPs) for human studies.

The assays:

- Use TaqMan® reagents to amplify and detect specific SNP alleles in purified genomic DNA samples.
- Are designed using Applied Biosystems bioinformatics system and software, as well as genomic information from Celera Genomics and public databases.
- Are designed and optimized to work with an Applied Biosystems TaqMan® master mix, using universal thermal cycling conditions.

Product Requirements

All TaqMan SNP Genotyping Assays require:

- Three components:
  - 1 to 20 ng of purified genomic DNA sample.
  - 20×, 40×, or 80× SNP Genotyping Assay Mix (specific for each polymorphism). Each assay consists of sequence-specific forward and reverse primers to amplify the SNP of interest and two TaqMan MGB probes: One probe labeled with VIC® dye detects the Allele 1 sequence; one probe labeled with FAM™ dye detects the Allele 2 sequence.
  - TaqMan® Genotyping Master Mix or TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG).
- PCR amplification and an endpoint read to obtain results.

Available Assays

TaqMan SNP Genotyping Assays are available as TaqMan® Pre-Designed SNP Genotyping Assays:

- Over 4.5 million predesigned genome-wide assays, including 3.5 million human HapMap SNPs, 70,000 human cSNPs, 160,000 human validated, and 10,000 mouse assays.
- Available in small, medium, and large scale.
- Made to Order (that is, manufactured at the time of order).

For More Information

- For information on the latest available products and specific product uses, go to:

  http://www.allsnps.com/

  and/or

  http://www.appliedbiosystems.com/

  a. In the Home page, under TaqMan® Products, select **TaqMan® SNP Genotyping Assays**.

  b. In the SNP Genotyping Assays page, under Pre-Designed/Validated Assays, select **TaqMan® SNP Genotyping Assays**.
- For information on preparing PCR reactions using the TaqMan SNP Genotyping Assays, refer to the *TaqMan® SNP Genotyping Assays Protocol*.

TaqMan® Drug Metabolism Genotyping Assays

Product Description

TaqMan® Drug Metabolism Genotyping Assays are a comprehensive collection of Inventoried primer and probe sets for genotyping SNPs, insertions and deletions (indels), and multiple nucleotide polymorphisms (MNPs) in drug metabolism related genes.

The assays:

- Use TaqMan reagents to amplify and detect specific polymorphisms in purified genomic DNA samples.
- Are designed using Applied Biosystems bioinformatics system and software, as well as genomic information from public SNP databases and public genome assemblies.
- Are designed and optimized to work with an Applied Biosystems TaqMan® master mix.

Product Requirements

All TaqMan Drug Metabolism Genotyping Assays require:

- Three components:
  - 3 to 20 ng of purified genomic DNA sample per well.
  - 20× Drug Metabolism Genotyping Assay Mix (specific for each polymorphism). Each assay consists of sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest and two TaqMan MGB probes: One probe labeled with VIC dye detects the Allele 1 sequence; one probe labeled with FAM dye detects the Allele 2 sequence.
  - TaqMan® Genotyping Master Mix or TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG).
- PCR amplification and an endpoint read to obtain results.

For More Information

- For information on the latest available products and specific product uses, go to:

  http://www.allsnps.com/

  and/or

  http://www.appliedbiosystems.com/

  a. In the Home page, under TaqMan® Products, select **TaqMan® SNP Genotyping Assays**.

  b. In the SNP Genotyping Assays page, under Pre-Designed/Validated Assays, select **TaqMan® Drug Metabolism Genotyping Assays**.
- For information on preparing PCR reactions using the TaqMan SNP Genotyping Assays, refer to the *TaqMan® Drug Metabolism Genotyping Assays Protocol*.

Pre-Developed TaqMan® Assay Reagents for Allelic Discrimination

Product Description

Pre-Developed TaqMan® Assay Reagents for Allelic Discrimination (TaqMan® PDARs for AD) are Inventoried assays optimized for the discrimination of specific alleles.

The assays:

- Use TaqMan reagents to amplify and detect specific polymorphisms in purified genomic DNA samples.
- Are designed and optimized to work with an Applied Biosystems TaqMan® master mix, using universal thermal cycling conditions.

Product Requirements

TaqMan PDARs for AD require three components:

- 2 to 20 ng of purified genomic DNA sample.
- 10× Allelic Discrimination Assay Mix (specific for each polymorphism). Each assay consists of sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest and two TaqMan MGB probes: One probe labeled with VIC dye detects the Allele 1 sequence; one probe labeled with FAM dye detects the Allele 2 sequence.
- TaqMan® Genotyping Master Mix or TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG).

Note: Allele 1 and 2 control DNA is included with each assay to allow each homozygote signal to be generated on each run.

For More Information

- For information on the latest available products and specific product uses, go to:

  http://www.allsnps.com/

  and/or

  http://www.appliedbiosystems.com/

  a. In the Home page, under TaqMan® Products, select **TaqMan® SNP Genotyping Assays**.

  b. In the SNP Genotyping Assays page, under Pre-Designed/Validated Assays, select **TaqMan® Pre-Developed Assay Reagents for Allelic Discrimination (TaqMan® PDARs for AD)**.

- For information on preparing PCR reactions using the TaqMan PDARs for Allelic Discrimination, refer to the *Pre-Developed TaqMan® Assay Reagents Allelic Discrimination Protocol*.

Custom TaqMan® SNP Genotyping Assays

Product Description

Custom TaqMan® SNP Genotyping Assays are TaqMan probe and primer sets that are designed, synthesized, and formulated by the Custom TaqMan® Genomic Assays service based on sequence information that you submit. Custom TaqMan SNP Genotyping Assays allow you to:

Action	Example
Perform genotyping studies with any possible single-nucleotide polymorphism (SNP) in any organism	AGTTCATCCATGGTCA --> AGTTCATACATGGTCA Annotated as: AGTTCAT[C/A]CATGGTCA
Detect insertions/deletions (in/dels) of up to six bases for genotyping studies	AGTTCATCCATGGTCA --> AGTTCATGGTCA Annotated as: AGTTCAT[CCAT/*]GGTCA
Detect multiple nucleotide polymorphisms (MNPs) of up to six bases for genotyping studies	AGTTCATCCGGTCA --> AGTTCATATGGTCA Annotated as: AGTTCAT[CC/AT]GGTCA

The assays:

- Use TaqMan reagents to amplify and detect specific polymorphisms in purified genomic DNA (gDNA).
- Are developed using proprietary assay-design software.
- Are designed and optimized to work with an Applied Biosystems TaqMan® master mix, using universal thermal cycling conditions.

Product Requirements

All Custom TaqMan SNP Genotyping Assays require:

- A submission file that includes your target SNP sequence. You create the submission file using free File Builder software, then submit the file to the Custom TaqMan® Genomic Assays service.
- Three components:
  - 1 to 20 ng of purified gDNA sample per well.
  - 40× SNP Genotyping Assay or 80× SNP Genotyping Assay (specific for each polymorphism). Each assay consists of sequence-specific forward and reverse primers to amplify the SNP of interest and two TaqMan MGB probes: One probe labeled with VIC dye detects the Allele 1 sequence; one probe labeled with FAM dye detects the Allele 2 sequence.
  - TaqMan® Genotyping Master Mix or TaqMan® Universal PCR Master Mix (with or without AmpErase® UNG).
- PCR amplification and an endpoint read to obtain results.

For More Information

- For information on the latest available products and specific product uses, go to:

  http://www.allsnps.com/

  and/or

  http://www.appliedbiosystems.com/

  a. In the Home page, under TaqMan® Products, select **TaqMan® SNP Genotyping Assays**.

  b. In the SNP Genotyping Assays page, under Custom Assays, select **Custom TaqMan® SNP Genotyping Assays**.
- For information on ordering Custom TaqMan SNP Genotyping Assays, refer to the *Custom TaqMan® Genomic Assays Protocol: Submission Guidelines*.
- For information on preparing PCR reactions using the Custom TaqMan SNP Genotyping Assays, refer to the *Custom TaqMan® SNP Genotyping Assays Protocol*.

Select the Master Mix

Available Master Mixes

For genotyping experiments, Applied Biosystems Pre-Designed/Validated assays are designed to work with the master mixes listed below.

- TaqMan SNP Genotyping Assays, TaqMan Drug Metabolism Genotyping Assays, and Custom TaqMan SNP Genotyping Assays can be used with:

Master Mix	Part Number
TaqMan® Genotyping Master Mix, 1-Pack (1 × 10 mL), 400 reactions	4371355
TaqMan® Genotyping Master Mix, 1 Bulk Pack (1 × 50 mL), 2000 reactions	4371357
TaqMan® 2× Universal PCR Master Mix, 200 reactions	4304437
TaqMan® 2× Universal PCR Master Mix, 2000 reactions	4326708
10-Pack, TaqMan® 2× Universal PCR Master Mix	4305719
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 200 reactions	4324018
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 2000 reactions	4326614
10-Pack, TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG	4324020

- TaqMan PDARs for AD contain TaqMan® 2× Universal PCR Master Mix (with AmpErase® UNG).

Note: Genotyping experiments are not supported for Fast or SYBR® Green master mixes and protocols.

For More Information

For information on using the TaqMan master mixes, refer to the:

- *TaqMan® Genotyping Master Mix Protocol*
- *TaqMan® Universal PCR Master Mix Protocol*

Design the Experiment

For information on designing genotyping experiments on the Real-Time PCR Systems, refer to:

System	Document
7300 System	*Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Allelic Discrimination Getting Started Guide*
7500/7500 Fast System	*Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Genotyping Experiments*
7900HT Fast System	*Applied Biosystems 7900HT Fast Real-Time PCR System Allelic Discrimination Getting Started Guide*
StepOne™ and StepOnePlus™ Systems	*Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Genotyping Experiments*

Note: For document part numbers, see "Related Documentation" on page viii. For information on obtaining these documents, see "How to Obtain Support" on page xii.

Presence/Absence Experiments 5

This chapter covers:

Section 5.1 About Presence/Absence Experiments

This section covers:

Overview

What Is a Presence/ Absence Experiment?

A presence/absence experiment is an endpoint experiment that indicates the presence or absence of a specific nucleic acid sequence (target) in a sample. The actual quantity of target is not determined.

Presence/absence experiments are commonly used to detect the presence or absence of a pathogen, such as a viral or bacterial pathogen. For example, a presence/absence experiment might be used to determine if *Salmonella* bacteria are present in hamburger meat. The results show if *Salmonella* bacteria are present or are not present; the quantity of bacteria is not determined.

Components

PCR reactions for presence/absence experiments include the following components:

- **Sample**– The sample in which the presence of a target is unknown.
- **Replicates** – Identical reactions containing identical components and volumes.
- **Internal positive control (IPC)** – A short synthetic DNA template that is added to PCR reactions. You can use the IPC to distinguish between true negative results and reactions affected by PCR inhibitors, incorrect assay setup, or a reagent or instrument failure.

