

# Applied Biosystems Real-Time PCR Rapid Assay Development Guidelines

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## Description

This tutorial will discuss recommended guidelines for designing and running real-time PCR quantification and SNP Genotyping (Allelic Discrimination) assays. Throughout this tutorial there are many hyperlinks to additional sites and documents. When you go to one of these hyperlinks simply click on the back button on your browser to return to your original location within this document.

## Introduction

Applied Biosystems Real-Time PCR Rapid Assay Development Guidelines are a series of design and experimental guidelines aimed towards maximizing success while reducing upfront time and costs of running real-time PCR. The Rapid Assay Development Guidelines consist of the following:

- **Selection or Design of Primer and Probe Sets:** the use of Applied Biosystems TaqMan® Genomic Assays or custom primers and probes designed using the Primer Express® Software
- **Universal Thermal Cycling Conditions:** enables multiple assays, designed following the guidelines, to be run on the same plate successfully
- **Universal PCR Master Mixes:** provides optimized component concentrations in a single vial, and simplifies assay set-up
- **Default Primer and Probe Concentrations:** eliminates assay optimization.

Traditionally researchers have had to empirically determine optimal reaction parameters for each assay, such as enzyme amounts, Mg<sup>2+</sup> concentrations, annealing temperatures and times. This kind of traditional optimization adds both time and cost to the development of PCR assays. The Applied Biosystems Rapid Assay Development Guidelines standardize primer and probe design, thermal cycling parameters and reaction components, thereby reducing costs and eliminating time-consuming optimization steps.

**Please Note:** It is expected that the custom 5' nuclease quantification assays designed with the Applied Biosystems Rapid Assay Development Guidelines will provide comparable results when using the default **Fast** thermal cycling conditions and the [TaqMan® Fast Universal PCR Maser Mix \(2X\), No AmpErase® UNG](#), as compared to running the standard thermal cycling conditions and the TaqMan® Universal PCR Master Mix.

## Selection or Design of Primer and Probe Sets

When deciding on a primer/probe set there are 4 options:

- TaqMan<sup>®</sup> Gene Expression and SNP Genotyping Assays
- Custom TaqMan<sup>®</sup> Gene Expression and SNP Genotyping Assays
- Pre-Developed TaqMan<sup>®</sup> Assay Reagents (TaqMan<sup>®</sup> PDARs)
- Primer and Probe Design using the Primer Express<sup>®</sup> Software.

### TaqMan<sup>®</sup> Genomic Assays

[TaqMan<sup>®</sup> Gene Expression Assays](#) are biologically informative, pre-formulated gene expression assays for rapid, reliable detection and quantification of human, mouse and rat mRNA transcripts. [TaqMan<sup>®</sup> SNP Genotyping Assays](#) are validated primer and TaqMan<sup>®</sup> MGB probe sets for the detection of human SNPs. Each product is delivered as pre-mixed primers and TaqMan<sup>®</sup> MGB probe(s) at a 20X concentration.

### Custom TaqMan<sup>®</sup> Genomic Assays

[Custom TaqMan<sup>®</sup> Gene Expression Assays](#) and [Custom TaqMan<sup>®</sup> SNP Genotyping Assays](#) are custom assays that are designed, synthesized, formulated, and delivered as analytically quality-controlled primer and probe sets based on sequence information submitted by the customer for your gene expression and SNP genotyping needs. Each product is delivered as pre-mixed primers and TaqMan<sup>®</sup> MGB probe(s) in a single tube. The concentration will vary by the scale of the assay ordered.

A free download of the [Custom Taqman<sup>®</sup> Genomic Assays File Builder software](#) makes it easy to format and submit sequences for design.

### TaqMan<sup>®</sup> Pre-Developed Assay Reagents (TaqMan<sup>®</sup> PDARs)

TaqMan<sup>®</sup> Pre-developed Assay Reagents are available for both [Gene Expression](#) and [Allelic Discrimination](#). TaqMan<sup>®</sup> PDARs are primer and probe sets designed to amplify specific target and control sequences in cDNA samples using the 5' nuclease assay. They are pre-optimized for potential use in either multiplex or separate reactions providing flexibility. Each product is delivered as pre-mixed primers and TaqMan<sup>®</sup> MGB or TaqMan<sup>®</sup> TAMRA<sup>™</sup> dye-labeled probes at a 20X concentration.

### Primer and Probe Design using Primer Express<sup>®</sup> Software

Primer Express<sup>®</sup> Software is a primer and probe design software that comes with the purchase of an Applied Biosystems Real-Time PCR instrument. It can be used for the design of custom TaqMan<sup>®</sup> assays, to include TaqMan<sup>®</sup> MGB and TAMRA dye labeled probes as well as primers designed for SYBR<sup>®</sup> Green reagent assays.

