TaqMan® PCR Reagent Kit with Controls

With AmpliTaq Gold® DNA Polymerase

Protocol

Applied Biosystems

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

Information in this document is subject to change without notice. Applied Biosystems assumes no responsibility for any errors that may appear in this document.

APPLIED BIOSYSTEMS DISCLAIMS ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. IN NO EVENT SHALL APPLIED BIOSYSTEMS BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF.

NOTICE TO PURCHASER: LIMITED LICENSE

Use of this product is covered by US patent claims and patent claims outside the US. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim (such as the patented 5' Nuclease Process claims) and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

The use of uracil-N-glycosylase for carryover prevention is licensed by Invitrogen Corporation 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 Invitrogen Corporation are covered by the purchase of this product.

Printed in the USA, 07/2010
Part Number 402823 Rev. G

Contents

1 Introduction

Overview

About This Chapter

This chapter describes the TaqMan® PCR Reagent Kit with Controls and provides important information about safety.

In This Chapter

The following topics are discussed in this chapter:

Purpose of the Kit

Overview

The TaqMan® PCR Reagent Kit with Controls (PN N8080230) provides a system for determining the presence or absence of a specific sequence. Direct detection of PCR product with no downstream processing is accomplished within minutes of PCR completion by monitoring the increase of fluorescence of a dye-labeled DNA probe. This method permits the analysis of thousands of samples per day with high sample-to-sample reproducibility.

This method can be incorporated into an established PCR system by designing a fluorescent DNA probe specific to the PCR target sequence flanked by PCR primers. The established PCR system should be optimized for yield and contain AmpErase® uracil-N-glycosylase (UNG) for PCR product carryover prevention.

Basics of the 5´ Nuclease Assay

The 5´ nuclease reaction exploits the 5´ nuclease activity of the AmpliTaq Gold® DNA Polymerase to cleave a TaqMan probe during PCR. The TaqMan probe contains a reporter dye at the 5´ end of the probe and a quencher dye at the 3´end of the probe. During the reaction, the reporter dye and quencher dye become separated, resulting in increased fluorescence of the reporter (see Figure 1-1 on page 3). Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye (see Figure 1-2 on page 4).

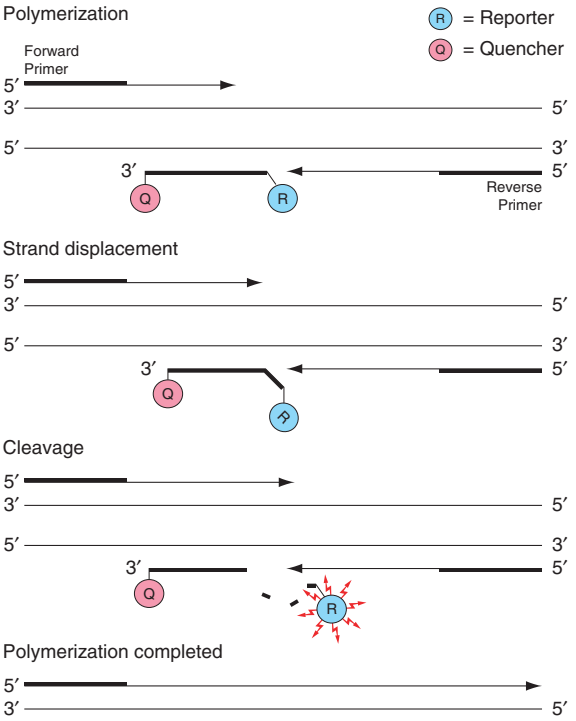


Figure 1-1 The fork-like-structure-dependent, polymerization associated, 5′ to 3′ nuclease activity of AmpliTaq Gold DNA Polymerase during PCR.

When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Förster, 1948; Lakowicz, 1983). During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites.

The 5′ to 3′ nucleolytic activity of the AmpliTaq Gold DNA Polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3′ end of the probe is blocked to prevent extension of the probe during PCR.

This process occurs in every cycle and does not interfere with the exponential accumulation of product.

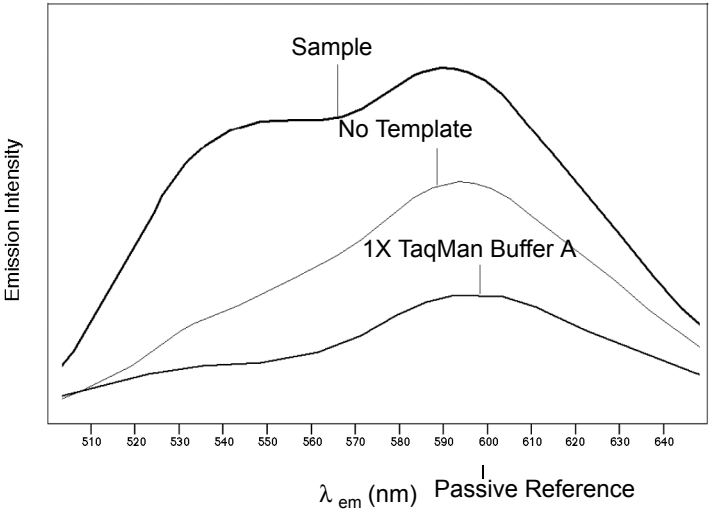


Figure 1-2 Overlay of three emission scans, post-PCR.

The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, nonspecific amplification is not detected.

TaqMan Probe

The TaqMan probe consists of an oligonucleotide with a 5´-reporter dye and a 3´-quencher dye. A fluorescent reporter dye, such as FAM™ dye, is covalently linked to the 5´ end of the oligonucleotide.

TET™ dye and VIC® dye have also been used as reporter dyes. Each of the reporters is quenched by TAMRA™ dye, or non-fluorescent quencher attached via a linker arm that is usually located at the 3´end.

Kit Reagents

The control primers and probe, supplied with the reagent kit, detect the amplification of a region in exon 3 of the human β-actin gene (du Breuil *et al.*, 1993). During amplification, the β-actin control generates a reporter fluorescence signal that is at least three times greater than that of the No Template Control.

The FAM dye-labeled β-actin TaqMan probe supplied with the TaqMan PCR Reagent Kit with Controls can only be used to detect amplification of the specific template included in the kit. A custom probe must be designed for detection of any other template.

Materials and Equipment

Kit Components

The TaqMan PCR Reagent Kit with Controls (PN N8080230) contains enough PCR reagents for up to 200 50-µL reactions. The kit includes enough primers, probe, and template for 100 control reactions, and comprises two boxes:

- TaqMan PCR Core Reagents Kit (PN N8080228)
- TaqMan β-actin Detection Reagents (PN 401846)

Both the TaqMan PCR Reagent Kit with Controls and the TaqMan PCR Core Reagents Kit can be purchased with or without this protocol booklet.

