TaqMan® Genotyping Master Mix

Protocol

Applied Biosystems

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Preface

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Safety

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below.

Definitions

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

CAUTION – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD.** Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" on page vi.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

The MSDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field of the MSDS Search page:

a. Type in the chemical name, part number, or other information that you expect to appear in the MSDS of interest.

b. Select the language of your choice.

c. Click **Search**.

3. To view, download, or print the document of interest:

   a. Right-click the document title.

   b. Select:

      - **Open** – To view the document
      - **Save Target As** – To download a PDF version of the document to a destination that you choose
      - **Print Target** – To print the document

4. To have a copy of an MSDS sent by fax or e-mail, in the Search Results page:

   a. Select **Fax** or **Email** below the document title.

   b. Click **RETRIEVE DOCUMENTS** at the end of the document list.

   c. Enter the required information.

   d. Click **View/Deliver Selected Documents Now**.

Note: For the MSDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Chemical Waste Hazards

CAUTION **HAZARDOUS WASTE.** Refer to Material Safety Data Sheets and local regulations for handling and disposal.

WARNING **CHEMICAL WASTE HAZARD.** Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

WARNING **CHEMICAL STORAGE HAZARD.** Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological Hazard Safety

BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories*
  http://bmbl.od.nih.gov
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/nara/cfr/waisidx_01/ 29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

http://www.cdc.gov

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

Purpose

TaqMan® Genotyping Master Mix is a convenient 2× mixture of the components (except primers, probes, template, and water) required to perform the polymerase chain reaction (PCR) for SNP genotyping, including genotyping single nucleotide polymorphisms (SNPs). Use TaqMan Genotyping Master Mix with the genomic DNA target of your choice and any TaqMan® genotyping assay including:

- TaqMan® SNP Genotyping Assays
- Custom TaqMan® SNP Genotyping Assays
- TaqMan® Drug Metabolism Genotyping Assays
- TaqMan® Pre-Developed Assay Reagents for Allelic Discrimination

TaqMan genotyping assays, when used with TaqMan Genotyping Master Mix:

- Are designed and optimized to work using the same universal thermal cycling conditions
- Require only:
  - 1 to 10 ng of purified genomic DNA sample per plate well
  - 2× TaqMan Genotyping Master Mix
  - 20×, 40×, or 80× SNP Genotyping Assay (specific for each polymorphism)
  - One amplification step and an endpoint reading to obtain results

PCR amplification and plate read analysis for any TaqMan genotyping assay can be performed using any of the following systems:

- Applied Biosystems 7300/7500 Real-Time PCR Systems
- Applied Biosystems 7500 Fast/7900HT Fast Real-Time PCR Systems (in standard or 9600 emulation mode)
- ABI PRISM® 7000 Sequence Detection System

For only the PCR amplification step of your genotyping experiment, you can use the:

- GeneAmp® PCR System 9700 thermal cycler
- Applied Biosystems 9800 Fast Thermal Cycler (in standard mode)
- Applied Biosystems Veriti™ Thermal Cycler

About this Protocol

This protocol provides:

- Background information about genotyping assays
- A list of materials required to perform genotyping assays using the TaqMan Genotyping Master Mix
- Instructions for preparing reaction plates and performing PCR
- An overview of procedures for performing endpoint plate reads and analyzing results

For details about specific procedures in this protocol, see the appropriate genotyping assay protocol or instrument user guide. A procedural overview is also provided in the *TaqMan® Genotyping Master Mix Quick Reference Card* (PN 4371130).

Chemistry Overview

About TaqMan Genotyping Master Mix

TaqMan Genotyping Master Mix is optimized for use with TaqMan genotyping assays, but it can also be used with any appropriately designed primer and probe to detect any genomic DNA sequence. The mix contains:

- AmpliTaq Gold® DNA Polymerase, Ultra Pure (UP)
- Deoxyribonucleotide triphosphates (dNTPs)
- ROX™ Passive Reference
- Buffer components optimized for tight endpoint fluorescence clusters, reproducible allelic discrimination, and bench top stability

AmpliTaq Gold DNA Polymerase, Ultra Pure (UP)

AmpliTaq Gold® DNA Polymerase, a chemically modified form of AmpliTaq® DNA Polymerase, is a key ingredient in an automated, convenient, and efficient Hot Start PCR. The thermal incubation step required for activation generates active enzyme only at temperatures where the DNA is fully denatured.

When AmpliTaq Gold DNA Polymerase is added to the reaction mixture at room temperature, the primer cannot be extended because the enzyme is inactive. Therefore, any low-stringency mispriming events that may occur are not enzymatically extended and subsequently amplified.

The AmpliTaq Gold® DNA Polymerase, UP enzyme is identical to AmpliTaq Gold DNA Polymerase, but the enzyme is further purified to reduce bacterial DNA introduced from the host organism. The purification process ensures that nonspecific, false positive DNA products due to bacterial DNA contamination are minimized during PCR.

ROX Passive Reference

The ROX™ Passive Reference provides an internal reference to which the reporter-dye signal can be normalized during data analysis on Applied Biosystems instruments. Normalization is necessary to correct for fluorescence fluctuations due to changes in concentration or volume.

R_n Values

Normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the ROX Passive Reference. The resulting ratio is the R_n (normalized reporter) for a given reaction tube.

where:

$$R_n = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$$

About TaqMan Genotyping Assays

TaqMan® genotyping assays genotype single nucleotide polymorphisms (SNPs) using the 5′ nuclease assay for amplifying and detecting specific SNP alleles in purified genomic DNA samples. Each assay allows you to genotype individuals for a specific SNP.