If there is not an assay already available from Applied Biosystems through the TaqMan<sup>®</sup> Genomic Assays or TaqMan<sup>®</sup> PDARs, another option is to design your own custom primers and probes using the Primer Express<sup>®</sup> Software. Primers can be designed in conjunction with TaqMan<sup>®</sup> MGB probes or

TaqMan<sup>®</sup> TAMRA dye labeled probes. If you will be using SYBR<sup>®</sup> Green reagent chemistry, and only need to design primers, we recommend designing primers in conjunction with a TaqMan<sup>®</sup> TAMRA dye labeled probe and saving the probe sequence in case you want to convert the assay to a 5' nuclease assay later.

When choosing a reporter dye for a TaqMan<sup>®</sup> probe, our general recommendation is to choose FAM<sup>™</sup> dye as a reporter dye. If you plan to use two TaqMan<sup>®</sup> probes in one assay, a multiplex reaction, VIC<sup>®</sup> dye would be a good choice for the second reporter dye since FAM<sup>™</sup> and VIC<sup>®</sup> dyes are spectrally well separated.

### **TaqMan<sup>®</sup> MGB Probes**

TaqMan<sup>®</sup> MGB probes contain a minor groove binding moiety which enhances the  $T_m$  differential between matched and mismatched probes. In addition, TaqMan<sup>®</sup> MGB probes contain a non-fluorescent quencher that enhances spectral resolution when using multiple dyes in a reaction. TaqMan<sup>®</sup> MGB probes are ideal for use in both gene expression and SNP Genotyping assays. When designing a TaqMan<sup>®</sup> MGB probe assay it is recommended to use the Primer Express<sup>®</sup> Software. The design of TaqMan<sup>®</sup> MGB probes can only be done using Primer Express<sup>®</sup> Software v1.5 and above. There is a separate document in the Primer Express<sup>®</sup> Software for designing TaqMan<sup>®</sup> MGB primer/probe sets.

For detailed instructions on designing TaqMan<sup>®</sup> MGB assays, please refer to the following tutorials:

[Designing TaqMan<sup>®</sup> MGB Probe and Primer Sets for Gene Expression Using Primer Express<sup>®</sup> Software Version 2.0](#)

and

[Designing TaqMan<sup>®</sup> MGB Probe and Primer Sets for Allelic Discrimination Assays Using Primer Express<sup>®</sup> Software Version 2.0.](#)

### **TaqMan<sup>®</sup> TAMRA Probes**

When designing a TaqMan<sup>®</sup> TAMRA dye labeled probe assay it is recommended to use the Primer Express<sup>®</sup> Software. Any version of the Primer Express<sup>®</sup> Software can be used to design TaqMan<sup>®</sup> TAMRA dye labeled probes.

For step-by-step instructions, please see the following tutorial for designing TaqMan<sup>®</sup> TAMRA dye labeled probes:

[Primer Express<sup>®</sup> Software v2.0: Designing Primers and TaqMan<sup>®</sup> TAMRA<sup>™</sup> Probes for 5' Nuclease Assays and Primers for Real-Time PCR Assays using SYBR<sup>®</sup> Green Dye.](#)

## SYBR® Green Primers

The use of SYBR® Green reagents in real-time PCR is based on the exceptionally high affinity of SYBR® Green dye for double-stranded DNA. The progress of a real-time run using SYBR® reagent chemistry can be measured by monitoring this increase in fluorescence as SYBR® Green dye binds to PCR products. Designing primers in line with our guidelines, to be used with the [SYBR® Green PCR Master Mix](#), will increase the success of these assays.

When designing a SYBR® Green primer set, it is recommended to use the Primer Express® Software. Any version of the Primer Express® Software can be used to design SYBR Green primers.