Table 1-1 TaqMan PCR Core Reagents Kit contents–200 reactions, 50 µL each

Item	Volume	Description
AmpErase® UNG	100 µL	One tube of 1 U/µL uracil N-glycosylase (PN N8080096 for AmpErase UNG alone; PN N8080068 for AmpErase UNG with dUTP)
dUTP	320 µL	One tube of 20 mM deoxyuridine triphosphate dissolved in autoclaved, deionized, ultrafiltered water; titrated with NaOH to pH 7.0
dATP	320 µL	One tube each of 3.2 µmol of dATP, dCTP, or dGTP at 10 mM concentration in autoclaved, deionized, ultrafiltered water; titrated with NaOH to pH 7.0
dCTP	320 µL	
dGTP	320 µL	
AmpliTaq Gold® DNA Polymerase	50 µL	One tube containing 5 U/µL AmpliTaq Gold® DNA Polymerase (PN N8080240)
10X TaqMan Buffer A	1.2 mL	One tube of 500 mM KCl, 100 mM Tris-HCl, 0.1 mM EDTA, 600 nM Passive Reference 1, pH 8.3 at room temperature (solution has been autoclaved)
25 mM $MgCl_2$	3.0 mL	Two tubes of 25 mM $MgCl_2$, 1.5 mL each
TaqMan® PCR Reagent Kit with Controls Protocol		Applied Biosystems (PN 402823)

Table 1-2 TaqMan β-actin Detection Reagents contents–100 reactions, 50 μL each

Reagent	Volume (μL)	Description
β-actin Forward Primer	500	One tube of 3 μM primer in 10 M Tris-HCl, pH 8.0 (at room temperature); 1 mM EDTA (ethylenediaminetetraacetic acid)
β-actin Reverse Primer	500	One tube of 3 μM primer in 10 mM Tris-HCl, pH 8.0 (at room temperature);1 mM EDTA
β-actin Probe (FAM)	500	One tube of 2 μM FAM dye-labeled probe in 10 mM Tris-HCl, pH 8.0 (at room temperature); 1 mM EDTA
Human DNA Male	100	One tube of 10 ng/μL of human genomic DNA formulated in 10 mM Tris-HCl, pH 8.0 (at room temperature); 1 mM EDTA

Materials Required but Not Supplied

Table 1-3 and Table 1-4 show items required when using the TaqMan PCR Reagent Kit with Controls, but are not supplied. See each table for source information.

Table 1-3 User-Supplied Materials

Item	Source
Probes and Primers	
Sequence Detection primers ♦ 10,000 pmoles ♦ 80,000 pmoles ♦ 130,000 pmoles	Applied Biosystems ♦ PN 4304970 ♦ PN 4304971 ♦ PN 4304972
TaqMan® MGB Probe ♦ 6000 pmoles ♦ 20,000 pmoles ♦ 50,000 pmoles	Applied Biosystems ♦ PN 4316034 ♦ PN 4316033 ♦ PN 4316032
TaqMan® TAMRA Probe ♦ 5000 to 6000 pmoles ♦ 20,000 pmoles ♦ 50,000 pmoles	Applied Biosystems ♦ PN 450025 ♦ PN 450024 ♦ PN 450003
Reaction Plates, Tubes, Caps and Covers	
MicroAmp™ Optical 96-Well Reaction Plate with Barcode and Optical Caps	Applied Biosystems (PN 403012)
MicroAmp™ Optical 96-Well Reaction Plate	Applied Biosystems (PN N8010560)
MicroAmp™ 384-Well Clear Optical Reaction Plate with Barcode	Applied Biosystems (PN 4309849)
MicroAmp™ 96-Well Tray/Retainer Set (10 sets)	Applied Biosystems (PN 403081)
MicroAmp® Optical Tubes without Cap	Applied Biosystems (PN N8010933)

Table 1-3 User-Supplied Materials *(continued)*

Item	Source
The MicroAmp Optical 96-Well Reaction Plate may be sealed with: ♦ MicroAmp® Optical Caps or ♦ MicroAmp™ Optical Adhesive Film Kit containing 20 optical adhesive covers, one applicator, and one compression pad	 ♦ Applied Biosystems (PN 4323032) ♦ Applied Biosystems (PN 4313663)
Other Equipment and Supplies	
Centrifuge with adapter for 96-well plate	Major laboratory supplier (MLS)
Disposable gloves	MLS
Microcentrifuge	MLS
NuSieve 4% (3:1) agarose gels for DNA <1 kb	Lonza Bioscience (PN 57235)
Pipette tips, with filter plugs	MLS
Pipettors	MLS
Polypropylene tubes	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Vortexer	MLS

Table 1-4 User-Supplied Instruments, Software and Calibration Kits

Item	Source
7900HT Sequence Detection System 7000 Sequence Detection System	See your local Applied Biosystems representative for the instrument or software best suited to meet your needs.
Primer Express® Software (single-use license)	
Calibration Kits	
Sequence Detection Systems Spectral Calibration Kit, for ABI PRISM® 7700 instruments	Applied Biosystems (PN 4305822)
Sequence Detection Systems 384-Well Spectral Calibration Kit	Applied Biosystems (PN 4323977)
ABI PRISM® 7900 Sequence Detection Systems 96-Well Spectral Calibration Kit	Applied Biosystems (PN 4328639)

Table 1-4 User-Supplied Instruments, Software and Calibration Kits

Item	Source
ABI PRISM® 7000 Sequence Detection Systems Spectral Calibration Kit	Applied Biosystems (PN 4328895)

Storage and Stability

Upon receipt, store the TaqMan PCR Reagent Kit with Controls or its components at –15 to –25 °C in a constant temperature freezer. If stored under the recommended conditions, the product will maintain performance through the control date printed on the label.

Safety

Documentation User Attention Words

Five user attention words appear in the text of all Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below.

Note Calls attention to useful information.

IMPORTANT Indicates information that is necessary for proper instrument operation.

⚠ CAUTION Indicates a potentially hazardous situation which, 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 which, if not avoided, could result in death or serious injury.

⚠ DANGER Indicates an imminently hazardous situation which, 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.

- 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.

- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* 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 (*e.g.*, 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 on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Chemical Waste Hazard Warning

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

- 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.
- Handle chemical wastes in a fume hood.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* 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 (*e.g.*, fume hood). For additional safety guidelines, consult the MSDS.
- 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.

Site Preparation and Safety Guide

A site preparation and safety guide is a separate document sent to all customers who have purchased an Applied Biosystems instrument. Refer to the guide written for your instrument for information on site preparation, instrument safety, chemical safety, and waste profiles.

About MSDSs

Some of the chemicals used with this instrument may be listed as hazardous by their manufacturer. When hazards exist, warnings are prominently displayed on the labels of all chemicals.

Chemical manufacturers supply a current material safety data sheet (MSDS) before or with shipments of hazardous chemicals to new customers and with the first shipment of a hazardous chemical after an MSDS update. MSDSs provide you with the safety information you need to store, handle, transport and dispose of the chemicals safely.

