Applied Biosystems Real-Time PCR Rapid Assay Development Guidelines

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^{2+} 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® Gene Expression and SNP Genotyping Assays
- Custom TaqMan® Gene Expression and SNP Genotyping Assays
- Pre-Developed TaqMan® Assay Reagents (TaqMan® PDARs)
- Primer and Probe Design using the Primer Express® Software.

TaqMan® Genomic Assays

TaqMan® 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® SNP Genotyping Assays are validated primer and TaqMan® MGB probe sets for the detection of human SNPs. Each product is delivered as pre-mixed primers and TaqMan® MGB probe(s) at a 20X concentration.

Custom TaqMan® Genomic Assays

Custom TaqMan® Gene Expression Assays and Custom TaqMan® 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® 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® Genomic Assays File Builder software makes it easy to format and submit sequences for design.

TaqMan® Pre-Developed Assay Reagents (TaqMan® PDARs)

TaqMan® Pre-developed Assay Reagents are available for Allelic Discrimination. TaqMan® PDARs are primer and probe sets designed to amplify specific target and control sequences in cDNA samples using the 5' nuclease assay. Each product is delivered as pre-mixed primers and TaqMan® MGB dye-labeled probes at a 20X concentration.

Primer and Probe Design using Primer Express® Software

Primer Express® 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® assays, to include TaqMan® MGB and TAMRA dye labeled probes as well as primers designed for SYBR® Green reagent assays.

If there is not an assay already available from Applied Biosystems through the TaqMan® Genomic Assays or TaqMan® PDARs, another option is to design your own custom primers and probes using the Primer Express® Software. Primers can be designed in conjunction with TaqMan® MGB probes or TaqMan® TAMRA dye labeled probes. If you will be using SYBR® Green reagent chemistry, and only need to design primers, we recommend

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

TaqMan® MGB Probes

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

For detailed instructions on designing TaqMan® MGB assays, please refer to the following tutorials:
Designing TaqMan® MGB Probe and Primer Sets for Gene Expression Using Primer Express® Software Version 2.0
and
Designing TaqMan® MGB Probe and Primer Sets for Allelic Discrimination Assays Using Primer Express® Software Version 2.0.

TaqMan® TAMRA Probes

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

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

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.

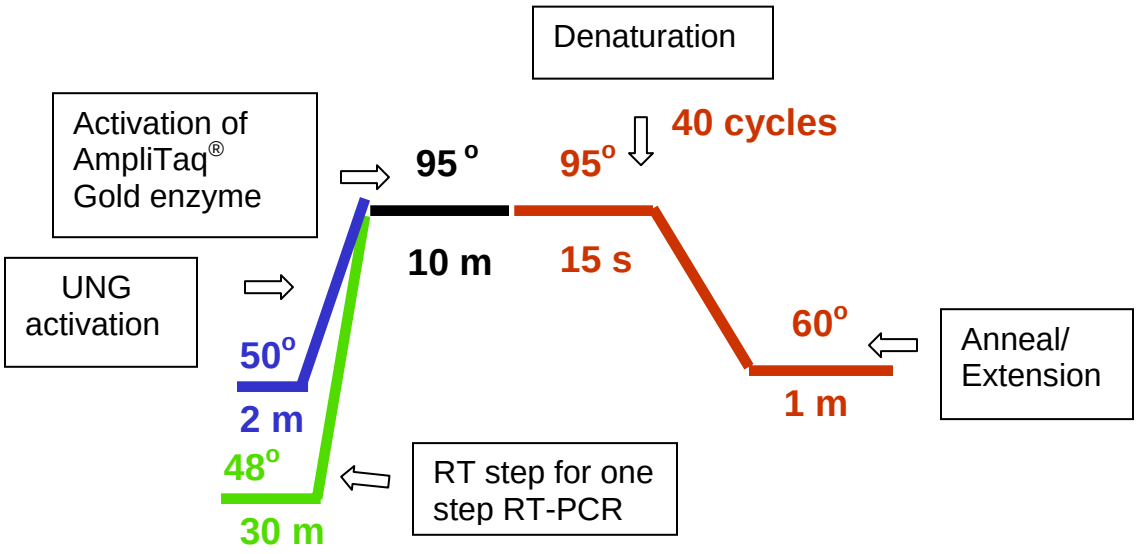
When designing a SYBR® Green primer set, it is recommended to use the Primer Express® Software. Any version of the Primer Express® Software can be used to design SYBR Green primers.