  Note: Presence/absence experiments can be performed without an IPC; however, the IPC ensures that a failed PCR is not mistaken for a negative test result.

- **Negative Controls** – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

In the Real-Time PCR System software, you can set up the PCR reactions for presence/absence experiments three different ways:

Setup	Well Types
IPC setup	• **Unknown-IPC wells** – Wells that contain sample template and IPC template; the presence of the target is not known. • **Negative control-IPC wells** – Wells that contain IPC template and water or buffer instead of sample template in the PCR reaction. Only the IPC template should amplify in negative control-IPC wells because the reaction contains no sample template. Also called *IPC+*. • **Negative control-blocked IPC wells** – Wells that contain IPC blocking agent instead of sample template in the PCR reaction. No amplification should occur in negative control-blocked IPC wells because the reaction contains no sample template and amplification of the IPC is blocked. Also called *no amplification control (NAC)*.
No IPC, singleplex setup	• **Unknown wells** – Wells that contain sample template; the presence of the target is not known. • **Negative controls** –Wells that contain water or buffer instead of sample template.

Setup	Well Types
No IPC, multiplex setup	• **Unknown-Unknown wells** – Wells that contain sample template; the presence of the target is not known. • **Negative control-Negative control wells** – Wells that contain water or buffer instead of sample template.

Endpoint Detection and Post-PCR Plate Read

Presence/absence experiments are endpoint experiments in which fluorescence data are collected after the PCR is complete.

To aid in troubleshooting presence/absence experiments, you can use an Applied Biosystems Real-Time PCR System to perform real-time PCR. If you use a Real-Time PCR System for PCR amplification, perform the pre-PCR read and post-PCR read runs separately.

How Presence/Absence Experiments Work

During the PCR, the fluorogenic probes anneal specifically to the complementary target between the forward and reverse primer sites on the template DNA. During extension, a DNA polymerase cleaves the hybridized probes in each sample containing the target. The cleavage of each matched probe separates the reporter dye from the quencher dye, resulting in increased fluorescence by the reporter.

After PCR cycling, the instrument reads the fluorescence generated during the PCR amplification. The fluorescence signals are used to determine the presence or absence of the target in each sample. Reporter signals are normalized to the emission of a passive reference, as follows:

$$R_{n\,(TT)} = \frac{\text{Emission Intensity of Target Template Sequence}}{\text{Emission Intensity of Passive Reference}}$$

$$R_{n\,(IPC)} = \frac{\text{Emission Intensity of Internal Positive Control}}{\text{Emission Intensity of Passive Reference}}$$

Incorporating an IPC

An IPC is a second TaqMan® probe and primer set added to the reaction plate to detect a low-copy, constitutive nucleic acid. The IPC and the target are amplified simultaneously in the same reaction well. If a well does not exhibit amplification, the Real-Time PCR System software uses the positive signal from the IPC to confirm that the well failed to amplify because of a lack of target template, rather than because of a pipetting error or inhibition.

Note: Presence/absence experiments can be performed without an IPC; however, the IPC ensures that a failed PCR is not mistaken for a negative test result.

Workflow

Before performing presence/absence experiments on an Applied Biosystems Real-Time PCR System, you need to:

1. Select the assay type (page 5-7).
2. Review the design guidelines for the assay type you selected (Section 5.2 on page 5-11).

Select the Assay Type

You can select the following assay types for presence/absence experiments:

- Inventoried/Made to Order (below)
- Custom (page 5-8)
- User-Designed (page 5-9)

Inventoried/Made to Order Assays

The table below lists the products available for Applied Biosystems Inventoried/Made to Order assay type. The assays are specific to the target of interest. The master mixes contain the remaining components needed for the PCR reaction.

Note: Presence/absence experiments are not supported for Fast or SYBR® Green master mixes and protocols.

Note: For guidelines on designing your experiments with Inventoried/Made to Order assays, see page 5-12.

Product		Attributes
Assay	TaqMan® Gene Expression Assays	• Predesigned, gene-specific primer/probe sets for human, mouse, rat, *Arabidopsis*, *Drosophila*, *C. elegans*, *C. familiares* (dog), and *M. mulatta* (Rhesus) genes. • Probe is a FAM™ dye-labeled TaqMan® MGB probe. • Provided in a convenient, single 20× tube. • Available as Inventoried or Made to Order assays.
	TaqMan® Endogenous Control Assays **Note:** FAM™ dye-labeled TaqMan® Endogenous Control Assays are available as TaqMan® Gene Expression Assays.	• Optimized, preformulated, ready-to-use endogenous control assays. • Cost-effective, gene-specific primer/probe sets for human, mouse, and rat species, and 18S rRNA. (18S rRNA detects any eukaryotic species.) • Choice of: – FAM™ dye-labeled TaqMan® MGB probes – VIC® dye-labeled TaqMan® MGB probes – VIC® dye-labeled TaqMan® TAMRA™ probes **Note:** The VIC® dye-labeled probes are primer limited.
Master mix	TaqMan® Gene Expression Master Mix	Designed for precise quantitation by real-time PCR for routine and challenging experiments: • Sensitive detection down to 1 copy of target. • Multiplex PCR for co-amplifying two targets in a single reaction. • Specificity for differentiation between gene family members. • Validated with TaqMan® Gene Expression Assays. • Uses one reagent for all assays to simplify assay preparation.
	TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG)	• Provides optimal performance for TaqMan® assays that use cDNA or DNA as a template. • Contains components that ensure excellent assay performance. • Uses one reagent for all assays to simplify assay preparation.

Custom Assays

The table below lists the products available for the Applied Biosystems Custom assay type. The assays are specific to the target of interest. The master mixes contain the remaining components needed for the PCR reaction.

Note: Presence/absence experiments are not supported for Fast or SYBR® Green master mixes and protocols.

Note: For guidelines on designing your experiments with Custom assays, see page 5-12.

Product		Attributes
Assay	Custom TaqMan® Gene Expression Assays	• Any species or organism. • Target of your choice. • Probe is a FAM™ dye-labeled TaqMan® MGB probe. • Provided in a convenient, single 20× tube.
Master mix	TaqMan® Gene Expression Master Mix	Designed for precise quantitation by real-time PCR for routine and challenging experiments: • Sensitive detection down to 1 copy of target. • Multiplex PCR for co-amplifying two targets in a single reaction. • Specificity for differentiation between gene family members. • Validated with TaqMan® Gene Expression Assays. • Uses one reagent for all assays to simplify assay preparation.
	TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG)	• Provides optimal performance for TaqMan® assays that use cDNA or DNA as a template. • Contains components that ensure excellent assay performance. • Uses one reagent for all assays to simplify assay preparation.

User-Designed Assays

The table below lists the products available from Applied Biosystems for designing your own assays. The primers/probes are specific to the target of interest. The master mixes contain the remaining components needed for the PCR reaction.

Note: Presence/absence experiments are not supported for Fast or SYBR® Green master mixes and protocols.

Note: For guidelines on designing your experiments with user-designed assays, see page 5-18.

Product		Attributes
Assay design	Custom TaqMan® MGB probes	• Any species or organism. • Choice of dye labels, quenchers, and synthesis scales. • For use with Applied Biosystems Assay Design Guidelines.
	Custom TaqMan® TAMRA™ probes	
	Sequence Detection Primers	
	Primer Express® Software	Software that designs primers and probes for real-time PCR using the Applied Biosystems Assay Design Guidelines.
Master mix	TaqMan® Gene Expression Master Mix	Designed for precise quantitation by real-time PCR for routine and challenging experiments: • Sensitive detection down to 1 copy of target. • Multiplex PCR for co-amplifying two targets in a single reaction. • Specificity for differentiation between gene family members. • Validated with TaqMan® Gene Expression Assays. • Uses one reagent for all assays to simplify assay preparation.
	TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG)	• Provides optimal performance for TaqMan® assays that use cDNA or DNA as a template. • Contains components that ensure excellent assay performance. • Uses one reagent for all assays to simplify assay preparation.

Section 5.2 Design Guidelines

This section covers:

Inventoried/Made to Order and Custom Assays

Workflow

The recommended workflow for performing presence/absence experiments with Applied Biosystems Inventoried/Made to Order and Custom assay types is the same. Applied Biosystems recommends that you:

1. Select the assay:
   - TaqMan® Gene Expression Assays (below).
   - Custom TaqMan® Gene Expression Assays (page 5-14).
2. Use the TaqMan® Exogenous Internal Positive Control Reagents (page 5-15).

   Note: Presence/absence experiments can be performed without an IPC; however, the IPC ensures that a failed PCR is not mistaken for a negative test result.
3. Select the master mix (page 5-16).
4. Design the experiment (page 5-17).

TaqMan® Gene Expression Assays

Product Description

TaqMan® Gene Expression Assays are a comprehensive collection of Inventoried and Made to Order probe and primer sets that you can use to perform presence/absence experiments on human, mouse, rat, *Arabidopsis*, *Drosophila*, *C. elegans*, *C. familiares* (dog), and *M. mulatta* (Rhesus) genes.

The assays:

- Use TaqMan® reagents to amplify and detect the target in cDNA samples.
- Are designed using an automated design and quality-controlled system. Inventoried assays are manufactured and placed in inventory; Made to Order assays are predesigned and manufactured when ordered.
- Are designed and optimized to work with an Applied Biosystems TaqMan® master mix, using universal thermal cycling conditions.
- When possible, amplify target cDNA without amplifying genomic DNA (*m* suffix in assay ID) by designing probes that cross exon-exon junctions.

Product Requirements

All TaqMan Gene Expression Assays require:

- Three components:
  - 1 to 100 ng of cDNA sample (converted from RNA) per well, with all wells in a study having the same amount of cDNA.
  - 20× Gene Expression Assay Mix (specific for each target). Each assay mix consists of two unlabeled PCR primers and a FAM™ dye-labeled TaqMan® MGB (minor groove binder) probe in a preformulated 20× mix. 1× final concentrations are 250 nM for the probe and 900 nM for each primer.
  - TaqMan® Gene Expression Master Mix or TaqMan Universal PCR Master Mix (with or without AmpErase UNG).

Available Assays

TaqMan Gene Expression Assays are available for human, mouse, rat, *Arabidopsis*, *Drosophila*, *C. elegans*, *C. familiares* (dog), and *M. mulatta* (Rhesus) genes. The part numbers are:

- 4331182 for Inventoried assays
- 4351372 for Made to Order assays

The prefix of the assay name indicates the species for which the assay was designed: *Hs* for *Homo sapiens* (human), *Mm* for *Mus musculus* (mouse), *Rn* for *Rattus norvegicus* (rat), *At* for *Arabidopsis thaliana*, *Dm* for *Drosophila melanogaster*, Ce for *C. elegans*, Cf for *C. familiares* (dog), and Rh for *M. mulatta* (Rhesus).