We strongly recommend that you replace the appropriate MSDS in your files each time you receive a new MSDS packaged with a hazardous chemical.

⚠ WARNING CHEMICAL HAZARD. Be sure to familiarize yourself with the MSDSs before using reagents or solvents.

Obtaining MSDSs

The MSDSs for many chemicals supplied by Applied Biosystems are available to you free 24 hours a day.

To obtain MSDSs:

Step	Action
1	Go to **www.appliedbiosystems.com**, click **Support**, then click **MSDS Search**.
2	In the Keyword Search field, enter the chemical name, product name, MSDS part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3	Find the document of interest, right-click the document title, then select any of the following: **Open** – To view the document **Print Target** – To print the document **Save Target As** – To download a PDF version of the document to a destination that you choose

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

Reagent Preparation

Preparation of Reagents Required but Not Supplied

Use reagent grade chemicals unless otherwise noted. Prepare all solutions using deionized or glass distilled water.

0.1 M EDTA

To prepare 0.1 M EDTA (100 mL):

Step	Action
1	Add 3.72 g of disodium ethylenediaminetetraacetic acid dihydrate ($Na_2EDTA•2H_2O$) to 80 mL of deionized water.
2	Stir vigorously until dissolved.
3	Adjust the pH to 8.0 (±0.2) by adding 1N NaOH.
4	Adjust the final volume to 100 mL with deionized water and mix. Autoclave or filter through a 0.2 μm Nalgene filter.
5	Discard after 6 months.

0.1 M Tris-HCl, pH 8.0

To prepare one liter of 0.1 M Tris-HCl:

Step	Action
1	Dissolve 12.11 g Tris base in 800 mL deionized water.
2	Adjust the pH to 8.0 (±0.2) at room temperature by adding 1N HCl.
3	Adjust the final volume to one liter with deionized water and mix. Autoclave or filter through a 0.2 μm Nalgene filter.
4	Discard after 6 months.

TE Buffer

10 mM Tris-HCl, 1 mM EDTA, pH 8.0

To prepare one liter of TE buffer:

Step	Action
1	Combine 100 mL of 0.1M Tris-HCl, pH 8.0, and 10 mL of 0.1 M EDTA 800 mL of deionized water.
2	Bring the volume to one liter with deionized water and mix. Autoclave or filter through a 0.2 μm Nalgene filter.
3	Dispense into 100 mL aliquots.
4	Discard after 6 months.

Preparation of Supplied Reagents

Prior to use, allow the kit components to thaw on ice. When the reagents are thawed, mix each tube component by vortexing gently. Using a microcentrifuge, collect the tube contents at the bottom of the tube. Store the tube on ice until ready for use. When finished with the kit, return it to a constant-temperature freezer (–15 to –25 °C).

Procedural Considerations

2

Overview

About This Chapter

This chapter discusses important considerations for protocol success, including contamination and storage conditions prior to analysis.

In This Chapter

The following topics are discussed in this chapter:

Important Considerations for Protocol Success

PCR Formulations

The PCR formulations and thermal cycling conditions described in this protocol have been optimized for the β-actin controls found in the TaqMan PCR Reagent Kit with Controls. When other PCR applications are used, the PCR formulations and thermal cycling condition must be optimized for each specific application. To avoid risk of PCR product carryover, use dUTP during optimization. Before using a custom fluorescent-labeled probe, verify that the target DNA is being amplified by performing gel electrophoresis and ethidium bromide staining of the PCR product.

Applied Biosystems strongly recommends using a Master Mix cocktail to reduce tube-to-tube variability.

Passive Reference

Passive Reference 1 is a dye, included in the 10X TaqMan Buffer A, that does not participate in the 5´ nuclease PCR. Passive Reference 1 provides an internal reference to which the reporter dye signal can be normalized. When a PCR system employs two or more reporter dyes, data analysis requires this normalization step.

In single-probe systems, results may be calculated directly from measurements of absolute emission values. One of the signals in this calculation represents the scan of 1X TaqMan Buffer A containing Passive Reference 1 and having an emission maximum of 602 nm (see Figure 1-2 on page 4).

AmpliTaq Gold DNA Polymerase

AmpliTaq Gold® DNA Polymerase is a modified version of recombinant AmpliTaq® DNA Polymerase. It is provided in an inactive state and activated through a pre-PCR step of 10 minutes at 95 °C. Once activated, AmpliTaq Gold® DNA Polymerase has the same enzyme unit activity, thermal stability, and half-life at 95 °C as AmpliTaq DNA Polymerase. AmpliTaq Gold® DNA Polymerase may be substituted unit-for-unit for AmpliTaq DNA Polymerase in any optimized PCR system that uses Applied Biosystems buffers, generally without further optimization.

Because the enzyme is supplied in an inactive state, complete reaction premixes can be made in advance and pipetted easily into reaction tubes or 96-Well Microplates with a multi-channel pipettor. This is a very important point for high-throughput applications. Since the enzyme does not become active until a temperature well above primer

annealing is reached, primer dimer (primer oligomerization) formation is minimized.

Hot Start

The initial inactive state of AmpliTaq Gold® DNA Polymerase allows a Hot Start PCR to be performed without the cumbersome, time-consuming, and expensive methods of conventional Hot Start techniques. A Hot Start PCR prevents primer dimer formation and other non-specific amplifications. Hot Start PCR results in higher specificity, higher sensitivity, increased yield and decreased background.

Contamination

Because the interpretation of results depends upon the comparison of a Sample (containing DNA) to a No Template Control (without DNA), it is extremely important that all sources of contamination be anticipated and controlled. All reaction mixes should be set up in an area isolated from PCR product analysis and sample preparation. Use dedicated or disposable vessels, solutions, and pipettors (preferably positive displacement pipettors with disposable tips) for DNA preparation, reaction assembly, and sample analysis to minimize PCR product carryover and sample cross-contamination (Kwok, 1990; Orrego 1990).

Since sample protein and fluorescent contaminants may interfere with this assay and give false positive results, it may be necessary to include a No Enzyme Control tube that contains target, but no enzyme. If the fluorescence of the No Enzyme Control is greater than that of the No Template Control after PCR, fluorescent contamination may be present in the sample.

Storage Conditions Prior to Analysis

Since AmpErase UNG has no effect on the fluorescence signal generated during a TaqMan assay, its use in prevention of PCR product carryover is ideal. AmpErase UNG is active below 55 °C, so the annealing temperature should be kept at or above this temperature. To prevent UNG digestion of amplicons before gel electrophoresis, hold the reaction at 72 °C until analyzed, or rapidly cool the reaction and store it at –15 to –25 °C.