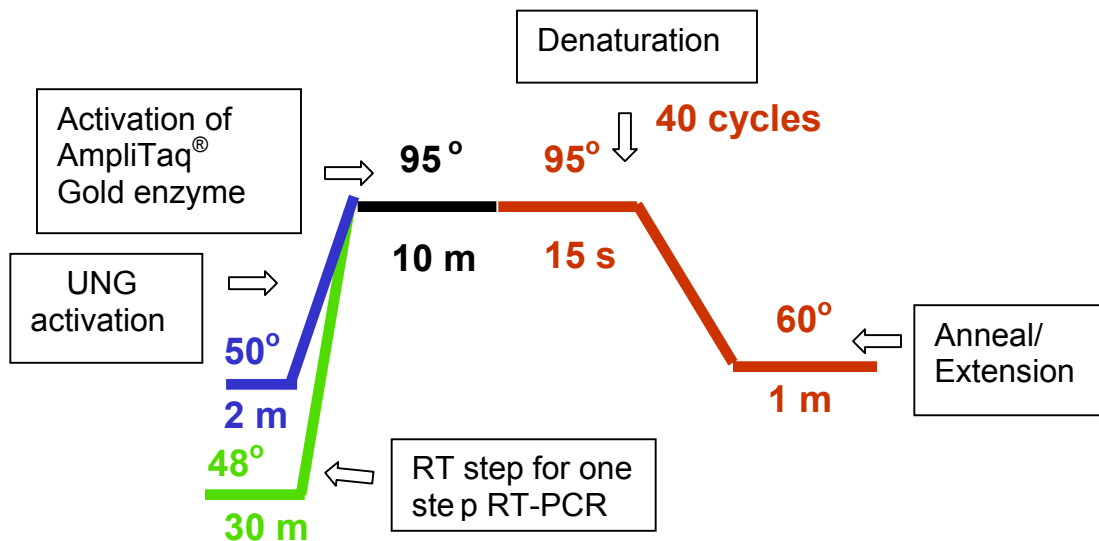
For detailed instructions on designing SYBR Green primers, please refer to the following tutorial:

[Primer Express® Software v2.0: Designing Primers and TaqMan® TAMRA™ Probes for 5' Nuclease Assays and Primers for Real-Time PCR Assays using SYBR® Green Dye.](#)

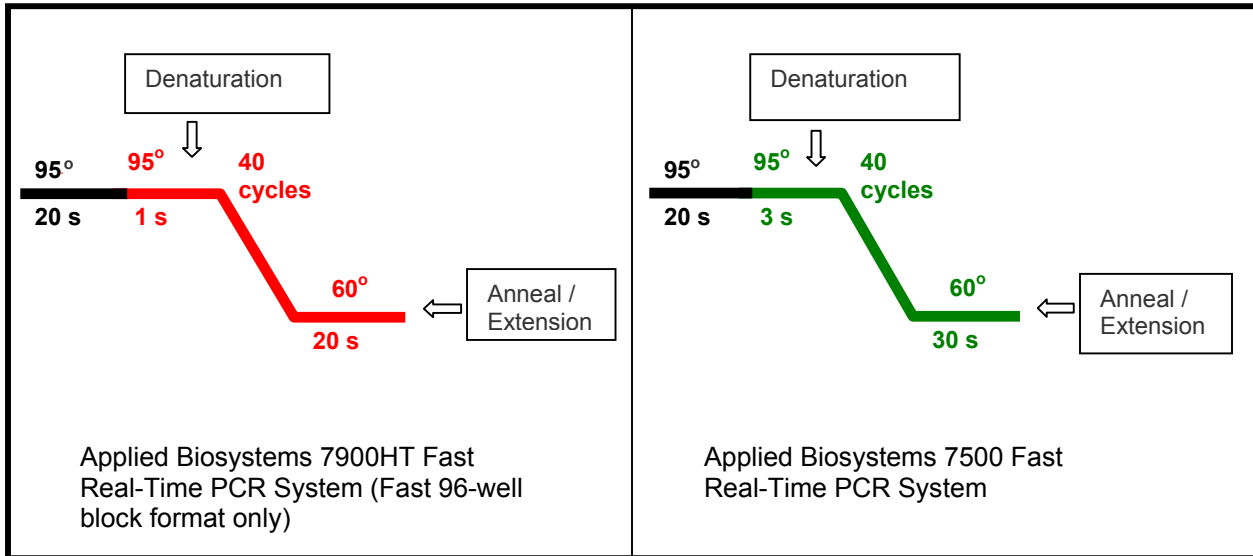
## Universal Thermal Cycling Conditions

For a standard quantitative Real-Time PCR reaction, the conditions below are the recommended default conditions on all Applied Biosystems Real-Time PCR instruments. The default thermal cycling conditions are comprised of a 50 °C UNG activation step, a 95 °C AmpliTaq Gold® enzyme activation, and 40 cycles of 95 °C denaturation and 60 °C anneal/extension.

For one-step RT-PCR, you can substitute the RT step for the UNG activation step.