Each TaqMan genotyping assay contains two primers for amplifying the sequence of interest and two TaqMan® MGB probes for detecting alleles. The presence of two probe pairs in each reaction allows genotyping of the two possible variant alleles at the SNP site in a DNA target sequence. The genotyping assay determines the presence or absence of a SNP based on the change in fluorescence of the dyes associated with the probes (see Table 1 on page 5).

TaqMan MGB Probes

The TaqMan® MGB Probes consist of target-specific oligonucleotides with:

- A reporter dye at the 5′ end of each probe:
  - VIC® dye is linked to the 5′ end of the Allele 1 probe.
  - 6FAM™ dye is linked to the 5′ end of the Allele 2 probe.
- A minor groove binder (MGB), which increases the melting temperature (T_m) without increasing probe length (Afonina *et al.,* 1997; Kutyavin *et al.,* 1997), thereby allowing the design of shorter probes. Shorter probes result in greater differences in T_m values between matched and mismatched probes, resulting in accurate allelic discrimination.
- A nonfluorescent quencher (NFQ) at the 3′ end of the probe.

Table 1 below shows the correlation between fluorescence signals and sequences in the sample.

Table 1 Fluorescence signal correlations

Fluorescence Increase	Indication
VIC dye fluorescence only	Homozygosity for allele 1
6FAM dye fluorescence only	Homozygosity for allele 2
Fluorescence signals for both dyes	Heterozygosity for allele 1-allele 2

Basics of the 5´ Nuclease Assay

During PCR:

- Genomic DNA is introduced into a reaction mixture consisting of TaqMan Genotyping Master Mix, forward and reverse primers and two TaqMan MGB Probes.
- Each TaqMan MGB Probe anneals specifically to a complementary sequence, if present, between the forward and reverse primer sites.

  When the probe is intact, the proximity of the quencher dye to the reporter dye suppresses the reporter fluorescence.
- As shown in Figure 1 on page 7, AmpliTaq Gold DNA Polymerase, UP cleaves only probes that are hybridized to the target.

  Cleavage separates the reporter dye from the quencher dye, increasing fluorescence by the reporter.

  The increase in fluorescence occurs only if the amplified target sequence is complementary to the probe. Thus, the fluorescence signal generated by PCR amplification indicates which alleles are in the sample.

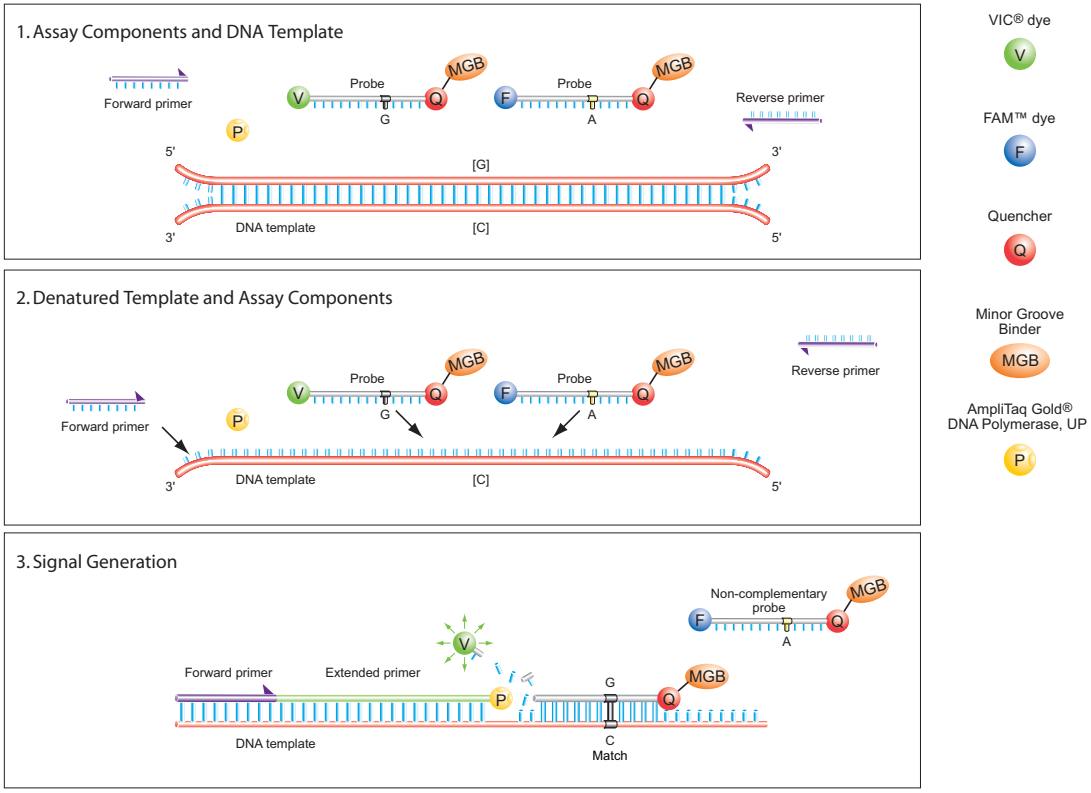

Figure 1 The complementary TaqMan probe fluoresces after it anneals to the template and after cleavage by AmpliTaq Gold DNA Polymerase, Ultra Pure (UP).

Materials and Equipment

Contents

TaqMan Genotyping Master Mix, supplied in a 2× concentration, is available from Applied Biosystems in the following volumes:

Item	Contents‡	Part Number
1-Pack	One 10-mL bottle (400 reactions)	4371355
2-Pack	Two 10-mL bottles (800 reactions)	4381656
Single Bulk Pack	One 50-mL bottle (2000 reactions)	4371357
Multi Bulk Pack	Two 50-mL bottles (4000 reactions)	4381657

‡ Based on a 50-µL reaction size.