AmpErase UNG for Prevention of PCR Product Carryover

False Positives

Special laboratory practices are necessary in order to avoid false positive amplifications (Higuchi and Kwok, 1989). This is because of the capability for single DNA molecule amplification provided by the PCR process (Saiki *et al.*, 1985; Mullis *et al.*, 1987; Saiki *et al.*, 1988). Because of the enormous amplification possible with PCR, amplicon carryover can result in sample contamination. Other sources of contamination could be samples with high DNA levels or positive control templates.

When dUTP replaces dTTP as a dNTP substrate in PCR and the method described below is used, AmpErase UNG treatment can prevent the reamplification of carryover PCR products in subsequent experiments (Sninsky and Gelfand, pers. comm.) This method uses enzymatic and chemical reactions analogous to the restriction-modification and excision-repair systems of cells to degrade specifically PCR products from previous PCR amplifications or to degrade mis-primed, non-specific products produced before specific amplifications, but not degrade native nucleic acid templates.

The method used to make PCR products susceptible to degradation involves substituting dUTP for dTTP in the PCR mix and treating subsequent PCR mixes with the enzyme uracil N-glycosylase (UNG, EC 3.2.2-) before amplification (Longo *et al.*, 1990).

The AmpErase UNG provided in this product is a pure, nuclease-free, 26-kDa enzyme encoded by the *Escherichia coli* uracil N-glycosylase gene which has been inserted into an *E. coli* host to direct the expression of the native form of the enzyme (Higuchi and Kwok, 1989).

Although the protocol and reagents described here can degrade or eliminate large numbers of carried over PCR products, Applied Biosystems encourages users to continue using the specific devices and suggestions described in this protocol and in Kwok (1990) and Higuchi and Kwok (1989) to minimize cross-contamination from non-dU-containing PCR products or other samples.

AmpErase UNG Inactivation

A ten minute hold cycle at 95 °C is necessary to cleave the dU-containing PCR products that are carried over from an earlier PCR. Because UNG is not completely deactivated during the 95 °C incubation, it is important to keep the reaction temperatures greater than 55 °C, to prevent amplicon degradation.

Prevention of PCR Product Carryover

Use primers that contain dA nucleotides near the 3´ ends so that any primer-dimer generated is efficiently degraded by AmpErase UNG at least as well as any dU-containing PCR products. The farther a dA nucleotide is from the 3´ end, the more likely that partially degraded primer-dimer molecules may serve as templates for a subsequent PCR amplification.

Production of primer dimer could lower the amplification yield of the desired target region. If primers cannot be selected with dA nucleotides near the ends, the use of primers with 3' terminal dU-nucleotides should be considered. Single-stranded DNA with terminal dU nucleotides are not substrates for AmpErase UNG (Delort *et al.*, 1985) and thus the primers will not be degraded. Biotin-dUMP derivatives are not substrates for AmpErase UNG.

The concentration of AmpErase UNG and the time of the incubation step necessary to prevent amplification of contaminating dU-containing PCR product depend on the PCR conditions necessary to amplify your particular DNA sequence and the level of contamination expected. In most cases, using AmpErase UNG at 1 U/l00 μL reaction and incubation at 50 °C for two minutes is sufficient.

Do not use AmpErase UNG in subsequent amplification of dU-containing PCR template, such as in nested-PCR protocols. The UNG degrades the dU-containing PCR product, preventing further amplification.

TaqMan PCR Reagent Kit 3

Overview

About This Chapter

This chapter describes the protocol for the TaqMan PCR Reagent Kit.

In This Chapter

The following topics are discussed in this chapter:

Protocol for TaqMan PCR Reagent Kit

Procedural Overview

The procedure, which has been optimized for the β-actin control, involves a PCR amplification of the target DNA, followed by fluorescence analysis.

Controls

Analysis requires at least three No Template Controls run concurrently with the Test Samples. The No Template Control is the complete PCR formulation without the target DNA. Changes in any of the components other than the target DNA in the PCR formulation require a separate No Template Control.

Preparing the Master Mix

For this protocol, Applied Biosystems strongly recommends preparation of a Master Mix containing all the kit components except the Control (target) DNA.

Table 3-1 Master Mix Preparation for 12 Reactions

Component	Volume (µL)	Final Concentration (in 50 µL)
Deionized water	207	—
10X TaqMan Buffer A	60	1X
25 mM $MgCl_2$ Solution	84	3.5 mM
dATP	12	200 µM
dCTP	12	200 µM
dGTP	12	200 µM
dUTP	12	400 µM
β-actin Forward Primer	60	300 nM
β-actin Reverse Primer	60	300 nM
β-actin probe (FAM)	60	200 nM
AmpliTaq Gold® DNA Polymerase (5 U/µL)	3	0.025 U/µL
AmpErase UNG (1U/µL)	6	0.01 U/µL
Total	588	

Performing the Assay

To perform TaqMan 5´ to 3´ nuclease assay:

Step	Action
1	Prepare the Master Mix, allowing seven volumes of Master Mix for each set of six Samples and four volumes of Master Mix for each set of three No Template Controls.
2	Aliquot 343 µL (seven volumes) of Master Mix into a microcentrifuge tube labeled Sample.
3	Aliquot 196 µL (four volumes) of Master Mix into a microcentrifuge tube labeled No Template Control.
4	Add 7 µL of Human Male DNA to the Sample tube. Cap the tube immediately and vortex gently to mix.
5	Add 4 µL of TE Buffer to the No Template Control for each test. Cap the tube immediately to prevent DNA contamination and vortex gently to mix.
6	Aliquot 50 µL of the Master Mix into the appropriate number of reaction tubes (six Sample, three No Template). If you use the DNA Thermal Cycler or the DNA Thermal Cycler 480, add 50 to 75 µL mineral oil as a vapor barrier.
7	Perform amplification by PCR as soon as all the tubes have been prepared. See “Thermal Cycling Conditions” on page 3-4.
8	Read fluorescence. When using a Sequence Detection System, measure fluorescence directly in the closed tubes. Refer to the instrument’s user manual for specific instructions.

Thermal Cycling Conditions

Thermal Cycling Parameters

The TaqMan PCR Reagent Kit is optimized and quality-control tested for performance on the Applied Biosystems Sequence Detection Systems.

Step	UNG Incubation	AmpliTaq Gold Activation	PCR	
	Hold	Hold	Cycle (40 cycles)	
			Denature	Anneal/Extend
Time	2 min.	10 min.	15 sec.	1 min.
Temp	50 °C	95 °C	95 °C	60 °C
Volume	50 µL			

Control for PCR Amplification

4

Overview

About This Chapter

This chapter describes the protocol for PCR amplification using the TaqMan® β-actin Detection Reagents.