For a Fast Real-Time PCR reaction, the conditions below are the recommended default thermal cycling conditions, and are comprised of a 95 °C initial template denaturation, and 40 cycles of 95 °C denaturation and 60 °C anneal/extension. *Note: Cycling times are different for each Fast System.*



The universal cycling conditions eliminate optimization of the thermal cycling conditions and allow multiple assays to be run on the same plate. These conditions are optimal for TaqMan® Gene Expression Assays, Custom TaqMan® Gene Expression Assays, TaqMan® Gene Expression PDARs, and primer/probe sets designed using the Primer Express Software. Please consult with the appropriate Applied Biosystems reagent protocol to identify the recommended thermal cycling conditions for each 5' nuclease assay. Protocols can be obtained from the Applied Biosystems [Product and Service Literature](#) web site.

## Universal PCR Master Mixes

### TaqMan® Universal PCR Master Mix

The [TaqMan® Universal PCR Master Mix](#) contains all of the necessary PCR components, fully optimized, to work with TaqMan® Genomic Assays, Custom TaqMan® Genomic Assays, TaqMan® PDARs, and primer/probe sets designed via the Primer Express® Software. It contains AmpliTaq Gold® DNA Polymerase, dNTPs, optimized buffer components, MgCl<sub>2</sub>, and Passive Reference Dye (ROX™ dye). TaqMan® Universal PCR Master Mix is available with [AmpErase® UNG](#) (P/N 4304437) or without AmpErase® UNG (P/N 426708).

### TaqMan® Fast Universal PCR Master Mix

The [TaqMan® Fast Universal PCR Master Mix](#) is uniquely engineered to significantly reduce overall run time. The use of Hot-Start DNA Polymerase enzyme system in the master mix minimizes non-specific amplification products and primer-dimers so you get high-quality results. The TaqMan® Fast Master Mix (P/N 4352042) contains dUTP for use with an optional decontamination protocol. The use of Amperase® UNG prevents subsequent reamplification of PCR products containing dUTP.

### SYBR Green PCR Master Mix

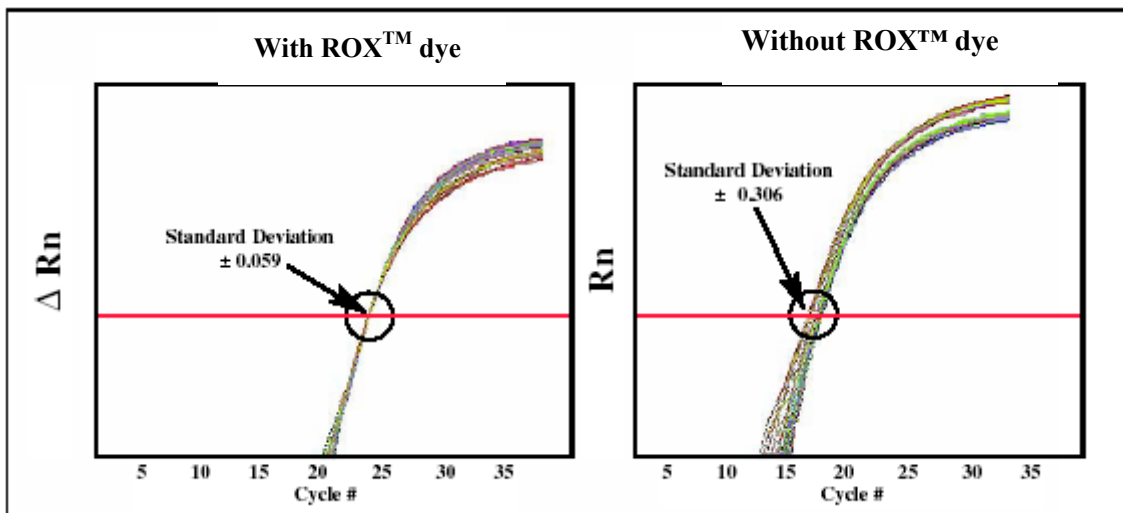
When using SYBR® Green chemistry, it is recommended that the [SYBR® Green PCR Master Mix](#) (P/N 4309155) be used. It contains SYBR® Green I dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, optimized buffer components, MgCl<sub>2</sub>, Passive Reference (ROX™ dye), and is compatible with the use AmpErase® UNG (P/N 4304437).

*Note: SYBR® Green reagent chemistry cannot be used with TaqMan® assays.*

### What is ROX™ Passive Reference Dye?

The Passive Reference Dye, ROX™, is used to normalize real-time PCR reactions. ROX™ dye performs the following functions:

- Normalizes most fluorescent fluctuations
- Compensates for well-to-well volume variations
- Regulates minor volume differences and changes in concentration
- Allows for high precision.



The passive reference, ROX dye, provides an internal reference to which the reporter dye signal can be normalized. This signal normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume. Normalization of the reporter dye signal results in increased data precision.