Storage and Stability

When you receive TaqMan Genotyping Master Mix, store it at 2 to 8 °C. TaqMan Genotyping Master Mix is stable through the date on the package and bottle label when stored at 2 to 8 °C.

If TaqMan Genotyping Master Mix is stored at –20 °C, transfer it to 2 to 8 °C. Applied Biosystems does not recommend storing TaqMan Genotyping Master Mix at temperatures other than 2 to 8 °C or using TaqMan Genotyping Master Mix after the date printed on the package and bottle label.

Before using the Master Mix, make sure it is thoroughly thawed and mixed.

Recommended Instruments

The following instruments are recommended when using TaqMan Genotyping Master Mix.

Instrument	Source
Applied Biosystems 7300 Real-Time PCR System	Contact your Applied Biosystems sales representative.
Applied Biosystems 7500 Real-Time PCR System	
Applied Biosystems 7500 Fast Real-Time PCR System	
Applied Biosystems 7900HT Fast Real-Time PCR System	
ABI PRISM® 7000 Sequence Detection System	
GeneAmp® PCR System 9700 thermal cycler	
Applied Biosystems 9800 Fast Thermal Cycler	
Applied Biosystems Veriti™ Thermal Cycler	

Reagents and Plastics Not Supplied

The following items are not supplied with the TaqMan Genotyping Master Mix.

Reagents and plastics from Applied Biosystems

Material	Applied Biosystems Part Number
Sequence Detection Primers • 10,000 pmol • 80,000 pmol • 130,000 pmol	4304970 4304971 4304972
TaqMan® MGB Probe • 6,000 pmol • 20,000 pmol • 50,000 pmol	4316034 4316033 4316032

Reagents and plastics from Applied Biosystems *(continued)*

Material	Applied Biosystems Part Number
Custom TaqMan® SNP Genotyping Assays	
• Small-Scale, human 40× concentration (1,000 × 5-µL reactions)	4331349
• Small-Scale, non-human 40× concentration (1,000 × 5-µL reactions)	4332077
• Medium-Scale, human 40× concentration (3,000 × 5-µL reactions)	4332072
• Medium-Scale, non-human 40× concentration (3,000 × 5-µL reactions)	4332075
• Large-Scale, human 80× concentration (12,000 × 5-µL reactions)	4332073
• Large-Scale, non-human 80× concentration (12,000 × 5-µL reactions)	4332076
TaqMan® Pre-Designed SNP Genotyping Assays	
• Small-Scale, 40× concentration (1,500 × 5-µL reactions)	4351379
• Medium-Scale, 40× concentration (5,000 × 5-µL reactions)	4351376
• Large-Scale, 80× concentration (12,000 × 5-µL reactions)	4351374
TaqMan® Validated and Coding Genotyping Assays	
Small-Scale, 20× concentration (750 × 5-µL reactions)	4331183
TaqMan® Pre-Developed Assay Reagents for Allelic Discrimination	
• CYP2C19*2, (400 reactions)	4312561
• CYP2C9*2, (400 reactions)	4312559
• CYP2C9*3, (400 reactions)	4312560
• CYP2D6*3, (400 reactions)	4312554
• CYP2D6*4, (400 reactions)	4312555
• CYP2D6*6, (400 reactions)	4312556
• CYP2D6*7, (400 reactions)	4312557

Reagents and plastics from Applied Biosystems *(continued)*

Material	Applied Biosystems Part Number
• CYP2D6*8, (400 reactions)	4312558
TaqMan® Drug Metabolism Genotyping Assays • Includes CD with protocol, Assay Information File (AIF), DME Assay Index, and Troubleshooting Guide. • For a list of assays go to: **http://www.appliedbiosystems.com** and search TaqMan Drug Metabolism Assay	appliedbiosystems.com
PrepMan® Ultra Sample Preparation Reagent 100 purifications	4318930
MicroAmp™ Optical 96-Well Reaction Plate with Barcode, (quantity 500)	4326659
MicroAmp™ Optical 96-Well Reaction Plate with Barcode, (quantity 20)	4306737
MicroAmp™ Optical 384-Well Reaction Plate with Barcode, (quantity 50)	4309849
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1-mL (quantity 20)	4346906
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (quantity 200)	4366932
MicroAmp™ Optical 8-Tube Strip, 0.2-mL (quantity 1000 tubes in strips of 8)	4316567
MicroAmp™ Optical 8-Cap Strip (300 strips)	4323032
MicroAmp™ Optical Adhesive Film (quantity 100)	4311971
MicroAmp™ Optical Adhesive Film Kit	4313663
MicroAmp™ Optical Adhesive Film (quantity 25)	4360954
MicroAmp™ Clear Adhesive Films (quantity 100)	4306311
MicroAmp™ Optical Film Compression Pad **Note:** This is not compatible with the Applied Biosystems 7300, 7500, 7500 Fast, and 7900HT Fast Real-Time PCR Systems.	4312639

Reagents and plastics from Applied Biosystems *(continued)*

Material	Applied Biosystems Part Number
MicroAmp™ Snap-On Optical Film Compression Pad	4333292
MicroAmp™ Multi Removal Tool	4313950

User-Supplied Materials from Other Sources

Material	Part Number
Accessories for tubes of assay mixes • Decapper for single caps • Decapper for eight caps • TPE cap cluster for simultaneously capping 96 individual polypropylene tubes, 50 capmats/bag	Micronic BV‡ PO Box 604 8200 AP Lelystad Netherlands Telephone: 0031.320.277.090 Fax: 0031.320.277.088 United States Telephone: 724.941.6411 Fax: 724.941.8662 www.micronic.com
DNase-free, RNase-free sterile-filtered water	major laboratory supplier (MLS)
Centrifuge with plate adapter	MLS
Disposable gloves	MLS
Microcentrifuge	MLS
Microsoft Excel® or equivalent spreadsheet and analysis software	Software suppliers
Pipette tips, aerosol resistant	MLS
Pipettors: • Positive-displacement • Air-displacement • Multichannel	MLS