In This Chapter

The following topics are discussed in this chapter:

TaqMan® β-Actin Detection Reagents

Overview

TaqMan® β-actin Detection Reagents can be used as a control for assessing the PCR amplification of 10 gene copies of human DNA. For this purpose, the protocol requires a series of dilutions of the provided DNA and control primers, and visualization of the end-point PCR product as a band on an agarose gel. The FAM™ dye-labeled probe included in the kit is not required to complete the assay described above; however, it can be used if real-time PCR data acquisition is required. If real-time data acquisition is preferred, note that the FAM dye-labeled β-actin TaqMan probe supplied with the kit can only be used to detect amplification of the specific template included in the kit. A custom probe must be designed for detection of any other template.

Note TaqMan β-actin Detection Reagents (PN 401846) is a replacement product for the discontinued GeneAmp® HIV-1 Control Reagents Kit (PN N8080015). The protocol for TaqMan β-actin Detection Reagents is similar to the GeneAmp HIV-1 kit protocol.

Materials and Equipment

The following items are required when using the TaqMan β-actin Detection Reagents Kit but are not supplied.

Refer to “Materials and Equipment” on page 1-5 for additional supplies.

Table 4-1 Supplies

Item	Source
Nuclease-free water	Major laboratory supplier (MLS)
Tris-EDTA (TE) Buffer, pH 8.0	MLS
10X TBE Buffer	MLS
3% agarose/1XTBE gels containing ethidium bromide	MLS
Horizontal electrophoresis apparatus	MLS
Camera apparatus	MLS
96-Well GeneAmp® PCR System 9700	Applied Biosystems (PN N8050200)

For real-time PCR instruments, see **www.appliedbiosystems.com**.

Preparation of Supplied Reagents

Replicate Samples

This protocol uses triplicates for each dilution. Applied Biosystems recommends triplicates as the minimum number of replicates to minimize the variability in results that is characteristic of PCR amplification with low number of DNA copies.

Controls

Analysis requires at least three “No Template Controls” (or “NTCs”) to be run concurrently with the Test Samples. An NTC is the complete PCR formulation without the target DNA. Changes in any components other than the target DNA in the PCR formulation require a separate NTC.

Preparing the Master Mix

For best results with this protocol, prepare a Master Mix, containing all the PCR components except the Control (target) DNA

⚠ WARNING CHEMICAL HAZARD. **AmpErase UNG** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ WARNING CHEMICAL HAZARD. **AmpliTaq Gold DNA Polymerase** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Table 4-2 Master Mix Preparation for 1 or 28 Reactions‡

Component	Volume (µL)		Final Concentration (in 50 µL)
	1 Reaction	28 Reactions	
Deionized water	18.5	511	—
10X TaqMan Buffer A	5	140	1X
25 mM $MgCl_2$ Solution	7	196	3.5 mM
dATP	1	28	200 µM
dCTP	1	28	200 µM
dGTP	1	28	200 µM
dUTP	1	28	400 µM
β-actin Forward Primer	5	140	300 nM
β-actin Reverse Primer	5	140	300 nM
β-actin Probe (FAM)	5	140	200 nM

Table 4-2 Master Mix Preparation for 1 or 28 Reactions‡ *(continued)*

Component	Volume (µL)		Final Concentration (in 50 µL)
	1 Reaction	28 Reactions	
AmpliTaq Gold® DNA Polymerase (5 U/µL)	0.25	7	0.025 U/µL
AmpErase UNG (1 U/µL)	0.5	14	0.01 U/µL
Total	50	1400	

‡. If you perform PCR and agarose gel electrophoresis, replace the probe volume with H_2O. (For example, instead of 5 µL of probe, add an extra 5 µL of H_2O.) Also, replace the 10X TaqMan® Buffer A with 10X GeneAmp® PCR Gold Buffer.

Thermal Cycling Conditions

Table 4-3 Thermal Cycling Parameters

Step	UNG Incubation	AmpliTaq Gold Activation	PCR	
	Hold	Hold	Cycle (40 cycles)	
			Denature	Anneal/Extend
Time	2 min.	10 min.	15 sec.	1 min.
Temperature	50 °C	95 °C	95 °C	60 °C
Volume	50 µL			

Amplification of Ten Copies of the β-Actin Gene

Serial Dilution

Perform a serial 10-fold dilution of the supplied DNA, and a dilution to obtain 10 copies/μL with TE buffer, pH 8 using the information provided in Table 4-4.

Table 4-4 Serial dilutions of template DNA

	Dilution					
	Neat	**1:10**	**1:100**	**1:1000**	**1:30.3**	**NTC**
Human Male DNA (ng)	10	1	0.1	0.01	0.033	0
Human Male DNA (μL)	10	10 of Neat	10 of 1:10	10 of 1:100	10 of 1:10	—
TE Buffer (μL)	—	90	90	90	293	100
Copies/μL	3030	303	30.3	3	10	0

Performing the Reaction

Prepare the Master Mix following the instructions in Table 4-2 on page 4-3.

To perform the reaction:

Step	Action
1	Prepare the Master Mix allowing four volumes of Master Mix for each set of three dilution sample replicates and four volumes of Master Mix for each set of three No Template Control (NTC).
2	Aliquot 196 μL of Master Mix into six microcentrifuge tubes labeled with the respective serial dilution Sample ID.
3	Aliquot 196 μL of Master Mix into a microcentrifuge tube labeled NTC.
4	To Sample tubes, add 4 μL of Human Male DNA to each of the serial dilution tubes (from serial dilution in Table 4-4). Cap the tubes immediately and vortex gently to mix.
5	To the NTC tube, add 4 μL of TE Buffer, pH 8. Cap the tube immediately to prevent DNA contamination and mix by gentle vortexing.
6	Aliquot 50 μL of the Master Mix plus DNA into the appropriate number of wells of a 96-well reaction plate (three replicates of each of the serial diluted sample).
7	Aliquot 50 μL of the Master Mix plus TE buffer into the appropriate number of wells of a 96-well reaction plate (three replicates of the NTCs).

To perform the reaction: *(continued)*

Step	Action
8	Cover the 96-well plate. ♦ If performing the reaction with the TaqMan probe, use an optical plastic cover. ♦ If performing the PCR, use a plastic cover.
9	Perform amplification by PCR as soon as all samples have been dispensed into the reaction plate, using the thermal cycler parameters in Table 4-3 on page 4-4.
10	If performing the reaction with the TaqMan probe, read the fluorescence. When using a real-time PCR instrument, measure fluorescence directly in the closed tubes. Refer to the instrument's user manual for specific instructions.

Preparing Samples for Electrophoresis

To prepare samples for agarose gel electrophoresis:

Step	Action
1	After PCR is completed, remove plates from the thermal cyclers.
2	Remove the plastic cover from each plate.
3	Add 3 µL of loading buffer to each well on both plates. You can use a multichannel pipettor, but be careful that the tips do not touch the buffer in any of the wells.
4	Cover each plate again and mix gently using a bench-top mixer.
5	Centrifuge the plates to force all liquid to the bottom of the wells. The samples are ready to be loaded on the gel.