The suffix of the assay name indicates the assay placement, as described in the table below.

Suffix	Description
_m	The assay's probe spans an exon junction; the assay does not detect genomic DNA.
_s	The assay's primers and probes are designed within a single exon; the assay detects genomic DNA.
_g	The assay may detect genomic DNA; the assay's primers and probes may be within a single exon.
_mH	The assay was designed to a transcript belonging to a gene family with high sequence homology. The assay provides between 10 C_T and 15 C_T difference between the target gene and the gene with the closest sequence homology. Therefore, the assay detects the target transcript with 1000- to 30,000-fold greater discrimination (sensitivity) than the closest homologous transcript, if both transcripts are present at the same copy number in a sample.
_sH	
_gH	
_u	The assay's amplicon spans an exon junction, and the probe sits completely in one of the spanned exons.
_ft	The assay is designed to detect fusion transcripts that result from chromosomal translocation. The probe and one primer are on one side of the fusion transcript breakpoint; the second primer is on the other side of the fusion transcript breakpoint. The assay does not detect genomic DNA.
_at	The assay is designed to detect a specific synthetic RNA transcript with a unique sequence that lacks homology to current annotated biological sequences.

TaqMan® Endogenous Control Assays

TaqMan® Endogenous Control Assays are available as:

- Inventoried/Made to Order TaqMan Gene Expression Assays (PN 4331182 and PN 4351372) – Each assay contains a FAM™ dye-labeled TaqMan® MGB probe in a single, preformulated 20× tube.
- Individual control assays for human, mouse, and rat species, and 18S rRNA; 18S rRNA detects any eukaryotic species (various part numbers) – Each assay contains either a FAM™ dye-labeled TaqMan® MGB probe, a VIC® dye-labeled TaqMan® MGB probe, or a VIC® dye-labeled TaqMan® TAMRA™ probe. TaqMan Endogenous Controls with VIC dye labels are primer limited.

For More Information

- For information on the latest available products and specific product uses, go to **http://www.appliedbiosystems.com/**:
  a. In the Home page, under TaqMan® Products, select **TaqMan® Gene Expression Assays**.
  b. In the Gene Expression Assays & Arrays page:
     - Under Individual Assays, select **TaqMan® Gene Expression Assays**. This option links to all TaqMan Gene Expression Assays.

       or
     - Under Individual Control Assays, select **TaqMan® Endogenous Controls**. This option links to the individual TaqMan Endogenous Control Assays (that contain FAM dye-labeled TaqMan MGB probes, VIC dye-labeled TaqMan MGB probes, or VIC dye-labeled TaqMan TAMRA probes).
- For information on Custom TaqMan Endogenous Control Assays, refer to the *Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies Application Note*.
- For information on preparing PCR reactions using the TaqMan Gene Expression Assays, refer to the *TaqMan® Gene Expression Assays Protocol*.

Custom TaqMan® Gene Expression Assays

Product Description

Custom TaqMan® Gene Expression Assays are TaqMan probe and primer sets that are designed, synthesized, and formulated by the Custom TaqMan® Genomic Assays service based on sequence information that you submit. Custom TaqMan Gene Expression Assays allow you to perform presence/absence experiments on any gene or splice variant in any organism.

The assays:

- Use TaqMan® reagents to amplify and detect the target in cDNA samples.
- Are developed using proprietary assay-design software.
- Are designed and optimized to work with an Applied Biosystems TaqMan® master mix, using universal thermal cycling conditions.

Product Requirements

All Custom TaqMan Gene Expression Assays require:

- A submission file that includes your target sequence. You create the submission file using free File Builder software, then submit the file to the Custom TaqMan® Genomic Assays service.
- Three components:
  – 1 to 100 ng of cDNA sample (converted from RNA) per well, with all wells in a study having the same amount of cDNA.
  – 20× Gene Expression Assay or 60× Gene Expression Assay (specific for each target). Each assay consists of two target-specific primers and a FAM™ dye-labeled TaqMan MGB probe in a preformulated 20× or 60× mix. 1× final concentrations are 250 nM for the probe and 900 nM for each primer.
  – TaqMan® Gene Expression Master Mix or TaqMan Universal PCR Master Mix (with or without AmpErase UNG).

For More Information

- For information on the latest available products and specific product uses, go to **http://www.appliedbiosystems.com/**:
  a. In the Home page, under TaqMan® Products, select **TaqMan® Gene Expression Assays**.
  b. In the Gene Expression Assays & Arrays page, under Individual Assays, select **Custom TaqMan® Gene Expression Assays**.
- For information on ordering Custom TaqMan Gene Expression Assays, refer to the *Custom TaqMan® Genomic Assays Protocol: Submission Guidelines*.
- For information on preparing PCR reactions using the Custom TaqMan Gene Expression Assays, refer to the *Custom TaqMan® Gene Expression Assays Protocol*.

TaqMan® Exogenous Internal Positive Control Reagents

Product Description

The Applied Biosystems TaqMan® Exogenous Internal Positive Control Reagents contain:

- An internal positive control (IPC) with predesigned primers and probe
- IPC DNA template
- IPC blocking control

The reagents are designed to:

- Distinguish types of negative results:
  – A negative call for the target and positive call for the IPC indicates that no target is present.
  – A negative call for the target and negative call for the IPC suggests PCR inhibition.
- Avoid amplification of endogenous controls.
- Permit co-amplification of the IPC and the target without compromising amplification of the target.
- Detect the IPC using a VIC® dye-labeled probe.
- Detect the target using a FAM™ dye-labeled probe.
- Work with the TaqMan® 2× Universal PCR Master Mix (with or without AmpErase® UNG), using universal thermal cycling conditions.

Product Requirements

The TaqMan Exogenous Internal Positive Control Reagents kits require the following components:

- DNA sample
- TaqMan assay for your target of interest
- TaqMan 2× Universal PCR Master Mix (with or without AmpErase UNG)

Available Kits

The following TaqMan Exogenous Internal Positive Control Reagents kits are available from Applied Biosystems:

Kits	Part Number
TaqMan® Exogenous Internal Positive Control Reagents with TaqMan® 2× Universal PCR Master Mix (with VIC® dye)	4308320
TaqMan® Exogenous Internal Positive Control Reagents **Note:** If you use this kit, you need to purchase one of the following TaqMan® reagents separately: • TaqMan® 2× Universal PCR Master Mix (PN 4304437) • TaqMan® PCR Core Reagents Kit (PN N808-0228)	4308323

For More Information

For information on preparing PCR reactions using the TaqMan Exogenous Internal Positive Control Reagents, refer to the *TaqMan® Exogenous Internal Positive Control Reagents Protocol*.

Select the Master Mix

Available Master Mixes

For presence/absence experiments, Applied Biosystems Inventoried/Made to Order and Custom assay types are designed to work with the following master mixes:

Master Mix	Part Number
TaqMan® Gene Expression Master Mix, 1-Pack (1 × 5 mL), 200 reactions	4369016
TaqMan® Gene Expression Master Mix, 10-Pack (10 × 5 mL), 2000 reactions	4369542
TaqMan® 2× Universal PCR Master Mix, 200 reactions	4304437
TaqMan® 2× Universal PCR Master Mix, 2000 reactions	4326708
10-Pack, TaqMan® 2× Universal PCR Master Mix	4305719
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 200 reactions	4324018
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 2000 reactions	4326614
10-Pack, TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG	4324020
TaqMan® PCR Core Reagents Kit	N808-0228

Note: If you purchase the TaqMan® Exogenous Internal Positive Control Reagents with TaqMan® 2× Universal PCR Master Mix kit (PN 4308320), you do not need to purchase the master mix separately.

Note: Presence/absence experiments are not supported for Fast or SYBR® Green master mixes and protocols.

For More Information

For information on using the TaqMan reagents, refer to the:

- *TaqMan® Gene Expression Master Mix Protocol*
- *TaqMan® Universal PCR Master Mix Protocol*

Design the Experiment

For information on designing presence/absence experiments on the Real-Time PCR Systems, refer to:

System	Document
7300 System	*Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Plus/Minus Getting Started Guide*
7500/7500 Fast System	*Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Presence/Absence Experiments*
7900HT Fast System	*Applied Biosystems 7900HT Fast Real-Time PCR System Plus-Minus Getting Started Guide*
StepOne™ and StepOnePlus™ Systems	*Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Presence/Absence Experiments*

Note: For document part numbers, see "Related Documentation" on page viii. For information on obtaining these documents, see "How to Obtain Support" on page xii.

User-Designed Assays

Workflow

If you are designing your own assays (primers and probes) for presence/absence experiments, Applied Biosystems recommends that you follow the workflow for the Applied Biosystems Assay Design Guidelines:

1. Design primers and probes using Primer Express® Software (page 3-23).
2. Select the appropriate reagents (page 3-26).

   IMPORTANT! Presence/absence experiments are not supported for Fast or SYBR® Green master mixes and protocols.
3. Use the recommended thermal cycling conditions (page 3-30).
4. Begin with default primer and probe concentrations. If needed, optimize the primer concentrations (page 3-33) and probe concentrations (page 3-37).

IMPORTANT! These steps provide a rapid and reliable system for assay design and optimization only when used in their entirety. Adopt the system as a whole to achieve the highest level of success. For a more detailed description of Applied Biosystems Assay Design Guidelines, see Appendix C.

For More Information

For information on:

- Using the TaqMan reagents, refer to:
  - *TaqMan® Gene Expression Master Mix Protocol*
  - *TaqMan® Universal PCR Master Mix Protocol*
- Performing presence/absence experiments on the Real-Time PCR Systems, refer to:

System	Document
7300 System	*Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Plus/Minus Getting Started Guide*
7500/7500 Fast System	*Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Presence/Absence Experiments*
7900HT Fast System	*Applied Biosystems 7900HT Fast Real-Time PCR System Plus-Minus Getting Started Guide*
StepOne™ and StepOnePlus™ Systems	*Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Presence/Absence Experiments*

Note: For document part numbers, see "Related Documentation" on page viii. For information on obtaining these documents, see "How to Obtain Support" on page xii.

Formulas A

This appendix covers:

Standard Deviation Calculation Using the Standard Curve Method

Example Comparing Samples with a Reference Sample

The normalized amount of target (c-myc$_N$ in the table below) is a unitless number that can be used to compare the relative amount of target in different samples. One way to make this comparison is to designate one of the samples as a reference sample. In the table below, brain is designated as the reference sample; brain is arbitrarily chosen because it has the lowest expression level of the target.