Material	Part Number
Polypropylene tubes	MLS
Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, made using DNase-free, RNase-free sterile-filtered water)	MLS
Vortexer	MLS

‡ Other vendors supply similar products

Optional User-Supplied Reagents

For a description of these reagents, go to:

www.ambion.com/techlib/index

Materials	Part Number
DNAZap™ Solution, 2, 250-mL bottles	AM9890
RT-PCR Grade Water, 10, 1.75-mL bottles	AM9935
TRI Reagent®, 100-mL	AM9738

Applied Biosystems Documents

You can download these and other documents from the Applied Biosystems Documents on Demand Web site at **http://docs.appliedbiosystems.com/search.taf**

Document	Applied Biosystems Part Number
TaqMan® Genotyping Master Mix Quick Reference Card	4371130
TaqMan® SNP Genotyping Assays Protocol	4332856
Real-Time PCR Systems Chemistry Guide	4348358
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Allelic Discrimination Getting Started Guide	4347822
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide	4347828
Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide	4351684
ABI PRISM® 7000 Sequence Detection System User Guide	4330228
GeneAmp® PCR System 9700 thermal cycler User's Manuals: • *Base Module* • *96-Well Sample Block Module* • *Dual 384-Well Sample Block Module* • *0.5-mL Sample Block Module* • *Auto-Lid Dual 96 Sample Block Module and Dual 96 Sample Block Module* • *Auto-Lid Dual 384 Sample Block Module*	 4303481 4316011 4304215 4307808 4343363 4310838
Applied Biosystems 9800 Fast Thermal Cycler User Guide	4350087
Applied Biosystems Veriti™ Thermal Cycler User Guide	4375799

PCR Amplification

The first step in a genotyping assay is PCR amplification, which involves:

- Preparing the reaction mix (page 21)
- Preparing an optical reaction plate (page 24)
- Performing PCR (page 28)

Before You Begin

Use the following sections to prepare for PCR amplification:

- Preventing Contamination
- Selecting a Method for Adding DNA
- Selecting an Instrument and Reaction Plate
- Quantifying Genomic DNA

Preventing Contamination

PCR assays require special laboratory practices to avoid false positive amplifications (Kwok and Higuchi, 1989). The high throughput and repetition of these assays can lead to amplification of a single DNA molecule (Saiki *et al.*, 1985; Mullis and Faloona, 1987). Follow these recommended general PCR procedures to avoid contamination:

- Wear a clean lab coat and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Keep reactions and components covered or capped as much as possible.

- Keep plates covered or capped between the PCR run and the allelic discrimination plate read.
- Use a positive-displacement pipette or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or 3% hydrogen peroxide.

Selecting a Method for Adding DNA

Table 2 describes the two methods for adding genomic DNA to the reaction. Decide which method to use based on your experimental needs.

Table 2 Methods for adding DNA

Method	Method Description	Experimental Use
Wet DNA delivery	1. Aliquot genotyping reaction mix to an optical reaction plate. 2. Deliver genomic DNA to the final reaction mix. **Note:** The liquid used to resuspend the DNA is used as a component of the final reaction.	For testing a limited number of SNP targets in many different DNA templates
DNA predelivery and dry-down	1. Deliver genomic DNA sample to the bottom surface of an optical reaction plate. 2. Dry-down the sample completely by evaporation. 3. Add the PCR reaction mix, and the DNA disperses in the final reaction mix.	• For repeated testing of different SNP targets on a limited number of DNA templates • For preparing a large number of DNA samples that, after drying down, can be stored before use

Selecting an Instrument and Reaction Plate

You can perform PCR amplification with any of the instruments and compatible plates shown in Table 3 on page 18. However, if you use a thermal cycler for PCR amplification, you must subsequently perform the endpoint plate read separately on a Real-Time PCR System in order to detect and record the fluorescent signals generated by the cleavage of TaqMan probes.

IMPORTANT! For the 7500 Fast System, TaqMan Genotyping Master Mix is supported for use with Fast Optical 96-Well Reaction Plates only with Standard mode thermal cycling conditions. TaqMan Genotyping Master Mix is not for use with Fast mode thermal cycling conditions.

IMPORTANT! For the 7900HT Fast System, TaqMan Genotyping Master Mix is *not* supported for thermal cycling with Fast Optical 96-Well Reaction Plates. Use either Optical 384-Well Reaction Plates or standard Optical 96-Well Reaction Plates with Standard mode thermal cycling conditions. TaqMan Genotyping Master Mix is not for use with Fast mode thermal cycling conditions.