Setting Up and Running the Gel

To perform gel electrophoresis:

Step	Action
1	Obtain or prepare a 3% agarose/TBE/EtBr gel.
2	Set it inside the electrophoresis apparatus.
3	Prepare the appropriate volume of 1X TBE buffer with EtBr for the gel apparatus.
4	Pour buffer over the gel until the gel is completely covered.
5	Load 10 µL of each sample and NTC in wells of the gel.
6	Place the cover on the gel apparatus.

To perform gel electrophoresis: *(continued)*

Step	Action
7	Run the gel at 125 V for 40 minutes or until the marker is 90% down the gel.
8	Turn off the power supply and carefully remove the gel.

Photographing the Gel

Use a camera apparatus to photograph the gel.

Results

Figure 4-1, below, shows a dilution series and Figure 4-2 on page 4-8 shows three replicates of the β-actin amplicon.

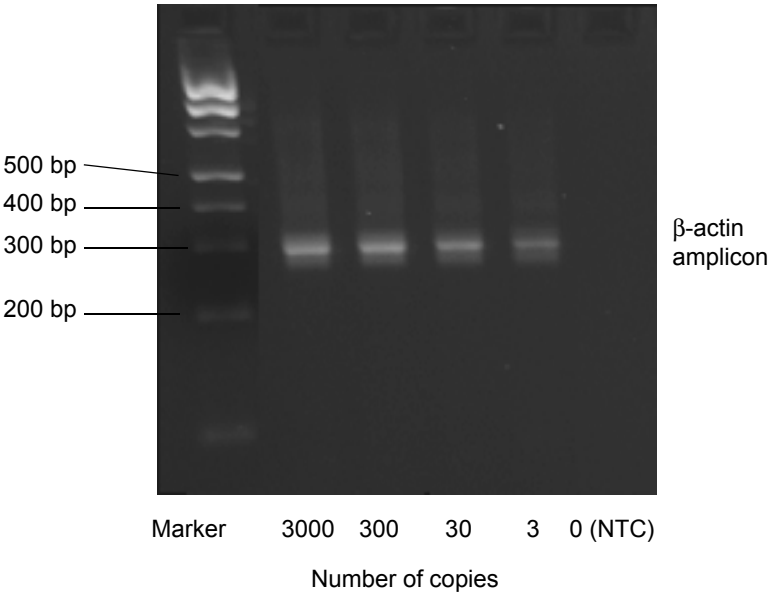


Figure 4-1 Dilution series of human DNA.

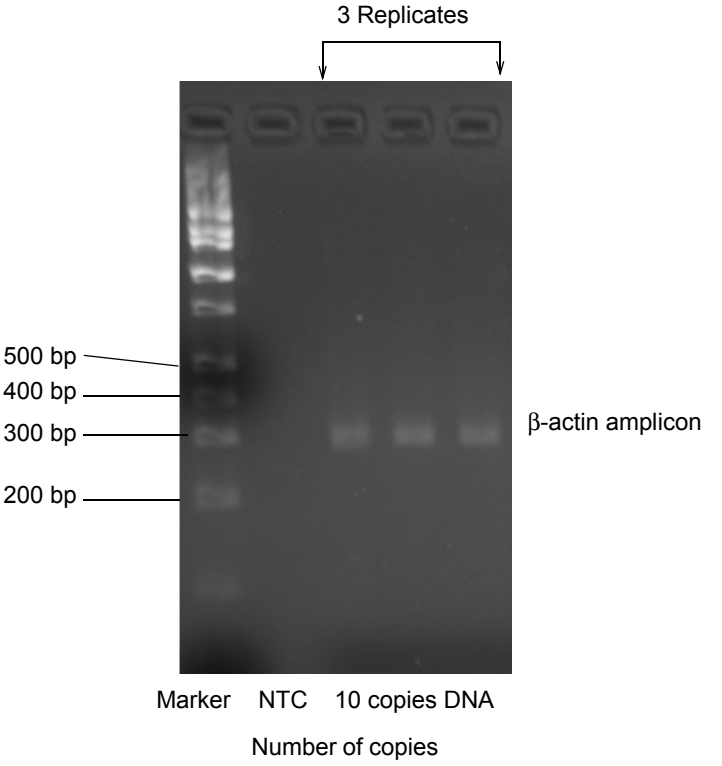


Figure 4-2 Amplification of 10 copies of the β-actin gene with three replicates.

PCR Optimization for Custom Probes

5

Overview

About This Chapter

This chapter describes PCR optimization for custom probes. The custom fluorescent probe can be added directly to an established, optimized PCR protocol. To improve performance of the system, consider the guidelines described in this section. For further guidelines for the design and use of TaqMan flurogenic probes, see the *Primer Express Version 1.5 and TaqMan MGB Probes for Allelic Discrimination User Bulletin* (PN 4317594).

In This Chapter

The following topics are discussed in this chapter:

Designing TaqMan Probes and Primers

Probes

Probes can be designed using Primer Express software as described in the Primer Express Software User Bulletin (PN 4317594). Follow these guidelines when designing probes:

- Keep the G-C content in the 20 to 80% range.
- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- Do not put a G on the 5´ end.
- Select the strand that gives the probe more Cs than Gs.
- For single-probe assays, the melting temperature (T_m) should be 68 to 70 °C when using Primer Express software.
- Use Primer Express software v 1.5A or v 2.0 when designing TaqMan MGB probes.

Primers

Primers can be designed using Primer Express software as described in the Primer Express Software User Bulletin. Follow these guidelines when designing primers:

- Choose the primers after the probe.
- Design the primers as close as possible to the probe without overlapping the probe.
- Keep the G-C content in the 20 to 80% range.
- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- When using Primer Express software, the T_m should be 58 to 60 °C.
- The five nucleotides at the 3´ end should have no more than two G and/or C bases.

Reagent Optimization

Overview

It may be necessary to optimize reactions for each primer-template pair. To optimize reactions, vary the cycling conditions and the concentrations of the following system components:

- Template
- Magnesium ion concentration
- dNTP

The effect of these variations can be evaluated by comparing the magnitude of ΔR_n after each change or by examining the intensity and distribution of bands after electrophoresis on Agarose for Separation of GeneAmp PCR Products (PN N9302774), followed by visualization with ethidium bromide staining of the gel (Saiki *et al.*, 1985).

Template

Start with enough copies of the template to be sure of obtaining a signal after 25 to 30 cycles (in a 40 cycle PCR reaction), preferably 10,000 copies, but less than 1 μg genomic DNA per 100 μL. Low concentrations of target DNA may require 35 or more cycles to produce sufficient product for analysis.

If proteases are present in the sample DNA (in impure genomic DNA, for example), inactivate the proteases by heating samples to 95 °C for five minutes before adding to PCR mix. When using AmpliTaq Gold® DNA Polymerase, this activation step also acts as the deactivation step for proteases.