## Real-Time PCR Primer/Probe Concentrations

For real-time PCR assays designed following our Rapid Assay Development Guidelines, we recommend final reaction concentrations of 900nM for each primer and 250nM of the probe for Gene Expression, and 900nM of each primer and 200nM of each probe for SNP Genotyping assays. These recommendations will provide highly reproducible and sensitive results when using cDNA or DNA as a template.

When using SYBR<sup>®</sup> Green chemistry, final concentrations of 50 nM of both reverse and forward primer should provide robust amplification results with a good level of specificity when using cDNA or DNA as a template. We recommend checking for nonspecific products by either dissociation curve or gel analysis.

While these are our default recommendations, the Applied Biosystems reagent protocols should be consulted to determine how to further optimize primer and probe concentrations for a real-time PCR assay. Protocols can be obtained from the Applied Biosystems [Products and Service Literature](#) web site.

## Summary

The Rapid Assay Development Guidelines consist of the following:

- The use of Applied Biosystems TaqMan<sup>®</sup> Genomic Assays or custom primers and probes designed using Primer Express<sup>®</sup> Software
- Universal Thermal Cycling Conditions
- The use of Universal PCR Master Mixes
- Default Primer and Probe Concentrations.

These guidelines were developed to provide the greatest chance for success in a 5' nuclease assay on an Applied Biosystems platform. Adopting the Rapid Assay Development Guidelines increases your chances of reaching 100% PCR efficiency. Most Assays will work under the universal conditions so optimizing reaction conditions will not be necessary.

For the best results, use Applied Biosystems platforms and reagents. For more information, continue to the following page for relevant documents.

## Related Documents

Document Title	Document Part Number
<a href="#">TaqMan® Gene Expression Assays Protocol</a>	4333458A
<a href="#">TaqMan® Gene Expression Assays: Product Quick Card</a>	127MI07-01
<a href="#">TaqMan® Universal PCR Master Mix Protocol</a>	4304449
<a href="#">High-Capacity cDNA Archive Kit Protocol</a>	4322169
<a href="#">Pre-Developed Taqman® Assay Reagents: Gene Expression Quantification</a>	4310255
<a href="#">User Bulletin #2 Relative Quantitation of Gene Expression</a>	4303859
<a href="#">User Bulletin #5: Rev B: Multiplex PCR with TaqMan® VIC Probes</a>	4306236B
<a href="#">Amplification Efficiency of TaqMan® Gene Expression Assays: Application Note</a>	127AP05-02
<a href="#">SYBR® Green PCR Master Mix</a>	4310251
<a href="#">TaqMan® Fast Universal PCR Master Mix (2X)</a>	4361968
<a href="#">Primer Express v1.5 and TaqMan® MGB Probes for Allelic Discrimination: All PCR Instruments: User Bulletin</a>	4317594A
<a href="#">TaqMan® SNP Genotyping Assays: Protocol</a>	4332856B
<a href="#">Assays-by-Design Service For Gene Expression Assays: Quick Reference Card</a>	4335636A
<a href="#">Assays-by-Design Service For SNP Assays: Quick Reference Card</a>	4335637A
<a href="#">Assays-by-Design Service For Gene Expression Assays: Protocol</a>	4334429C
<a href="#">Assays-by-Design Service For SNP Assays: Protocol</a>	4334431C
<a href="#">Assays-by-Design Service Submission Guidelines: Protocol</a>	4334672C
<a href="#">Pre-Developed TaqMan® Assay Reagents Allelic Discrimination</a>	4312214B



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#### TaqMan Universal PCR Master Mix -

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The use of uracil-N-glycosylase for carryover prevention is licensed by Life Technologies, Inc. under U.S. Patent 5,035,996 and foreign equivalents for research purposes only. No right for use in other applications, including the diagnosis of disease in humans, animals, or plants under any patents owned by Life Technologies, Inc. are covered by the purchase of this product.

TaqMan Universal Master Mix, No AmpErase UNG and TaqMan Fast Universal PCR Master Mix, No AmpErase UNG

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The SYBR® Green dye is sold pursuant to a limited license from Molecular Probes, Inc. under U.S. Patent No. 5,436,134 and 5,658,751 and corresponding foreign patents and patent applications.

TaqMan Gene Expression and SNP Genotyping products -

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**TaqMan® probes are covered by U.S. Patent 5,723,591 and foreign counterparts and patents pending owned by Applied Biosystems, and may be covered by U.S. Patents 5,801,155 and 6,084,102 and foreign counterparts licensed to Applied Biosystems.**

Custom TaqMan Gene Expression and SNP Genotyping products -

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## TaqMan Pre-Developed Assay Reagents for Allelic Discrimination -

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### Primer Express software -

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