Relative Standard Curve Results

Each c-myc$_N$ value in the table below is divided by the brain c-myc$_N$ value to give the values in the final column. These results indicate that the kidney sample has 5.5× as much c-myc mRNA as the brain sample, the liver sample has 34.2× as much, and the lung sample has 15.7× as much.

To determine relative values:

1. Average the c-myc and GAPDH values from the table below.
2. Divide the c-myc average by the GAPDH average.
3. Designate the reference sample.
4. Divide the averaged sample value by the averaged reference sample value.

Tissue	c-myc ng Total Raji RNA	GAPDH ng Total Raji RNA	c-myc$_N$ Norm. to GAPDH‡	c-myc$_N$ Rel. to Brain§
Brain	0.033	0.51		
	0.043	0.56		
	0.036	0.59		
	0.043	0.53		
	0.039	0.51		
	0.040	0.52		
Average	0.039±0.004	0.54±0.034	0.07±0.008	1.0±0.12
Kidney	0.40	0.96		
	0.41	1.06		
	0.41	1.05		
	0.39	1.07		
	0.42	1.06		
	0.43	0.96		
Average	0.41±0.016	1.02±0.052	0.40±0.025	5.5±0.35

Tissue	c-myc ng Total Raji RNA	GAPDH ng Total Raji RNA	$c\text{-}myc_N$ Norm. to GAPDH‡	$c\text{-}myc_N$ Rel. to Brain§
Liver	0.67	0.29		
	0.66	0.28		
	0.70	0.28		
	0.76	0.29		
	0.70	0.26		
	0.68	0.27		
Average	0.70±0.036	0.28±0.013	2.49±0.173	34.2±2.37
Lung	0.97	0.82		
	0.92	0.88		
	0.86	0.78		
	0.89	0.77		
	0.94	0.79		
	0.97	0.80		
Average	0.93±0.044	0.81±0.041	1.15±0.079	15.7±1.09

‡ The $c\text{-}myc_N$ value is determined by dividing the average c-myc value by the average GAPDH value. The standard deviation of the quotient is calculated from the standard deviations of the c-myc and GAPDH values. See “Formula” on page A-4.

§ The calculation of $c\text{-}myc_N$ relative to brain involves division by the reference sample value. This is division by an arbitrary constant, so the cv (coefficient of variance) of this result is the same as the cv for $c\text{-}myc_N$.

Formula

The c-myc$_N$ value is determined by dividing the average c-myc value by the average GAPDH value. The standard deviation of the quotient is calculated from the standard deviations of the c-myc and GAPDH values using the following formula:

$$cv = \sqrt{cv_1^{\,2} + cv_2^{\,2}}$$

where:

$$cv = \frac{s}{\bar{X}} = \frac{stddev}{meanvalue}$$

Using the brain sample from table on page A-2 as an example:

$$cv_1 = \frac{0.004}{0.039}$$

and

$$cv_2 = \frac{0.034}{0.54}$$

$$cv = \sqrt{\left(\frac{0.004}{0.039}\right)^2 + \left(\frac{0.034}{0.54}\right)^2} = 0.12$$

since

$$cv = \frac{s}{\bar{X}}$$

$$s = (cv)(\bar{X})$$

$$s = (0.12)(0.07)$$

$$s = 0.008$$

Standard Deviation Calculation Using the Comparative Method

Example The C_T data used to determine the amounts of c-myc and GAPDH mRNA shown in the table on page A-2 are used to illustrate the $\Delta\Delta C_T$ calculation. The table below shows the average C_T results for the human brain, kidney, liver, and lung samples and how these C_Ts are manipulated to determine ΔC_T, $\Delta\Delta C_T$, and the relative amount of c-myc mRNA. The results are comparable to the relative c-myc levels determined using the standard curve method.

Tissue	c-myc Average C_T	GAPDH Average C_T	ΔC_T c-myc–GAPDH‡	$\Delta\Delta C_T$ ΔC_T–$\Delta C_{T,\ Brain}$§	c-myc$_N$ Rel. to Brain#
Brain	30.49±0.15	23.63±0.09	6.86±0.17	0.00±0.17	1.0 (0.9 to 1.1)
Kidney	27.03±0.06	22.66±0.08	4.37±0.10	–2.50±0.10	5.6 (5.3 to 6.0)
Liver	26.25±0.07	24.60±0.07	1.65±0.10	–5.21±0.10	37.0 (34.5 to 39.7)
Lung	25.83±0.07	23.01±0.07	2.81±0.10	–4.05±0.10	16.5 (15.4 to 17.7)

‡ The ΔC_T value is determined by subtracting the average GAPDH C_T value from the average c-myc C_T value. The standard deviation of the difference is calculated from the standard deviations of the c-myc and GAPDH values. See “Formula” on page A-6.

§ The calculation of $\Delta\Delta C_T$ involves subtraction by the ΔC_T reference sample value. This is subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value.

The range for c-myc$_N$ relative to brain is determined by evaluating the expression: $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T$ + s and $\Delta\Delta C_T$ – s, where s = the standard deviation of the $\Delta\Delta C_T$ value.

Formula

The ΔC_T value is determined by subtracting the average GAPDH C_T value from the average c-myc C_T value. The standard deviation of the difference is calculated from the standard deviations of the c-myc and GAPDH values using the following formula:

$$s = \sqrt{s_1^2 + s_2^2}$$

where:

s = std dev

Using the brain sample from the table on page A-5 as an example:

$$s_1 = 0.15$$

and

$$s_2 = 0.09$$

$$s = \sqrt{(0.15)^2 + (0.09)^2} = 0.17$$

Formula for Comparative C_T ($\Delta\Delta C_T$) Experiments

Formula

The quantity of target, normalized to an endogenous control and relative to a reference sample, is calculated by:

$$2^{-\Delta\Delta C_T}$$

Derivation of the Formula

The equation that describes the exponential amplification of PCR is:

$$X_n = X_o \times (1 + E_X)^n$$

where:

- x_n = number of target molecules at cycle n
- x_o = initial number of target molecules
- E_x = efficiency of target amplification
- n = number of cycles
- X_o =initial number of target molecules

The threshold cycle (C_T) indicates the fractional cycle number at which the quantity of amplified target reaches a specified threshold. Thus,

$$X_T = X_o \times (1 + E_X)^{C_{T,X}} = K_X$$

where:

- x_T = threshold number of target molecules
- $C_{T, X}$ = threshold cycle for target amplification
- K_X = constant

A similar equation for the endogenous control reaction is:

$$R_T = R_o \times (1 + E_R)^{C_{T,R}} = K_R$$

where:

- R_T = threshold number of reference molecules
- R_o = initial number of reference molecules
- E_R = efficiency of reference amplification
- $C_{T, R}$ = threshold cycle for reference amplification
- K_R = constant

Dividing X_T by R_T yields the following expression:

$$\frac{X_T}{R_T} = \frac{X_O \times (1 + E_X)^{C_{T,X}}}{R_O \times (1 + E_R)^{C_{T,R}}} = \frac{K_X}{K_R} = K$$

The exact values of X_T and R_T depend on a number of factors, including:

- Reporter dye used in the probe
- Sequence context effects on the fluorescence properties of the probe
- Efficiency of probe cleavage
- Purity of the probe
- Setting of the fluorescence threshold.

Therefore, the constant K does not have to be equal to 1.

Assuming that efficiencies of the target and the reference are the same:

$E_X = E_R = E$

$$\frac{X_O}{R_O} \times (1 + E)^{C_{T,X} - C_{T,R}} = K$$

or

$$X_N \times (1 + E)^{\Delta C_T} = K$$

where:

- $X_N = X_O/R_O$, the normalized quantity of target
- $\Delta C_T = C_{T,X} - C_{T,R}$, the difference in threshold cycles for target and reference

Rearranging gives the following expression:

$$X_N = K \times (1 + E)^{-\Delta C_T}$$

The final step is to divide the X_N for any sample (q) by the X_N for the reference sample (cb):

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K \times (1 + E)^{-\Delta C_{T,q}}}{K \times (1 + E)^{-\Delta C_{T,cb}}} = (1 + E)^{-\Delta\Delta C_T}$$

where:

- $\Delta\Delta C_T = \Delta C_{T,q} - \Delta C_{T,cb}$

For amplicons designed and optimized according to Applied Biosystems Assay Design Guidelines (amplicon size <150 bp), the efficiency is close to 1. Therefore, the quantity of target, normalized to an endogenous control and relative to a reference sample, is given by:

$2^{-\Delta\Delta C_T}$

Primer Limiting in Multiplex PCR

B

To generate an accurate multiplex assay, it is important that the amplification of one species does not dominate amplification of the other. Otherwise, the amplification of a highly abundant species can prevent the less-abundant species from amplifying efficiently. If the less-abundant species does not amplify efficiently, your experiment may produce inaccurate results. Or, in severe cases, detection of the less-abundant species may be inhibited completely. You can avoid this situation by limiting the concentrations of the primers used to amplify the more abundant species, thereby "turning off" the amplification soon after the C_T has been established.

Primer limitation results in the reaction components common to both assays not being exhausted, allowing the amplification of the less-abundant species to continue at high efficiency. If the more abundant species is not known, you should determine it before performing multiplex PCR by running both targets in separate tubes. Both amplifications should be primer limited if neither species is consistently more abundant.

Considering Relative Abundance of the Target and Reference

In applying the primer limitation to target and endogenous control amplifications, the relative abundance of the two species must be considered. For quantitation experiments, it is possible to use rRNA as an endogenous control. The concentration of rRNA in total RNA is always greater than the concentration of any target mRNA. Therefore, in multiplex reactions amplifying both target and rRNA, only the concentrations of the rRNA primers need to be limited.

Limiting Primer Matrix

To define primer-limiting concentrations, run a matrix of forward and reverse primer concentrations using the value of the minimum initial template. The goal is to identify primer concentrations that reduce the ΔRn value of the assay without affecting the C_T value. The table below illustrates a recommended matrix of forward and reverse primers varying in concentration from 20 to 100 nM.

Primer	Concentration (nM)				
Forward: Reverse:	100 100	100 80	100 60	100 40	100 20
Forward: Reverse:	80 100	80 80	80 60	80 40	80 20
Forward: Reverse:	60 100	60 80	60 60	60 40	60 20
Forward: Reverse:	40 100	40 80	40 60	40 40	40 20
Forward: Reverse:	20 100	20 80	20 60	20 40	20 20

Note: Although following all design criteria helps to identify primer-limiting concentrations, it may not be possible for all assays. If a limiting primer matrix experiment does not enable the identification of primer-limiting concentrations, you must redesign at least one primer or run the reactions in separate tubes.