Magnesium Ion Concentration

The optimum magnesium ion concentration must be determined empirically by testing concentrations from 0.8 to 9.0 mM $MgCl_2$ for each primer/probe set. Too little or too much $MgCl_2$ could reduce amplification efficiency or result in amplification of non-target sequences (Williams, 1989). If the samples contain EDTA, citrate, or other chelators, raise the $MgCl_2$ concentration in the reaction mix proportionately. Magnesium chloride concentrations should also be adjusted in parallel with significant changes in the concentrations of sample DNA or dNTPs to keep free magnesium ion constant.

dNTPs

Keep concentrations of dNTPs in the reaction mix balanced. If the concentration of any one of these is significantly different from the rest, the AmpliTaq Gold® DNA Polymerase tends to mis-incorporate, slow down, and terminate prematurely (Innis *et al.*, 1988). Substitution of dUTP for dTTP for control of PCR product carryover, however, may require higher concentrations of dUTP (typically twice that of any other dNTP) for the best amplification (Kwok, 1990; Orrego 1990).

PCR Optimization

Volume

A range of volumes permit good amplification, depending upon the reaction volume of the thermal cycler sample block. If variable volumes of reagents or templates are used in the master mix, adjust the volume of water in the master mix by an equivalent amount to keep the concentrations of the other reagents constant.

Two Steps in a Typical Cycle

A typical cycle consists of two steps:

- A melting step (95 °C) to separate the complementary strands of DNA.
- A primer anneal/extension step (60 °C) to allow hybridization of the primers to the single stranded DNA, initiation of polymerization, and completion of primer extension.

Cycling temperatures must be greater than 55 °C due to the presence of AmpErase UNG.

High G+C Content DNA

Amplification of high G+C content DNA requires one or more of the following modifications to overcome secondary structure (McConlogue *et al.*, 1988; Smith *et al.*, 1990; Sarker *et al.*, 1990):

- High annealing (>65 °C) and melting temperatures
- Cosolvents
- The use of 7-deaza-2´-deoxy-GTP mixed with dGTP

The half life of AmpliTaq Gold® DNA Polymerase is less than 35 minutes at temperatures over 95 °C (Gelfand *et al.*, 1990), suggesting that the maximum practical melting temperature is 95 to 96 °C.

6 Data Analysis

Overview

About This Chapter

This chapter describes how to analyze the data generated in your experiments.

In This Chapter

The following topics are discussed in this chapter:

Interpreting the Results

Normalization

The Passive Reference 1, a dye included in the 10X TaqMan Buffer A, does not participate in the 5´ nuclease PCR. The Passive Reference provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume.

Multicomponenting

Multicomponenting is the term used to distinguish the contribution each individual dye makes to the fluorescent spectra. The overlapping spectra from the pure dye components generate the composite spectrum. This spectrum represents one fluorescent reading from one well. Dyes available for multicomponent analysis are:

Types of Dyes	Dyes
Reporters	FAM™, TET™, JOE™, VIC®
Quenchers	TAMRA™, NON-FLUORESCENT QUENCHER
Passive Reference	ROX™

R_n and ΔR_n Values

Normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the Passive Reference to obtain a ratio defined as the R_n (normalized reporter) for a given reaction tube.

R_n^+ is the R_n value of a reaction containing all components including the template.

R_n^- is the R_n value of an unreacted sample. This value may be obtained from the early cycles of a real-time run, those cycles prior to a detectable increase in fluorescence. This value may also be obtained from a reaction not containing template.

ΔR_n is the difference between the R_n^+ value and the R_n^- value. It reliably indicates the magnitude of the signal generated by the given set of PCR conditions.

The following equation expresses the relationship of these terms:

$\Delta R_n = (R_n^+) - (R_n^-)$

where:

$$R_n^+ = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$$ PCR with template

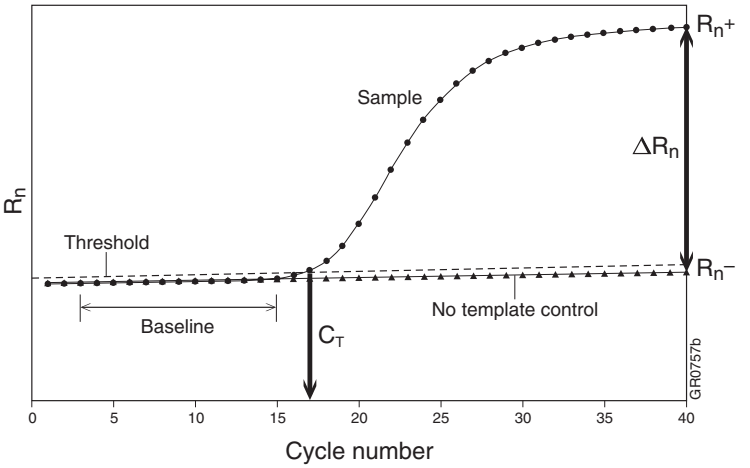
$$R_n^- = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$$ PCR without template or early cycles of a real-time reaction

Real-Time Detection

Threshold Cycle

The threshold cycle or C_T value is the cycle at which a statistically significant increase in ΔR_n is first detected. Threshold is defined as the average standard deviation of R_n for the early cycles, multiplied by an adjustable factor.

On the graph of R_n versus cycle number shown below, the threshold cycle occurs when the Sequence Detection System begins to detect the increase in signal associated with an exponential growth of PCR product.



Performance Characteristics

Positive Result Using the human DNA sample and the primers and probe provided with the TaqMan® β– actin Detection kit, you will achieve a positive result under the conditions recommended for the kit. A positive result is defined as any value greater than the threshold ΔR_n determined by analysis of three or more No Template Controls for a defined set of system conditions.

Troubleshooting

A

Possible Causes and Recommended Actions

Observation	Possible Cause	Recommended Action
$\Delta R_n \leq$ threshold ΔR_n and no PCR product generated in the reaction	PCR component omitted, incorrect primer(s) or template used	Troubleshoot PCR optimization, then repeat TaqMan PCR Reagent Kit with Controls reaction with β-actin control.
	Primer or template sequence incorrect	
	Inhibitor present in PCR	
$\Delta R_n \leq$ threshold ΔR_n and both R_n^+ and R_n^- reactions show PCR product	Negative Reaction (R_n^-) contaminated with DNA	Check technique and equipment to confine contamination. Use dUTP instead of dTTP. Incorporate UNG for control of carryover contamination.
$\Delta R_n \leq$ threshold ΔR_n and both R_n^+ and R_n^- reactions show PCR product	Failure of carryover contamination method	Troubleshoot carryover contamination method.
$\Delta R_n \leq$ threshold ΔR_n with PCR product in R_n^+ reaction but not in R_n^- reaction	Probe degradation (independent of AmpliTaq Gold® DNA Polymerase), incorrect mixing or probe accidentally omitted	Recheck probe concentration and rerun PCR. Use different batch of probe.
$\Delta R_n \leq$ threshold ΔR_n with PCR product in Rn^+ reaction but not in R_n^- reaction	Probe does not anneal	Verify probe sequence. Redesign if necessary.