Table 3 Instruments and reaction plates appropriate for PCR amplification

Instrument	Compatible Plate	Reaction Volume (µL)
• Applied Biosystems 7300 Real-Time PCR System • Applied Biosystems 7500 Real-Time PCR System • ABI PRISM® 7000 Sequence Detection System	MicroAmp™ Optical 96-Well Reaction Plate	25
Applied Biosystems 7500 Fast Real-Time PCR System (in standard mode)	MicroAmp™ Fast Optical 96-Well Reaction Plate	10
Applied Biosystems 7900HT Fast Real-Time PCR System (in standard mode)	• MicroAmp™ Optical 384-Well Reaction Plate • MicroAmp™ Optical 96-Well Reaction Plate (with Standard 96-Well Block)	5 25
GeneAmp® PCR System 9700 thermal cycler	• MicroAmp™ Optical 384-Well Reaction Plate • MicroAmp™ Optical 96-Well Reaction Plate	5 25
Applied Biosystems 9800 Fast Thermal Cycler (in standard mode)	MicroAmp™ Fast Optical 96-Well Reaction Plate	10
Applied Biosystems Veriti™ Thermal Cycler Model 9901	MicroAmp™ Fast Optical 96-Well Reaction Plate‡	10
Applied Biosystems Veriti™ Thermal Cycler Model 9902	MicroAmp™ Optical 96-Well Reaction Plate‡	25

‡ Requires 96-well tray for VeriFlex™ systems (PN 4379983)

Quantifying Genomic DNA

For a TaqMan genotyping assay, add 1 to 10 ng of DNA template per reaction well. Quantitate genomic DNA using a reliable method such as A_{260} measurements or real-time quantification by RNase P. If you use the RNase P method, generate a standard curve using the DNA template standards in the TaqMan® DNA Template Reagents Kit (PN 401970) and the RNase P gene primers and probe in the TaqMan RNase P Detection Reagents Kit (PN 4316831). For additional information on generating a standard curve, refer to *Creating*

Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR at **http://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf**.

Genotyping Workflow

Figure 2 summarizes the procedures for performing a genotyping experiment.

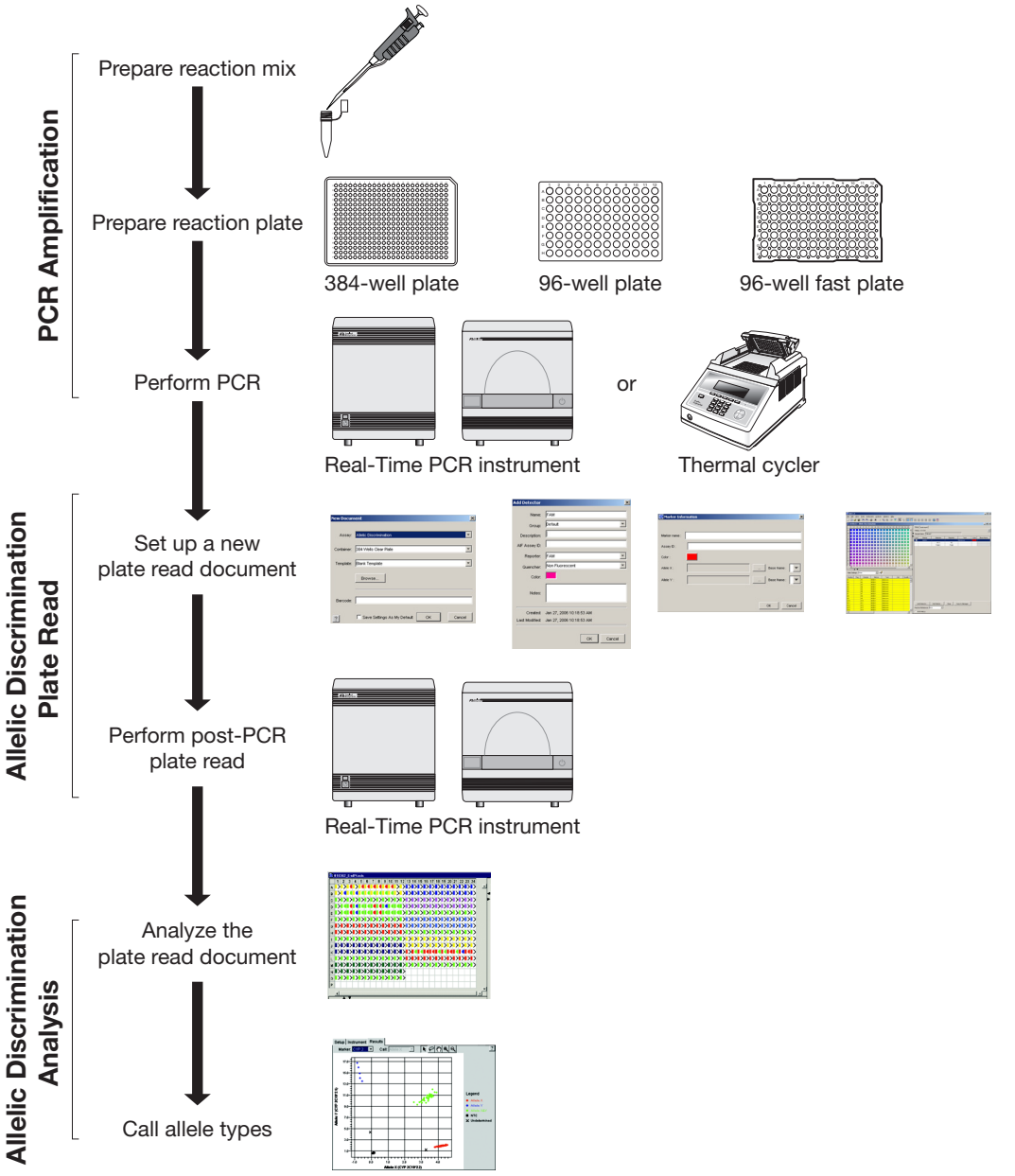

Figure 2 Overview of a genotyping experiment

Preparing the Reaction Mix

Guidelines

For optimal PCR performance:

- Keep all TaqMan reagents protected from light until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
- Minimize freeze-thaw cycles.
- Prior to use:
  - Mix the TaqMan Genotyping Master Mix thoroughly by swirling the bottle.
  - Thaw any frozen TaqMan reagents by placing them on ice. When thawed, resuspend the samples by vortexing, then centrifuge the tubes briefly.
  - Resuspend the TaqMan reagents (for example, the TaqMan genotyping assay mix) by vortexing, then centrifuge the tube briefly.
  - Thaw any frozen genomic DNA samples by placing them on ice. When thawed, resuspend the samples by vortexing, then centrifuge the tubes briefly.
- Prepare the PCR reaction mix for each assay before transferring it to the optical reaction plate for thermal cycling and fluorescence analysis.