Example

The results of a limiting primer matrix experiment are shown in Figure B-1 on page B-3:

- Figure B-1a shows that only when lowering the primer concentrations below approximately 50 nM is the C_T value significantly affected. The plateau area shows the region in which suitable primer-limiting concentrations can be found. In this area, the C_T (and therefore the corresponding quantitation value) is unchanged, whereas the ΔRn value and corresponding product yield are significantly reduced.
- Figure B-1b shows the corresponding relationship between primer concentrations and ΔRn. The figure demonstrates that lower product yields can be achieved by decreasing forward and reverse primer concentrations.

For this example, an appropriate selection of primer-limiting concentrations would be at least 50 nM of forward and reverse primer. Probe concentration should be kept at an optimal level even when an assay is primer limited to ensure that the fluorescence signal that is produced is large enough for accurate multicomponenting by the Real-Time PCR System software.

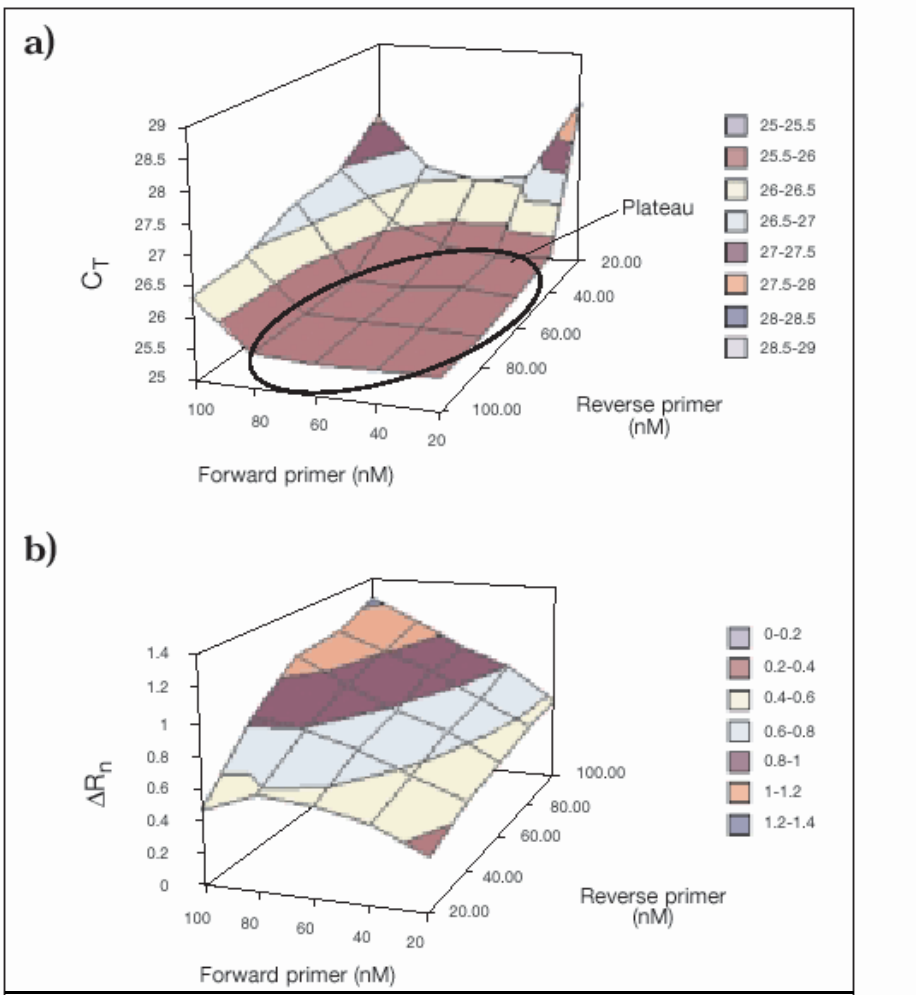


Figure B-1 Results from a limiting primer matrix experiment
(a) Shows how the C_T value is affected by variation in the forward and reverse primer concentrations. The plateau region indicated shows the area where the C_T value remains constant.
(b) Shows the reduction in the ΔRn values as the primer concentration decreases.

Assay Design Guidelines

C

About Assay Design Guidelines

If you are designing your own assays (primers and probes), Applied Biosystems recommends that you follow the Applied Biosystems Assay Design Guidelines. The Assay Design Guidelines specify that you:

1. **Design primers and probes using Primer Express® Software** – The Primer Express software uses a set of default parameters to automatically select primer and probe sets.
2. **Select the appropriate reagents** – There are several TaqMan® and SYBR® Green reagents available. The reagents you use depend on your assay type.
3. **Use the recommended thermal cycling conditions** – Use the thermal cycling conditions recommended for your sample (DNA/cDNA, RNA for 1-step PCR, and RNA for 2-step PCR).

   Note: Thermal cycling conditions for Fast reagents differ from thermal cycling conditions for standard reagents.
4. **Use default primer and probe concentrations or optimize primer and probe concentrations** – When you use Applied Biosystems Assay Design Guidelines, you can use default primer and probe concentrations for nonmultiplex optimized assays, or you can optimize primer and probe concentrations.

IMPORTANT! These steps provide a rapid and reliable system for assay design and optimization only when used in their entirety. Adopt the system as a whole to achieve the highest level of success.

Note: Applied Biosystems Assay Design Guidelines do not guarantee that all assays will provide the same level of performance and sensitivity. Even the most scrupulous design parameters cannot account for all the possible variations between two different assay systems.

Conclusions for Quantitation Experiments

In general, the following conclusions can be made when you use the Assay Design Guidelines for quantitation experiments:

- For most TaqMan assays, a concentration of 900-nM primers and 250-nM probe results in a highly reproducible and sensitive assay when using DNA or cDNA as a template.
- Due to the nonspecific nature of its detection, SYBR® Green I dye primer optimization should be bypassed only with caution. However, if all guidelines are followed, concentrations of 50-nM forward and reverse primers generally provide robust amplification with a good level of specificity when using DNA or cDNA as a template. Verify this assumption by checking for nonspecific product formation with either melt curve or gel analysis.
- Most TaqMan assays should enable detection and accurate quantitation to <50 copies of a target, with even greater sensitivity possible.

- SYBR Green assays are capable of similar performance; however, nonspecific product formation can potentially increase the minimum detection limit.

Conclusions for Genotyping Experiments

In general, the following conclusion can be made when you use the Assay Design Guidelines for genotyping experiments:

You can use 900-nM primers, a 200-nM probe, and 1 to 20 ng of genomic DNA to achieve reproducible and sensitive assay results.

Reagent Part Numbers D

This appendix covers:

Quantitation Experiments

Inventoried/Made to Order and Custom Assays

Assays

Product	Part Number
TaqMan® Gene Expression Assays	• Inventoried assays: 4331182 • Made to Order assays: 4351372 For information on the latest available products and specific product uses, go to: **http://www.appliedbiosystems.com/**
TaqMan® Endogenous Control Assays **Note:** FAM™ dye-labeled TaqMan® Endogenous Control Assays are available as TaqMan® Gene Expression Assays.	
Custom TaqMan® Gene Expression Assays	For information on the latest available products and specific product uses, go to: **http://www.appliedbiosystems.com/**

Master Mixes

Master Mix	Part Number
TaqMan® Gene Expression Master Mix, 1-Pack (1 × 5 mL), 200 reactions	4369016
TaqMan® Gene Expression Master Mix, 10-Pack (10 × 5 mL), 2000 reactions	4369542
TaqMan® Fast Universal PCR Master Mix (2X), No AmpErase® UNG, 250 reactions **IMPORTANT!** TaqMan® Fast Universal PCR Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.	4352042
TaqMan® 2X Universal PCR Master Mix, 200 reactions	4304437
TaqMan® 2X Universal PCR Master Mix, 2000 reactions	4326708
10-Pack, TaqMan® 2X Universal PCR Master Mix	4305719
TaqMan® 2X Universal PCR Master Mix, No AmpErase® UNG, 200 reactions	4324018
TaqMan® 2X Universal PCR Master Mix, No AmpErase® UNG, 2000 reactions	4326614
10-Pack, TaqMan® 2X Universal PCR Master Mix, No AmpErase® UNG	4324020

User-Designed Assays

Assay Design

Product	Part Number
Custom TaqMan® MGB probes	For information on the latest available products and specific product uses, go to: **http://www.appliedbiosystems.com/**
Custom TaqMan® TAMRA™ probes	
Sequence Detection Primers	

DNA or cDNA Quantitation

Reagent	Kit	Part Number
TaqMan® reagents	TaqMan® Gene Expression Master Mix, 1-Pack (1 × 5 mL), 200 reactions	4369016
	TaqMan® Gene Expression Master Mix, 10-Pack (10 × 5 mL), 2000 reactions	4369542
	TaqMan® Fast Universal PCR Master Mix (2X), No AmpErase® UNG, 250 reactions‡	4352042
	TaqMan® 2X Universal PCR Master Mix, 200 reactions	4304437
	TaqMan® 2X Universal PCR Master Mix, 2000 reactions	4326708
	10-Pack, TaqMan® 2X Universal PCR Master Mix	4305719
	TaqMan® 2X Universal PCR Master Mix, No AmpErase® UNG, 200 reactions	4324018
	TaqMan® 2X Universal PCR Master Mix, No AmpErase® UNG, 2000 reactions	4326614
	10-Pack, TaqMan® 2X Universal PCR Master Mix, No AmpErase® UNG	4324020
	TaqMan® PCR Core Reagents Kit, 200 reactions	N808-0228

Reagent	Kit	Part Number
SYBR® Green reagents	Fast SYBR® Green Master Mix (1 mL)§	4385610
	Fast SYBR® Green Master Mix (5 mL)	4385612
	Fast SYBR® Green Master Mix (10 × 5 mL)	4385618
	Fast SYBR® Green Master Mix (50 mL)	4385614
	Power SYBR® Green PCR Master Mix (1 mL), 40 reactions	4368577
	Power SYBR® Green PCR Master Mix (5-mL), 200 reactions	4367659
	Power SYBR® Green PCR Master Mix (10 × 5-mL), 2000 reactions	4368708
	Power SYBR® Green PCR Master Mix (50 mL), 2000 reactions	4367660
	SYBR® Green PCR Master Mix (1-mL), 40 reactions	4344463
	SYBR® Green PCR Master Mix (5-mL), 200 reactions	4309155
	SYBR® Green PCR Master Mix (50-mL), 2000 reactions	4334973
	SYBR® Green PCR Core Reagents, 200 reactions	4304886

‡TaqMan® Fast Universal PCR Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.

§Fast SYBR® Green Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.