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.

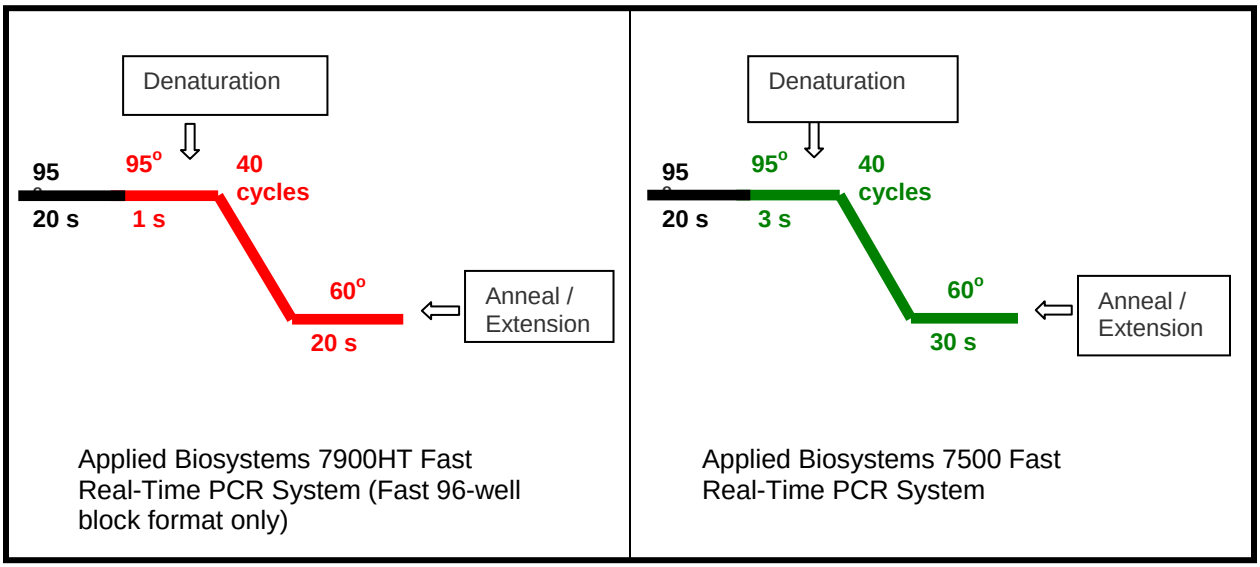
Universal Thermal Cycling Conditions

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 ºC UNG activation step, a 95 ºC AmpliTaq Gold® enzyme activation, and 40 cycles of 95 ºC denaturation and 60 ºC 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 ºC initial template denaturation, and 40 cycles of 95 ºC denaturation and 60 ºC anneal/extension. *Note: Cycling times are different for each Fast System*.



Applied Biosystems 7900HT Fast Real-Time PCR System (Fast 96-well block format only)

Applied Biosystems 7500 Fast Real-Time PCR 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, $MgCl_2$, 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