WARNING CHEMICAL HAZARD. SNP Genotyping Assay (<2% formamide). Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

CAUTION CHEMICAL HAZARD. TaqMan Genotyping Master Mix may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare the reaction mix:

1.	Calculate the number of reactions to be performed for each assay: • Include extra reactions (approximately one extra reaction for every 10 required reactions) in your calculation to provide excess volume for the loss that occurs during reagent transfers. For example, for a 96-well plate, prepare enough volume for approximately 110 reactions. • Include at least two no template controls (NTCs) and (if needed) at least one genomic DNA control of known genotype on each plate to ensure accurate genotype calling. **Note:** You can run multiple genotyping assays on one reaction plate. Include controls for each assay run on the plate.

To prepare the reaction mix: ***(continued)***

2. Calculate the volume of each reaction mix component needed for each assay by multiplying the appropriate volume from the table below by the number of reactions determined in step 1.

PCR Reaction Mix Volume (µL/Well)			
Component	**5-µL Reaction (384-well plate)**	**10-µL Reaction (96-well fast plate)‡**	**25-µL Reaction (96-well standard plate)**
Wet DNA Delivery Method			
TaqMan Genotyping Master Mix (2X)	2.50	5.0	12.50
TaqMan genotyping assay mix§ (20X)	0.25	0.5	1.25
DNase-free, RNase-free water	(none)	(none)	(none)
Total	2.75	5.5	13.75
DNA Predelivery and Dry-Down Method			
TaqMan Genotyping Master Mix (2X)	2.50	5.0	12.50
TaqMan genotyping assay mix§ (20X)	0.25	0.5	1.25
DNase-free, RNase-free water	2.25	4.5	11.25
Total	5.0	10.0	25.00

‡ When using TaqMan Genotyping Master Mix, use fast reaction plates only on the 7500 Fast Real-Time PCR System or 9800 Fast Thermal Cycler, and only with Standard mode thermal cycling conditions.

§ For ease of use, dilute 40X and 80X Assay Mixes to 20X working solutions with 1X TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Use DNase-free, RNase-free water.

IMPORTANT! If you use Custom TaqMan Probes and Sequence Detection Primers in place of a TaqMan genotyping assay, Applied Biosystems recommends concentrations of 900 nM for primers and 250 nM for probes.

To prepare the reaction mix: *(continued)*

3.	Swirl the bottle of 2× TaqMan Genotyping Master Mix gently to mix.
4.	Vortex and centrifuge the 20× genotyping assay mix briefly.
5.	Pipette the required volumes of 2× TaqMan Genotyping Master Mix, 20× genotyping assay mix, and (for dry-down method only) DNase-free, RNase-free water into a sterile tube.
6.	Cap the tube(s).
7.	Vortex the tube(s) briefly to mix the solutions.
8.	Centrifuge the tube(s) briefly to spin down the contents and to eliminate air bubbles from the solution.

Preparing the Reaction Plate with the Wet DNA Delivery Method

To prepare using the wet DNA delivery method:

1. Pipette the reaction mix (see "Preparing the Reaction Mix" on page 21) into each well of a reaction plate. Use the volumes in the following table.

Reaction Plate	Volume of Reaction Mix (µL)
MicroAmp™ Optical 384-Well Reaction Plate	2.75
MicroAmp™ Fast Optical 96-Well Reaction Plate	5.50
MicroAmp™ Optical 96-Well Reaction Plate	13.75

2. Inspect each well for volume uniformity, noting which wells do not contain the proper volume.
3. Cover the plate with MicroAmp Optical Adhesive Film.
4. Centrifuge the plate briefly to spin down the contents and eliminate any air bubbles from the solutions.
5. Dilute 1 to 10 ng of each purified genomic DNA sample into DNase-free, RNase-free water.

To prepare using the wet DNA delivery method: ***(continued)***

6. Pipette samples into the plate:

   a. Remove the clear adhesive film from the plate.

   b. Into each well of the plate, pipette one control or diluted DNA sample, using the volumes in the following table. Use a calibrated, positive-displacement pipettor to minimize contamination and error.

Reaction Plate	Volume of DNA Sample and DNase-Free, RNase-Free Water Per Reaction (µL)
MicroAmp™ Optical 384-Well Reaction Plate	2.25
MicroAmp™ Fast Optical 96-Well Reaction Plate	4.50
MicroAmp™ Optical 96-Well Reaction Plate	11.25

   IMPORTANT! Be sure that no cross-contamination occurs from well to well.

7. Cover the plate with MicroAmp Optical Adhesive Film or MicroAmp Optical Caps if you are using a MicroAmp™ Optical 96-well plate.

   IMPORTANT! Use a MicroAmp™ Optical Film Compression Pad when using MicroAmp™ Optical Adhesive Film with a:

   - MicroAmp™ Optical 96-well plate on the ABI Prism 7000 Sequence Detection System
   - MicroAmp™ Optical 96-well plate on the 7900HT or 9800 Fast Real-Time PCR System
   - A MicroAmp™ Optical 96- or 384-well plate on the GeneAmp® PCR System 9700 thermal cycler

8. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions.