RNA Quantitation Using 1-Step RT-PCR

Reagent	Kit	Part Number
TaqMan® reagents	TaqMan® One-Step RT-PCR Master Mix Reagents Kit	4309169
	TaqMan® EZ RT-PCR Core Reagents **IMPORTANT!** Use the TaqMan® EZ RT-PCR Core Reagents when a high-temperature RT step is required.	N808-0236
	TaqMan® Gold RT-PCR Reagents without Controls, 200 reactions	N808-0232
	TaqMan® Gold RT-PCR Reagents without Controls, 10-Pack, 2000 reactions	4304133
	TaqMan® Gold RT-PCR Reagents with Controls, 200 reactions	N808-0233
SYBR® Green reagents	*Power* SYBR® Green RT-PCR Reagents Kit	4368711
	SYBR® Green RT-PCR Reagents	4310179

RNA Quantitation Using 2-Step RT-PCR

Reagent	Step	Kit	Part Number
TaqMan® reagents	PCR step only	TaqMan® Gene Expression Master Mix, 1-Pack (1 × 5 mL), 200 reactions	4369016
		TaqMan® Gene Expression Master Mix, 10-Pack (10 × 5 mL), 2000 reactions	4369542
		TaqMan® 2X Universal PCR Master Mix	4304437
		TaqMan® Fast Universal PCR Master Mix (2X), No AmpErase® UNG‡	4352042
	RT step only	High-Capacity cDNA Reverse Transcription Kit	4374966
		TaqMan® Reverse Transcription Reagents	N808-0234
	Both RT and PCR steps	TaqMan® Gold RT-PCR Reagents without Controls, 200 reactions	N808-0232
		TaqMan® Gold RT-PCR Reagents without Controls, 10-Pack, 2000 reactions	4304133
		TaqMan® Gold RT-PCR Reagents with Controls, 200 reactions	N808-0233
SYBR® Green reagents	PCR step only	Fast SYBR® Green Master Mix (5 mL)§	4385612
		Power SYBR® Green PCR Master Mix	4367659
		SYBR® Green PCR Master Mix	4309155
	RT step only	High-Capacity cDNA Reverse Transcription Kit	4374966
		TaqMan® Reverse Transcription Reagents	N808-0234
	Both RT and PCR steps	*Power* SYBR® Green RT-PCR Reagents Kit	4368711
		SYBR® Green RT-PCR Reagents	4310179

‡TaqMan® Fast Universal PCR Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.

§Fast SYBR® Green Master Mix is designed for Real-Time PCR Systems that support Fast reagents and protocols.

Genotyping Experiments

Pre-Designed/Validated and Custom Assays

Assays

Product	Part Number
TaqMan® SNP Genotyping Assays	For information on the latest available products and specific product uses, go to: **http://www.appliedbiosystems.com/**
TaqMan® Drug Metabolism Genotyping Assays	
Pre-Developed TaqMan® Assay Reagents for Allelic Discrimination	
Custom TaqMan® SNP Genotyping Assays	

Master Mixes

- TaqMan SNP Genotyping Assays, TaqMan Drug Metabolism Genotyping Assays, and Custom TaqMan SNP Genotyping Assays can be used with:

Master Mix	Part Number
TaqMan® Genotyping Master Mix, 1-Pack (1 × 10 mL), 400 reactions	4371355
TaqMan® Genotyping Master Mix, 1 Bulk Pack (1 × 50 mL), 2000 reactions	4371357
TaqMan® 2× Universal PCR Master Mix, 200 reactions	4304437
TaqMan® 2× Universal PCR Master Mix, 2000 reactions	4326708
10-Pack, TaqMan® 2× Universal PCR Master Mix	4305719
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 200 reactions	4324018
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 2000 reactions	4326614
10-Pack, TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG	4324020

- TaqMan PDARs for AD contain TaqMan® 2× Universal PCR Master Mix (with AmpErase® UNG).

Note: Genotyping experiments are not supported for Fast or SYBR® Green master mixes and protocols.

Presence/Absence Experiments

Inventoried/Made to Order and Custom Assays

Assays

Product	Part Number
TaqMan® Gene Expression Assays	• Inventoried assays: 4331182 • Made to Order assays: 4351372 For information on the latest available products and specific product uses, go to: **http://www.appliedbiosystems.com/**
TaqMan® Endogenous Control Assays **Note:** FAM™ dye-labeled TaqMan® Endogenous Control Assays are available as TaqMan® Gene Expression Assays.	
Custom TaqMan® Gene Expression Assays	For information on the latest available products and specific product uses, go to: **http://www.appliedbiosystems.com/**

TaqMan Exogenous IPC Reagents

Kits	Part Number
TaqMan® Exogenous Internal Positive Control Reagents with TaqMan® 2X Universal PCR Master Mix (with VIC® dye)	4308320
TaqMan® Exogenous Internal Positive Control Reagents **Note:** If you use this kit, you need to purchase one of the following TaqMan® reagents separately: • TaqMan® 2X Universal PCR Master Mix (PN 4304437) • TaqMan® PCR Core Reagents Kit (PN N808-0228)	4308323

Master Mixes

Master Mix	Part Number
TaqMan® Gene Expression Master Mix, 1-Pack (1 × 5 mL), 200 reactions	4369016
TaqMan® Gene Expression Master Mix, 10-Pack (10 × 5 mL), 2000 reactions	4369542
TaqMan® 2× Universal PCR Master Mix, 200 reactions	4304437
TaqMan® 2× Universal PCR Master Mix, 2000 reactions	4326708
10-Pack, TaqMan® 2× Universal PCR Master Mix	4305719
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 200 reactions	4324018
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 2000 reactions	4326614
10-Pack, TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG	4324020
TaqMan® PCR Core Reagents Kit	N808-0228

Note: If you purchase the TaqMan® Exogenous Internal Positive Control Reagents with TaqMan® 2× Universal PCR Master Mix kit (PN 4308320), you do not need to purchase the master mix separately.

Note: Presence/absence experiments are not supported using Fast or SYBR® Green master mixes and protocols.

User-Designed Assays

Assay Design

Product	Part Number
Custom TaqMan® MGB probes	For information on the latest available products and specific product uses, go to: **http://www.appliedbiosystems.com/**
Custom TaqMan® TAMRA™ probes	
Sequence Detection Primers	

DNA or cDNA Quantitation

Kit	Part Number
TaqMan® Gene Expression Master Mix, 1-Pack (1 × 5 mL), 200 reactions	4369016
TaqMan® Gene Expression Master Mix, 10-Pack (10 × 5 mL), 2000 reactions	4369542
TaqMan® 2× Universal PCR Master Mix, 200 reactions	4304437
TaqMan® 2× Universal PCR Master Mix, 2000 reactions	4326708
10-Pack, TaqMan® 2× Universal PCR Master Mix	4305719
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 200 reactions	4324018
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG, 2000 reactions	4326614
10-Pack, TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG	4324020
TaqMan® PCR Core Reagents Kit, 200 reactions	N808-0228

Note: Presence/absence experiments are not supported using Fast or SYBR® Green master mixes and protocols.

RNA Quantitation Using 1-Step RT-PCR

Kit	Part Number
TaqMan® One-Step RT-PCR Master Mix Reagents Kit	4309169
TaqMan® EZ RT-PCR Core Reagents **IMPORTANT!** Use the TaqMan® EZ RT-PCR Core Reagents when a high-temperature RT step is required.	N808-0236
TaqMan® Gold RT-PCR Reagents without Controls, 200 reactions	N808-0232
TaqMan® Gold RT-PCR Reagents without Controls, 10-Pack, 2000 reactions	4304133
TaqMan® Gold RT-PCR Reagents with Controls, 200 reactions	N808-0233

Note: Presence/absence experiments are not supported using Fast or SYBR® Green master mixes and protocols.

RNA Quantitation Using 2-Step RT-PCR

Step	Kit	Part Number
PCR step only	TaqMan® Gene Expression Master Mix, 1-Pack (1 × 5 mL), 200 reactions	4369016
	TaqMan® Gene Expression Master Mix, 10-Pack (10 × 5 mL), 2000 reactions	4369542
	TaqMan® 2X Universal PCR Master Mix	4304437
RT step only	High-Capacity cDNA Reverse Transcription Kit	4374966
	TaqMan® Reverse Transcription Reagents	N808-0234
Both RT and PCR steps	TaqMan® Gold RT-PCR Reagents without Controls, 200 reactions	N808-0232
	TaqMan® Gold RT-PCR Reagents without Controls, 10-Pack, 2000 reactions	4304133
	TaqMan® Gold RT-PCR Reagents with Controls, 200 reactions	N808-0233

Note: Presence/absence experiments are not supported using Fast or SYBR® Green master mixes and protocols.

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Glossary

AIF — See assay information file (AIF).

allele — For a given target, any of the different sequences that occurs in the population.

allelic discrimination plot — Display of data collected during the post-PCR read. The allelic discrimination plot is a graph of the normalized reporter signal from the allele 1 probe plotted against the normalized reporter signal from the allele 2 probe.

amplicon — A segment of DNA amplified during PCR.

amplification — Part of the instrument run in which PCR produces amplification of the target. For quantitation experiments, fluorescence data collected during amplification are displayed in an amplification plot, and the data are used to calculate results. For genotyping or presence/absence experiments, fluorescence data collected during amplification are displayed in an amplification plot, and the data can be used for troubleshooting.

amplification efficiency (EFF%) — Calculation of efficiency of the PCR amplification. The amplification efficiency is calculated using the slope of the regression line in the standard curve. A slope close to –3.32 indicates optimal, 100% PCR amplification efficiency. Factors that affect amplification efficiency:

- **Range of standard quantities** – To increase the accuracy and precision of the efficiency measurement, use a broad range of standard quantities, 5 to 6 logs (10^5 to 10^6 fold).
- **Number of standard replicates** – To increase the precision of the standard quantities and decrease the effects of pipetting inaccuracies, include replicates.
- **PCR inhibitors** – PCR inhibitors in the reaction can reduce amplification and alter measurements of the efficiency.

amplification plot — Display of data collected during the cycling stage of PCR amplification. Can be viewed as:

- Baseline-corrected normalized reporter (ΔRn) vs. cycle
- Normalized reporter (Rn) vs. cycle
- Threshold cycle (C_T) vs. well

amplification stage Part of the instrument run in which PCR produces amplification of the target. The amplification stage is called a cycling stage in the thermal profile and consists of denaturing, primer annealing, and polymerization steps that are repeated.

For quantitation experiments, fluorescence data collected during the amplification stage are displayed in an amplification plot, and the data are used to calculate results. For genotyping or presence/absence experiments, fluorescence data collected during the amplification stage are displayed in an amplification plot, and the data can be used for troubleshooting. See also cycling stage.

assay In an Applied Biosystems Real-Time PCR System, a PCR reaction mix that contains primers to amplify a target and a reagent to detect the amplified target.