Observation	Possible Cause	Recommended Action
Control $\Delta R_n \geq$ threshold ΔR_n, but the user system's $\Delta R_n \leq$ threshold ΔR_n **or** User system's $\Delta R_n \geq$ threshold ΔR_n, but the Control $\Delta R_n \leq$ threshold ΔR_n	PCR cycling conditions used were incompatible with one system	Rerun system using conditions optimized for that specific system.
Post-PCR fluorescence at or near background fluorescence	Probe omitted	Rerun system with probe.
$\Delta R_n \geq$ threshold ΔR_n in No Enzyme control	Nuclease or fluorescence contaminatIon	Review template preparation protocol for possible sources of contamination.
Replicate sample coefficient of variation >10%	Template pipetting error	Use positive displacement pipettes. Dilute DNA with water or TE buffer, vortex, and adjust volume of Master Mix accordingly.
Replicate sample coefficient of variation >10%	Dirty reaction plate	Increase probe concentration in reactions.
	Too little signal	Check that all parameters (emission filter, emission and excitation wavelength, slit widths, integration time) are set correctly.
Directional trends across microplate in well-to-well fluorescent emissions	Thermal cycler out of calibration	Recalibrate thermal cycler. Contact your service engineer.
	Annealing temperature too high	Lower annealing temperature

Technical Support

B

Services & Support

Applied Biosystems Web Site

To access the Applied Biosystems Web site, go to:

http://www.appliedbiosystems.com

At the Applied Biosystems Web site, 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 Applied Biosystems Web site provides a list of telephone and fax numbers that can be used to contact Technical Support.

References

C

Beyer, W.H., ed., 1984. *CRC Standard Mathematical Tables*, 27th ed, CRC Press, Boca Raton, FL.

Delort, A.-M., Duplaa, A.-M., Molko, D. and Teoule, R., 1985. Excision of uracil residues in DNA: mechanism of action of *Escherichia coli* and *Micrococcus luteus* uracil-DNA glycosylases *Nucleic Acids Res.*13: 319–335.

du Breuil, R.M., Patel, J.M. and Mendelow B.V., 1993. Quantitation of β-actin-specific mRNA transcripts using xeno-competitive PCR. *PCR Methods Appl.* 3:57–59.

Förster, V.Th., 1948. Zwischenmolekulare Energiewanderung und Fluoreszenz," *Ann. Phys. (Leipzig)*. 2:55–75.

Gelfand, D.H. and White, T.J., 1990. Thermostable DNA Polymerases. In *PCR Protocols. A Guide to Methods and Applications,* edited by M.A. Innis *et al*. Academic Press, Inc., San Diego, CA, pp. 129–141.

Higuchi, R. and Kwok, S., 1989. Avoiding false positives with PCR. *Nature* 339:237–238.

Holland, P.M., Abramson, R.D., Watson, R. and Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5´ to 3´ exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* 88:7276–7280.

Innis, M.A., Myambo, K.B., Gelfand, D.H. and Brow, M.A.D., 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proc. Natl. Acad. Sci. U.S.A.* 85: 9436–9440.

Jeffreys, A.J., Wilson, V., Neumann, R. and Keyte, J., 1988. Amplification of human minisatellites by the polymerase chain reaction:

towards DNA fingerprinting of single cells. *Nucleic Acids Res.*16:10953–10971.

Kwok, S., 1990. Procedures to minimize PCR-product carry-over. In *PCR Protocols. A Guide to Methods and Applications,* edited by M.A. Innis *et al*. Academic Press, Inc., San Diego, CA.

Lakowicz, J.R., *Principles of Flourescent Spectroscopy*, Plenum Press, N.Y., 1983.

Lawyer, F.C., Stoffel, S., Saiki R.K., Myambo, K.B., Drummond R. and Gelfand, D.H., 1989. Isolation, characterization, and expression in *Escherichia coli* of the DNA polymerase gene from the extreme thermophile, *Thermus aquaticus. J. Biol. Chem.* 264:6427–6437.

Lee, L.G., Connell, C.R. and Bloch, W., 1993. Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic Acids Res.* 21: 3761–3766.

Longo, N., Berninger, N.S. and Hartley, J.L., 1990. Use of Uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* 93:125–128.

Lyamichev, V., Brow, M.A.D. and Dahlberg, J.E., 1993. Structure-specific endonucleolytic cleavage of nucleic acids by eubacterial DNA polymerases. *Science* 260:778–783.

McConlogue, L., Brow, M.A.D. and Innis, M.A., 1988. Structure-dependent DNA amplification by PCR using 7-deaza-2-deoxyguanosine. *Nucleic Acids Res.*16:9869.

Mullis, K.B. and Faloona, F. A., 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. In *Methods in Enzymology*, Vol. 155, edited by R. Wu. Academic Press, Inc., San Diego, CA, pp. 335–350.

Nakajima-Iijima, S., Hamada, H., Reddy, P. and Kakunaga, T., 1985. Molecular structure of the human cytoplasmic beta-actin gene: Interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. U.S.A.* 82:6133–6137.

Orrego, C., 1990. Organizing a laboratory for PCR work. In *PCR Protocols. A Guide to Methods and Applications*, edited by M.A. Innis et al. Academic Press, Inc., San Diego, CA.

Rychlik, W., Spencer, W.J. and Rhoads, R.E., 1990. Optimization of the annealing temperature for DNA amplification *in vitro*. *Nucleic Acids Res.*18:6409–6412.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487–491.

Saiki, R.K., Scharf, S.J., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N.,1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–1354.

Sarkar, G., Kapeiner, S. and Sommer, S.S., 1990. Formamide can dramatically improve the specificity of PCR. *Nucleic Acids Res.* 18:7465.

Smith, K.T., Long, C.M., Bowman, B. and Manos, M.M., 1990. Using cosolvents to enhance PCR amplification. *Amplifications* 5:16–17.

Varshney, U., Hutcheon, T. and van de Sande, J.H., 1988. Sequence analysis, expression, and conservation of *Escherichia coli* uracil DNA glycosylase and its gene (ung). *J. Biol. Chem.* 263: 7776–7784.

Williams, J.F., 1989. Optimization strategies for the polymerase chain reaction. *Bio Techniques* 7:762–768.

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

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.applied biosystems.com or to the Technical Support and Training appendix in this document.

www.appliedbiosystems.com

Applied Biosystems

Applied Biosystems is committed to providing the world's leading technology and information for life scientists.

Printed in the USA, 07/2010
Part Number 402823 Rev. G