Preparing the Reaction Plate with the DNA Dry-Down Method

To prepare the reaction plate using the DNA predelivery and dry-down method:

1. Pipette and dry the sample:
   a. Pipette one control or sample (1 to 10 ng of purified genomic DNA) into each well of a MicroAmp™ Optical 96- or 384-Well Reaction Plate. Use a calibrated, positive-displacement pipettor to minimize contamination and error.

      Note: Use sample volumes of 2 to 5 µL to minimize drying time.

   b. Dry down the samples completely by evaporation at room temperature in a dark, amplicon-free location.
2. Pipette the reaction mix (see "Preparing the Reaction Mix" on page 21) into each well using the volumes in the following table.

Reaction Plate	Volume of Reaction Mix (µL)
MicroAmp™ Optical 384-Well Reaction Plate	5
MicroAmp™ Fast Optical 96-Well Reaction Plate	10
MicroAmp™ Optical 96-Well Reaction Plate	25

3. Inspect each well for volume uniformity, noting which wells do not contain the proper volume.

To prepare the reaction plate using the DNA predelivery and dry-down method: *(continued)*

4.	Cover the plate with MicroAmp Optical Adhesive Film or MicroAmp Optical Caps if you are using a MicroAmp™ Optical 96-well plate. **IMPORTANT!** Use a MicroAmp™ Optical Film Compression Pad when using MicroAmp™ Optical Adhesive Film with a: • MicroAmp™ Optical 96-well plate on the ABI Prism 7000 Sequence Detection System • MicroAmp™ Optical 96-well plate on the 7900HT or 9800 Fast Real-Time PCR System • A MicroAmp™ Optical 96- or 384-well plate on the GeneAmp® PCR System 9700 thermal cycler
5.	Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions.

Performing PCR

Refer to the appropriate instrument user guide for help with programming the thermal cycling conditions or with running the plate.

To perform PCR:

1. Use the thermal cycling conditions specified in the following table:

   IMPORTANT! These conditions are optimized for use only with TaqMan genotyping assays on the instruments and reaction plates specified in Table 3, "Instruments and reaction plates appropriate for PCR amplification," on page 18. Because of differences in ramp rates and thermal accuracy, you may need to adjust the thermal cycling settings if you use a thermal cycler not listed in Table 3 on page 18.

Step	Temp (°C)	Duration	Cycles
AmpliTaq Gold, UP Enzyme Activation	95	10 min	HOLD
Denature	95	15 sec	40
Anneal/Extend	60	1 min	

2. In the plate document, select the **Standard** mode thermal cycling setting.

   IMPORTANT! TaqMan Genotyping Master Mix is not for use with Fast Mode thermal cycling conditions. If you use TaqMan Genotyping Master Mix on the 7500 Fast or 7900HT Fast instruments, or the 9800 Fast Thermal Cycler, use only Standard mode thermal cycling conditions. Also, if you use TaqMan Genotyping Master Mix with thermal cycling conditions other than those specified in this protocol or with assays other than the TaqMan genotyping assays, reoptimize your thermal cycling conditions and validate your assays. Refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4348358) for information on selecting thermal cycling conditions.

To perform PCR: ***(continued)***

3. Set the reaction volume according to the following table.

Plate Format	Reaction Volume (µL)
MicroAmp™ Optical 384-Well Reaction Plate	5
MicroAmp™ Fast Optical 96-Well Reaction Plate	10
MicroAmp™ Optical 96-Well Reaction Plate	25

4. Load the reaction plate into the thermal cycler, then start the run.

Additional PCR References

For more information about performing PCR amplification, refer to the appropriate user guide:

- *Real-Time PCR Systems Chemistry Guide* (PN 4348358)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Allelic Discrimination Getting Started Guide* (PN 4347822)
- *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684)
- *ABI PRISM® 7000 Sequence Detection System User Guide* (PN 4330228)
- *GeneAmp® PCR System 9700 thermal cycler User's Manuals* (see list on page 14)
- *Applied Biosystems 9800 Fast Thermal Cycler User Guide* (PN 4350087)
- *Applied Biosystems Veriti™ Thermal Cycler User Guide* (PN 4375799)

Allelic Discrimination Plate Read and Analysis

Overview

After PCR amplification, perform an endpoint plate read on a Real-Time PCR instrument. Using the fluorescence measurements made during the plate read, the SDS software plots R_n values based on the fluorescence signals from each well, then determines which alleles are in each sample.

The general process for analyzing data for genotyping involves:

- Creating and setting up an allelic discrimination plate read document
- Performing an allelic discrimination plate read on a real-time PCR instrument
- Analyzing the plate read document
- Making automatic or manual allele calls
- Verifying allele types

Note: Refer to the allelic discrimination section of the appropriate instrument user guide for instructions on how to use the system software to perform the plate read and analysis.

Viewing Assay Results

The SDS software plots the results of the allelic discrimination run on a scatter plot of Allele 1 versus Allele 2. Each well of the 96-well or 384-well reaction plate is represented as an individual point on the plot.

Figure 3 on page 31 shows variation in clustering due to the genotype of the target allele.