Assay ID Identifier assigned by Applied Biosystems to TaqMan® Gene Expression Assays and TaqMan® SNP Genotyping Assays.

assay information file (AIF) Data file on a CD shipped with each assay order. The file name includes the number from the barcode on the plate. The information in the AIF is provided in a tab-delimited format.

assay mix PCR reaction component in Applied Biosystems TaqMan® Gene Expression Assays and TaqMan® SNP Genotyping Assays. The assay mix contains primers designed to amplify a target and a TaqMan® probe designed to detect amplification of the target.

AutoDelta In the run method, a setting to increase or decrease the temperature and/or time for a step with each subsequent cycle in a cycling stage. When AutoDelta is enabled for a cycling stage, the settings are indicated by an icon in the thermal profile:

- AutoDelta on: ▲
- AutoDelta off: ▲

automatic baseline An analysis setting in which the Real-Time PCR System software calculates the baseline start and end values for the amplification plot. You can apply the automatic baseline setting to specific wells in the reaction plate. See also baseline.

automatic C_T An analysis setting in which the Real-Time PCR System software calculates the baseline start and end values and the threshold in the amplification plot. The software uses the baseline and threshold to calculate the threshold cycle (C_T). See also threshold cycle (C_T).

baseline In the amplification plot, a line fit to the fluorescence levels during the initial stages of PCR, when there is little change in fluorescence signal.

baseline-corrected normalized reporter (ΔRn)

The magnitude of normalized fluorescence signal generated by the reporter:

1. In experiments that contain data from real-time PCR, the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. In the ΔRn vs. Cycle amplification plot, ΔRn is calculated at each cycle as:

   ΔRn (cycle) = Rn (cycle) − Rn (baseline), where Rn = normalized reporter

2. In genotyping experiments and presence/absence experiments, the difference in normalized fluorescence signal generated by the reporter between the pre-PCR read and the post-PCR read. In the allelic discrimination plot (genotyping experiments) and the presence/absence plot (presence/absence experiments), ΔRn is calculated as:

   ΔRn = Rn (post-PCR read) − Rn (pre-PCR read), where Rn = normalized reporter

See also normalized reporter (Rn).

blocked IPC

In presence/absence experiments, a reaction that contains IPC blocking agent, which blocks amplification of the internal positive control (IPC). In the Real-Time PCR Systems software, the task for the IPC target in wells that contain IPC blocking agent. See also negative control-blocked IPC wells.

calibrator

See reference sample.

chemistry

See reagents.

comparative C_T ($\Delta\Delta C_T$) method

Method for determining relative target quantity in samples. With the comparative C_T ($\Delta\Delta C_T$) method, the Real-Time PCR System software measures amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the endogenous control. The software determines the relative quantity of target in each sample by comparing normalized target quantity in each sample to normalized target quantity in the reference sample.

C_T

See threshold cycle (C_T).

custom dye

Dye that is not supplied by Applied Biosystems. Custom dyes may be adapted for use in experiments on an Applied Biosystems Real-Time PCR System. When using custom dyes, the custom dye should be added to the Dye Library and a custom dye calibration performed.

cycle threshold

See threshold cycle (C_T).

cycling stage

In the thermal profile, a stage that is repeated. A cycling stage is also called an amplification stage. For cycling stages, you can enable AutoDelta settings. See also amplification stage.

data collection

A process during the instrument run in which an instrument component detects fluorescence data from each well of the reaction plate. The instrument transforms the signal to electronic data, and the data are saved in the experiment file. In the Real-Time PCR System software, a data collection point is indicated by an icon in the thermal profile:

- Data collection on: 
- Data collection off: 

delta Rn (ΔRn)

See baseline-corrected normalized reporter (ΔRn).

derivative reporter (–Rn′)

The negative first-derivative of the normalized fluorescence generated by the reporter during PCR amplification. In the derivative reporter (–Rn′) vs. temperature melt curve, the derivative reporter signal is displayed in the y-axis.

diluent

A reagent used to dilute a sample or standard before adding it to the PCR reaction. The diluent can be water or buffer.

dilution factor

See serial factor.

dissociation curve

See melt curve.

EFF%

See amplification efficiency (EFF%).

endogenous control

A target or gene that should be expressed at similar levels in all samples you are testing. Endogenous controls are used in relative standard curve and comparative C_T ($\Delta\Delta C_T$) experiments to normalize fluorescence signals for the target you are quantifying. Housekeeping genes can be used as endogenous controls. See also housekeeping gene.

endpoint read

See post-PCR read.

experiment

Refers to the entire process of performing a run using an Applied Biosystems Real-Time PCR System, including setup, run, and analysis. The types of experiments you can perform using a Real-Time PCR System are:

- Quantitation - standard curve
- Quantitation - relative standard curve
- Quantitation - comparative C_T ($\Delta\Delta C_T$)
- Melt curve
- Genotyping
- Presence/absence

experiment name

Entered during experiment setup, the name that is used to identify the experiment. Experiment names cannot exceed 100 characters and cannot include any of the following characters: forward slash (/), backslash (\), greater than sign (>), less than sign (<), asterisk (*), question mark (?), quotation mark ("), vertical line (|), colon (:), or semicolon (;).

experiment type The type of experiment you are performing using an Applied Biosystems Real-Time PCR System:

- Standard curve
- Comparative C_T ($\Delta\Delta C_T$)
- Relative standard curve
- Melt curve (not available in the Design Wizard)
- Genotyping
- Presence/absence

The experiment type you select affects the setup, run, and analysis.

forward primer Oligonucleotide that flanks the 5′ end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.

holding stage In the thermal profile, a stage that includes one or more steps. You can add a holding stage to the thermal profile to activate enzymes, to inactivate enzymes, or to incubate a reaction.

housekeeping gene A gene that is involved in basic cellular functions and is constitutively expressed. Housekeeping genes can be used as endogenous controls. See also endogenous control.

internal positive control (IPC) In presence/absence experiments, a short synthetic DNA template that is added to PCR reactions. You can use the IPC to distinguish between true negative results (that is, the target is absent in the samples) and negative results caused by PCR inhibitors, incorrect assay setup, or reagent or instrument failure.

inventoried assays TaqMan® Gene Expression Assays and TaqMan® SNP Genotyping Assays that have been previously manufactured, passed quality control specifications, and stored in inventory.

IPC In presence/absence experiments, abbreviation for internal positive control. In the Real-Time PCR Systems software, the task for the IPC target in wells that contain the IPC and do not contain IPC blocking agent. See also internal positive control (IPC).

IPC blocking agent Reagent added to PCR reactions to block amplification of the internal positive control (IPC).

IPC+ See negative control-IPC wells.

made-to-order assays TaqMan® Gene Expression Assays or TaqMan® SNP Genotyping Assays that are manufactured at the time of order. Only assays that pass manufacturing quality control specifications are shipped.

manual baseline An analysis setting in which you enter the baseline start and end values for the amplification plot. You can apply the manual baseline setting to specific wells in the reaction plate.

manual C_T An analysis setting in which you enter the threshold value and select whether to use automatic baseline or manual baseline values. The Real-Time PCR System software uses the baseline and the threshold values to calculate the threshold cycle (C_T).

melt curve A plot of data collected during the melt curve stage. Peaks in the melt curve can indicate the melting temperature (Tm) of the target or can identify nonspecific PCR amplification. Also called dissociation curve.

melt curve stage In the thermal profile, a stage with a temperature increment to generate a melt curve.

melting temperature (Tm) In melt curve experiments, the temperature at which 50% of the DNA is double-stranded and 50% of the DNA is dissociated into single-stranded DNA. The Tm is displayed in the melt curve.

multicomponent plot A plot of the complete spectral contribution of each dye for the selected well(s) over the duration of the PCR run.

negative control (NC) See no template control (NTC).

negative control-blocked IPC wells In presence/absence experiments, wells that contain IPC blocking agent instead of sample in the PCR reaction. No amplification should occur in negative control-blocked IPC wells because the reaction contains no sample and amplification of the IPC is blocked. Previously called no amplification control (NAC).

negative control-IPC wells In presence/absence experiments, wells that contain IPC template and buffer or water instead of sample. Only the IPC template should amplify in negative control-IPC wells because the reaction contains no sample. Previously called IPC+.

no amplification control (NAC) See negative control-blocked IPC wells.

no template control (NTC) In the Real-Time PCR Systems software, the task for targets or SNP assays in wells that contain water or buffer instead of sample. No amplification of the target should occur in no template control wells. Also called negative control (NC).

nonfluorescent quencher-minor groove binder (NFQ-MGB) Molecules that are attached to the 3′ end of TaqMan® probes. When the probe is intact, the nonfluorescent quencher (NFQ) prevents the reporter dye from emitting fluorescence signal. Because the NFQ does not fluoresce, it produces lower background signals, resulting in improved precision in quantitation. The minor groove binder (MGB) increases the melting temperature (Tm) without increasing probe length. It also allows the design of shorter probes.

normalized quantity Quantity of target divided by the quantity of endogenous control.

normalized reporter (Rn) Fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference.

omit well An action that you perform before reanalysis to omit one or more wells from analysis. Because no algorithms are applied to omitted wells, omitted wells contain no results.

outlier For a set of data, a datapoint that is significantly smaller or larger than the others.

passive reference A dye that produces fluorescence signal. Because the passive reference signal should be consistent across all wells, it is used to normalize the reporter dye signal to account for non-PCR related fluorescence fluctuations caused by minor well-to-well differences in concentrations or volume. Normalization to the passive reference signal allows for high data precision.

plate layout An illustration of the grid of wells and assigned content in the reaction plate.

point One standard in a standard curve. The standard quantity for each point in the standard curve is calculated based on the starting quantity and serial factor.

positive control In genotyping experiments, a DNA sample with a known genotype, homozygous or heterozygous. In the Real-Time PCR Systems software, the task for the SNP assay in wells that contain a sample with a known genotype.

post-PCR read Used in genotyping and presence/absence experiments, the part of the instrument run that occurs after amplification. In genotyping experiments, fluorescence data collected during the post-PCR read are displayed in the allelic discrimination plot and used to make allele calls. In presence/absence experiments, fluorescence data collected during the post-PCR read are displayed in the presence/absence plot and used to make detection calls. Also called endpoint read.

pre-PCR read Used in genotyping and presence/absence experiments, the part of the instrument run that occurs before amplification. The pre-PCR read is optional but recommended. Fluorescence data collected during the pre-PCR read can be used to normalize fluorescence data collected during the post-PCR read.

primer mix PCR reaction component that contains the forward primer and reverse primer designed to amplify the target.

primer/probe mix PCR reaction component that contains the primers designed to amplify the target and a TaqMan® probe designed to detect amplification of the target.

pure dye See custom dye and system dye.

quantitation method In quantitation experiments, the method used to determine the quantity of target in the samples. In an Applied Biosystems Real-Time PCR System, there are three types of quantitation methods: standard curve, relative standard curve, and comparative C_T ($\Delta\Delta C_T$).

quantity In quantitation experiments, the amount of target in the samples. Absolute quantity can refer to copy number, mass, molarity, or viral load. Relative quantity refers to the fold-difference between normalized quantity of target in the sample and normalized quantity of target in the reference sample.

quencher A molecule attached to the 3′ end of TaqMan® probes to prevent the reporter from emitting fluorescence signal while the probe is intact. With TaqMan® reagents, a nonfluorescent quencher-minor groove binder (NFQ-MGB) can be used as the quencher. With SYBR® Green reagents, no quencher is used.