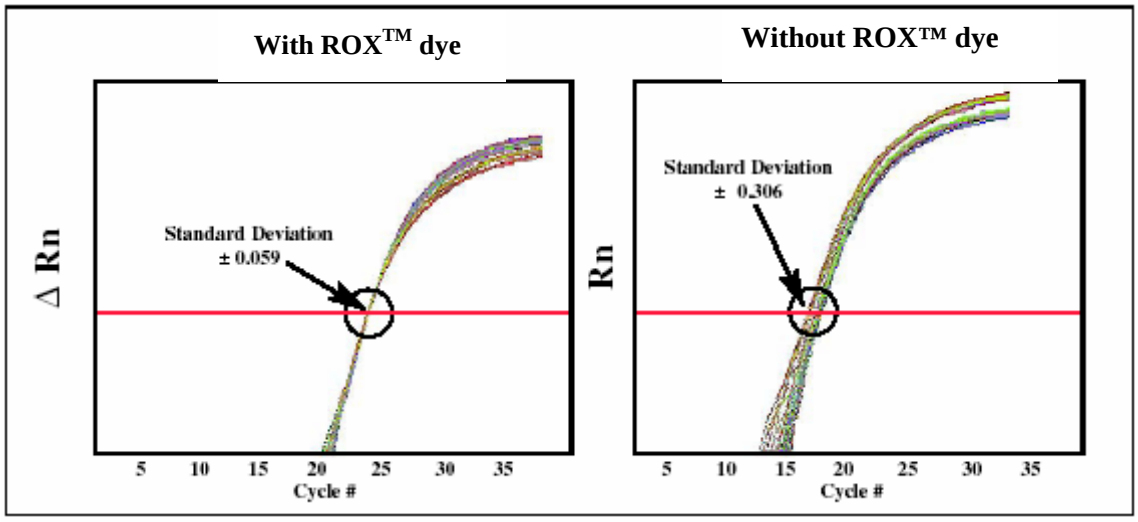
When using SYBR® Green chemistry, it is recommended that the SYBR® Green PCR Master Mix (P/N 4309155) be used. It contains SYBR® Green I dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, optimized buffer components, $MgCl_2$, Passive Reference (ROX™ dye), and is compatible with the use AmpErase® UNG (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® 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® Genomic Assays or custom primers and probes designed using Primer Express® 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
TaqMan® Gene Expression Assays Protocol	4333458
TaqMan® Gene Expression Assays: Product Quick Card	127MI07-01
TaqMan® Universal PCR Master Mix Protocol	4304449
High-Capacity cDNA Archive Kit Protocol	4322169
Pre-Developed TaqMan® Assay Reagents Allelic Discrimination	4312214
User Bulletin #2 Relative Quantitation of Gene Expression	4303859
User Bulletin #5: Rev B: Multiplex PCR with TaqMan® VIC Probes	4306236
Amplification Efficiency of TaqMan® Gene Expression Assays: Application Note	127AP05-02
SYBR® Green PCR Master Mix	4310251
TaqMan® Fast Universal PCR Master Mix (2X)	4361968
Primer Express v1.5 and TaqMan® MGB Probes for Allelic Discrimination: All PCR Instruments: User Bulletin	4317594
TaqMan® SNP Genotyping Assays: Protocol	4332856
Performing a Custom TaqMan Gene Expression Assay for 96-Well Plates: Quick Reference Card	4371398
Performing a Custom TaqMan Gene Expression Assay for 384-Well Plates: Quick Reference Card	4371393
Performing a Custom TaqMan SNP Genotyping Assay for 96-Well Plates: Quick Reference Card	4371394
Performing a Custom TaqMan SNP Genotyping Assay for 384-Well Plates: Custom TaqMan® SNP Genotyping Assays: Quick Reference Card	4371395
Custom TaqMan Gene Expression Assays Protocol	4334429
Custom TaqMan SNP Genotyping Assays Protocol	4334431
Custom TaqMan® Genomic Assays Submission Guidelines Protocol	4367671

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

TaqMan Universal PCR Master Mix -

Notice to Purchaser: Limited Label License

The use of uracil-N-glycosylase for carryover prevention is licensed by Life Technologies, Inc. under U.S. patents 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 Gene Expression and SNP Genotyping products -

Notice to Purchaser: Disclaimer of License

This product is optimized for use in the Polymerase Chain Reaction (PCR) and 5' nuclease detection methods covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd. No license under these patents to use the PCR process or 5' nuclease detection methods is conveyed expressly or by implication to the purchaser by the purchase of this product. A license to use the PCR process for certain research and development activities accompanies the purchase of certain Applied Biosystems reagents when used in conjunction with an authorized thermal cycler, or is available from Applied Biosystems. Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404.

Custom TaqMan Gene Expression and SNP Genotyping products -

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Primer Express software -

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