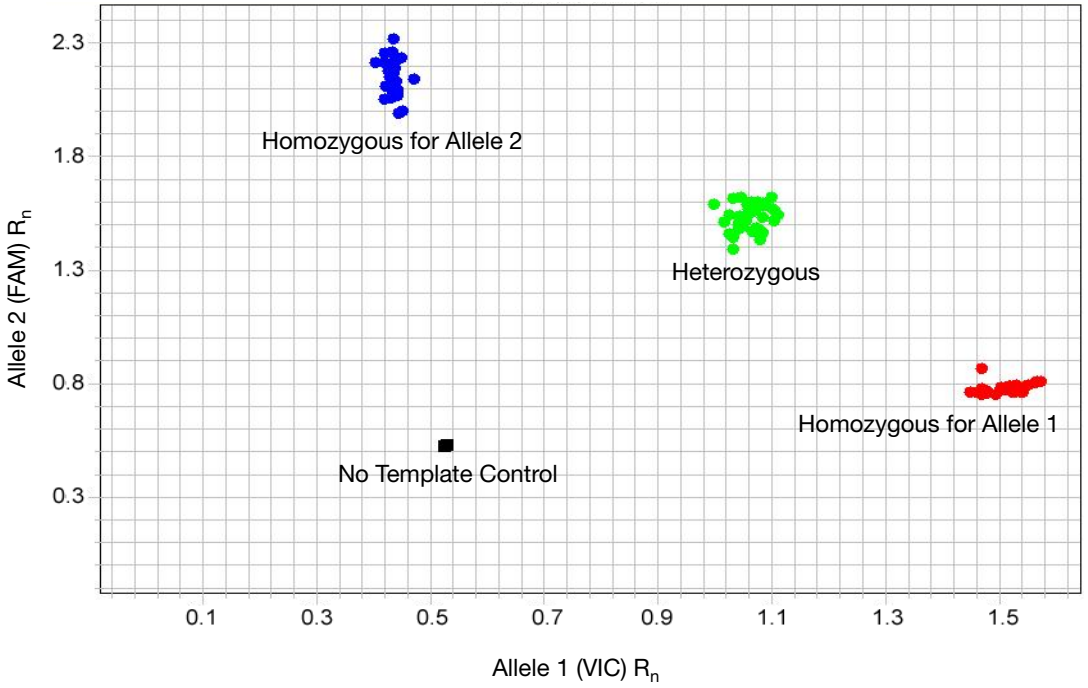

Figure 3 Allelic Discrimination plot

Resources for Data Analysis

Data analysis varies depending on the instrument. Refer to the following documents for more information about analyzing your data:

- The appropriate instrument user guide
- *Real-Time PCR Systems Chemistry Guide* (PN 4348358)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Allelic Discrimination Getting Started Guide* (PN 4347822)
- *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684)
- *ABI PRISM® 7000 Sequence Detection System User Guide* (PN 4330228)
- The Applied Biosystems web site for a variety of tutorials on performing TaqMan® genotyping assays, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

Appendix A Troubleshooting

Troubleshooting Endpoint Analysis

Observation	Possible Cause	Recommended Action
Distinct clusters are not observed or a sample does not cluster with one specific allele type	Inappropriately assigned reporter dyes or improperly selected quencher dye	1. Verify the dye settings. 2. Reanalyze the plate read.
	A sample may contain: • More or less DNA than other samples • A rare allelic variation or sequence duplication • Mixtures of multiple alleles or contamination • Copy number variation	1. Recheck the DNA concentrations of the samples, using one of the procedures referred to in "Quantifying Genomic DNA" on page 18. 2. Retest the sample to verify that clusters are now distinct. 3. Test the sample using a different genotyping assay.
	Inaccurate reagent delivery or evaporation occurred	• Check each well for a variation in volume, then redo any assay that did not contain the proper volume. • Use compression pads when using optical adhesive film with a: – standard 96-well plate on the ABI PRISM 7000 Sequence Detection System – standard 96-well plate on the 7900HT Fast Real-Time PCR System – standard 96-well plate or 384-well plate on the GeneAmp® PCR System 9700 thermal cycler – fast 96-well plate on the 9800 Fast Thermal Cycler
	Bubbles in the wells	Prepare a new reaction plate, making sure you centrifuge the plate before you perform PCR.
	ROX™ dye was not selected as the passive reference	Select ROX dye as the passive reference when you set up the plate document.
	Reaction components not mixed well	Follow mixing procedures in the protocol.

Troubleshooting Endpoint Analysis *(continued)*

Observation	Possible Cause	Recommended Action
Unknown genomic samples did not generate fluorescence signals	The sample may: • Contain no DNA • Contain PCR inhibitors • Be homozygous for a rare allelic variation • Amplify poorly • Be degraded	1. Recheck the DNA concentrations of the samples using: a. One of the procedures referred to in "Quantifying Genomic DNA" on page 18. b. The Quantifiler® Human DNA Quantification Kit (PN 4343895) 2. Test a positive control: Control DNA CEPH Individual 1347-02 (PN 403062). 3. Retest the sample to verify that samples now generate fluorescence. 4. Test the sample using a different genotyping assay if the problem still occurs.
Small R_n	PCR efficiency is poor	• If using non-Applied Biosystems assays, reoptimize for primer and probe concentration. • Increase annealing/extension time to 90 seconds and increase number of cycles to 50.
	Low copy number of target	Increase input template quantity.
Samples on plate edges fail to be called	Non-uniform thermal contact	Use compression pads when using optical adhesive film with a: • standard 96-well plate on the ABI PRISM 7000 Sequence Detection System • standard 96-well plate on the 7900HT Fast Real-Time PCR System • standard 96-well plate or 384-well plate on the GeneAmp® PCR System 9700 thermal cycler • fast 96-well plate on the 9800 Fast Thermal Cycler
Scale (x and y axis values) missing on Allelic Discrimination Plot	ROX dye was not selected as the passive reference when the plate document was set up	Select ROX dye as the passive reference when you set up the plate document.

Troubleshooting Endpoint Analysis *(continued)*

Observation	Possible Cause	Recommended Action
ROX signal is low	• Reaction volume too low • Poor mixing of reagents	1. Confirm reaction volumes are within recommended range. 2. Confirm pipettors are calibrated. 3. Increase reaction volume.
	Instrument is off calibration	Re-calibrate instrument.

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