R^2 value Regression coefficient calculated from the regression line in the standard curve. The R^2 value indicates the closeness of fit between the standard curve regression line and the individual C_T data points from the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points.

ramp The rate at which the temperature changes during the instrument run. Except for the melt curve step, the ramp is defined as a percentage. For the melt curve step, the ramp is defined as a temperature increment. In the graphical view of the thermal profile, the ramp is indicated by a diagonal line.

ramp speed Speed at which the temperature ramp occurs during the instrument run. Available ramp speeds include fast and standard.

- For optimal results using the fast ramp speed, Applied Biosystems recommends using TaqMan® Fast reagents in your PCR reactions.
- For optimal results using the standard ramp speed, Applied Biosystems recommends using standard reagents in your PCR reactions.

IMPORTANT! TaqMan Fast reagents are not supported for genotyping or presence/absence experiments.

raw data plot A plot of raw fluorescence signal (not normalized) for each optical filter.

reaction mix A solution that contains all components to run the PCR reaction, except for the template (sample, standard, or control).

reagents The PCR reaction components you are using to amplify the target and to detect amplification. Types of reagents used on the Applied Biosystems Real-Time PCR Systems:

- TaqMan® reagents
- SYBR® Green reagents
- Other fluorescence-based reagents

real-time PCR Process of collecting fluorescence data during PCR. Data from the real-time PCR are used to calculate results for quantitation experiments or to troubleshoot results for genotyping or presence/absence experiments.

reference sample In relative standard curve and comparative C_T ($\Delta\Delta C_T$) experiments, the sample used as the basis for relative quantitation results. Also called the calibrator.

refSNP ID Identifies the reference SNP (refSNP) cluster ID. Generated by the Single Nucleotide Polymorphism Database of Nucleotide Sequence Variation (dbSNP) at the National Center for Biotechnology Information (NCBI). The refSNP ID can be used to search the Applied Biosystems Store for an Applied Biosystems SNP Genotyping Assay. Also called an rs number.

regression coefficients Values calculated from the regression line in standard curves, including the R^2 value, slope, and y-intercept. You can use the regression coefficients to evaluate the quality of results from the standards. See also standard curve.

regression line In standard curve and relative standard curve experiments, the best-fit line from the standard curve. Regression line formula:

$C_T = m [\log (Qty)] + b$

where m is the slope, b is the y-intercept, and Qty is the standard quantity.

See also regression coefficients.

reject well An action that the Real-Time PCR System software performs during analysis to remove one or more wells from further analysis if a specific flag is applied to the well. Rejected wells contain results calculated up to the point of rejection.

relative standard curve method Method for determining relative target quantity in samples. With the relative standard curve method, the Real-Time PCR System software measures amplification of the target and of the endogenous control in samples, in a reference sample, and in a standard dilution series. Measurements are normalized using the endogenous control. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates target quantity in the samples and in the reference sample. The software determines the relative quantity of target in each sample by comparing target quantity in each sample to target quantity in the reference sample.

replicate group A set of identical reactions in an experiment.

replicates Total number of identical reactions containing identical components and identical volumes.

reporter Fluorescent dye used to detect amplification. If you are using TaqMan® reagents, the reporter dye is attached to the 5′ end. If you are using SYBR® Green reagents, the reporter dye is SYBR® Green I dye.

reverse primer An oligonucleotide that flanks the 3′ end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.

reverse transcriptase An enzyme that converts RNA to cDNA. Reverse transcriptase is added to the PCR reaction to perform 1-step RT-PCR.

Rn See normalized reporter (Rn).

ROX™ dye A dye supplied by Applied Biosystems and precalibrated on the Applied Biosystems Real-Time PCR Systems. ROX dye is used as the passive reference.

rs number See refSNP ID.

run method Definition of the reaction volume and the thermal profile for the instrument run.

sample The template that you are testing.

sample/SNP assay reaction In genotyping experiments, the combination of which sample to test and which SNP assay to perform in one PCR reaction. Each PCR reaction can contain only one sample and one SNP assay.

sample/target reaction In quantitation experiments, the combination of which sample to test and which target to detect and quantify in one PCR reaction. In the Design Wizard, you can detect and quantify only one target in one PCR reaction. Use Advanced Setup to detect and quantify more than one target in one PCR reaction.

serial factor A numerical value that defines the sequence of quantities in the standard curve. The serial factor and the starting quantity are used to calculate the standard quantity for each point in the standard curve. For example, if the standard curve is defined with a serial factor of 1:10 or 10×, the difference between any 2 adjacent points in the curve is 10-fold. Also called dilution factor.

series See standard dilution series.

slope Regression coefficient calculated from the regression line in the standard curve. The slope indicates the PCR amplification efficiency for the assay. A slope of –3.32 indicates 100% amplification efficiency. See also amplification efficiency (EFF%) and regression line.

SNP Abbreviation for single nucleotide polymorphism. The SNP can consist of a base difference or an insertion or deletion of one base.

SNP assay Used in genotyping experiments, a PCR reaction that contains primers to amplify the SNP and two probes to detect different alleles.

spatial calibration Type of system calibration in which the Applied Biosystems Real-Time PCR System maps the positions of the wells in the sample block. Spatial calibration data are used so that the Real-Time PCR System software can associate increases in fluorescence during a run with specific wells in the reaction plate.

stage In the thermal profile, a group of one or more steps. There are three types of stages: holding stage (including pre-PCR read and post-PCR read), cycling stage (also called amplification stage), and melt curve stage.

standard Sample that contains known standard quantities. Standard reactions are used in quantitation experiments to generate standard curves. See also standard curve and standard dilution series.

standard curve In standard curve and relative standard curve experiments:

- The best-fit line in a plot of the C_T values from the standard reactions plotted against standard quantities. See also regression line.
- A set of standards containing a range of known quantities. Results from the standard curve reactions are used to generate the standard curve. The standard curve is defined by the number of points in the dilution series, the number of standard replicates, the starting quantity, and the serial factor. See also standard dilution series.

standard curve method Method for determining absolute target quantity in samples. With the standard curve method, the Real-Time PCR System software measures amplification of the target in samples and in a standard dilution series. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates the absolute quantity of target in the samples. See also standard and standard curve.

standard dilution series In standard curve and relative standard curve experiments, a set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards. For example, the standard stock is used to prepare the first dilution point, the first dilution point is used to prepare the second dilution point, and so on. See also standard curve.

standard quantity A known quantity in the PCR reaction.

- In standard curve experiments, the quantity of target in the standard.
- In relative standard curve experiments, a known quantity in the standard. Standard quantity can refer to the quantity of cDNA or the quantity of standard stock in the PCR reaction. The units are not relevant for relative standard curve experiments because they cancel out in the calculations.

starting quantity When defining a standard curve in the Real-Time PCR System software, corresponds to the highest or lowest quantity.

step A component of the thermal profile. For each step in the thermal profile, you can set the ramp rate (ramp increment for melt curve steps), hold temperature, hold time (duration), and you can turn data collection on or off for the ramp or the hold parts of the step. For cycling stages, a step is also defined by the AutoDelta status.

SYBR® Green reagents PCR reaction components that consist of two primers designed to amplify the target and SYBR® Green I dye to detect double-stranded DNA.

system dye Dye supplied by Applied Biosystems and precalibrated on the Applied Biosystems Real-Time PCR Systems. Before you use system dyes in your experiments, make sure the system dye calibration of your instrument is current.

TaqMan® reagents PCR reaction components that consist of primers designed to amplify the target and a TaqMan® probe designed to detect amplification of the target.

target The nucleic acid sequence that you want to amplify and detect.

target color In the Real-Time PCR Systems software, a color assigned to a target to identify the target in the plate layout and analysis plots.

task

In the Real-Time PCR Systems software, the type of reaction performed in the well for the target or SNP assay. Available tasks:

- Unknown
- No Template Control or Negative Control
- Standard (standard curve and relative standard curve experiments)
- Positive control (genotyping experiments)
- IPC (presence/absence experiments)
- Blocked IPC (presence/absence experiments)

template

The type of nucleic acid to add to the PCR reaction. The recommended template varies according to experiment type:

- Quantitation experiments (standard curve, relative standard curve, and comparative C_T) – cDNA (complementary cDNA), RNA, or gDNA (genomic DNA)

  For quantitation experiments, the template type selection affects the run method, reaction setup, and materials list.
- Genotyping experiments – Wet DNA (gDNA or cDNA) or dry DNA (gDNA or cDNA)

  For genotyping experiments, the template type selection affects the reaction setup.
- Presence/absence experiments - DNA

  For presence/absence experiments, Applied Biosystems recommends adding DNA templates to the PCR reactions.

thermal profile

Part of the run method that specifies the temperature, time, ramp, and data collection points for all steps and stages of the instrument run.

threshold

1. In amplification plots, the level of fluorescence above the baseline and within the exponential growth region The threshold can be determined automatically (see automatic C_T) or can be set manually (see manual C_T).
2. In presence/absence experiments, the level of fluorescence above which the Real-Time PCR System software assigns a presence call.

threshold cycle (C_T)

The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.

Tm

See melting temperature (Tm).

unknown In the Real-Time PCR Systems software, the task for the target or SNP assay in wells that contain the sample you are testing:

- In quantitation experiments, the task for the target in wells that contain a sample with unknown target quantities.
- In genotyping experiments, the task for the SNP assay in wells that contain a sample with an unknown genotype.
- In presence/absence experiments, the task for the target in wells that contain a sample in which the presence of the target is not known.

unknown-IPC wells In presence/absence experiments, wells that contain a sample and internal positive control (IPC).

y-intercept In the standard curve, the value of y where the regression line crosses the y-axis. The y-intercept indicates the expected threshold cycle (C_T) for a sample with quantity equal to 1.

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Worldwide Sales and Support

Applied Biosystems vast distribution and service network, composed of highly trained support and applications personnel, reaches 150 countries on six continents. For sales office locations and technical support, please call our local office or refer to our Web site at **www.appliedbiosystems.com**.

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Headquarters
850 Lincoln Centre Drive
Foster City, CA 94404 USA
Phone: +1 650.638.5800
Toll Free (In North America): +1 800.345.5224
Fax: +1 650.638.5884

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