TaqMan® EZ RT-PCR Kit

Protocol

Applied Biosystems

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1 Introduction

Overview

About This Chapter

This chapter describes the TaqMan® EZ RT-PCR Kit and provides important information about safety.

In This Chapter

The following topics are discussed in this chapter:

Purpose of the Kit

RNA Detection

The TaqMan® EZ RT-PCR Kit provides a system for the detection and analysis of RNA. Direct detection of the reverse transcription polymerase chain reaction (RT-PCR) product with no downstream processing is accomplished by monitoring the increase in fluorescence of a dye-labeled DNA probe. This method makes it possible to analyze thousands of samples per day with high sample-to-sample reproducibility.

Using a single-tube, single-enzyme system, RNA is reverse transcribed to cDNA and amplified using the PCR process (Figure 1-1). Reverse transcription and DNA polymerization take place without the addition of subsequent enzymes or buffers.

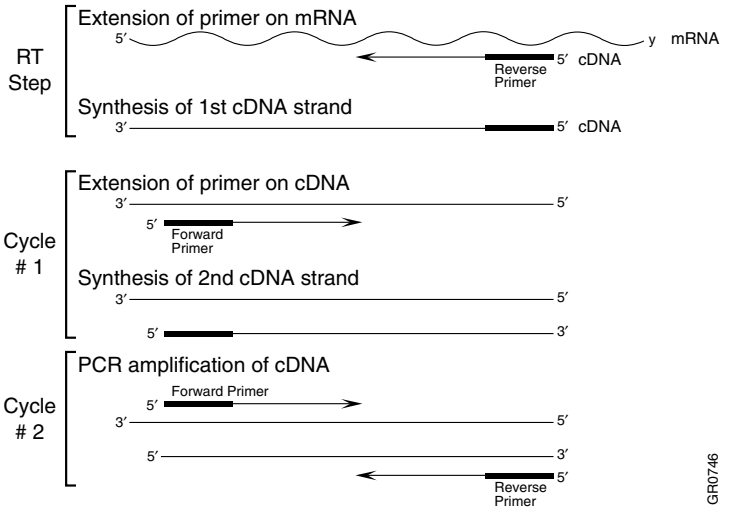


Figure 1-1 Schematic representation of RT-PCR using the TaqMan EZ RT-PCR Kit. Hybridization of the TaqMan probe is not shown.

Basics of the 5´ Nuclease Assay

The RT-PCR reaction exploits the 5´ nuclease activity of the r*Tth* 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. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.

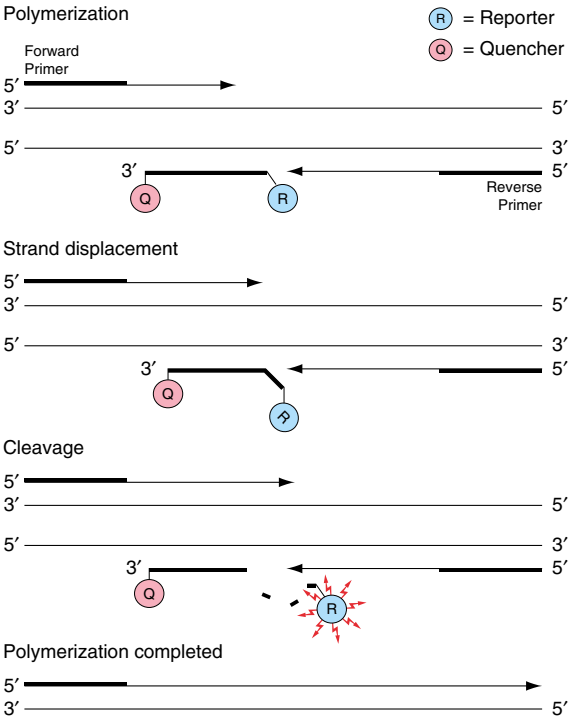


Figure 1-2 The fork-like-structure-dependent, polymerization associated, 5'–3' nuclease activity of r*Tth* DNA Polymerase during PCR.

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 r*Tth* 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 (see Figure 1-2).

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

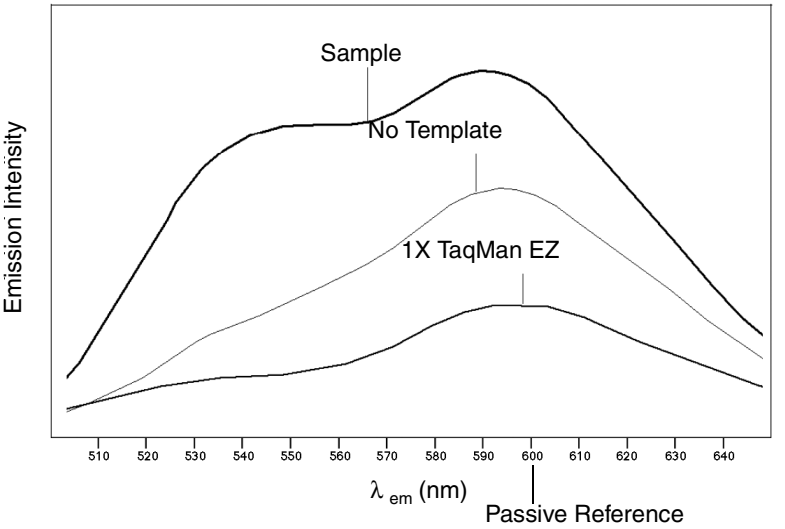


Figure 1-3 An overlay of three emission scans, post-PCR.

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 a non-fluorescent quencher attached via a linker arm that is usually located at the 3´end.

r*Tth* DNA Polymerase

When using the TaqMan EZ RT-PCR reagents and protocol, the r*Tth* DNA Polymerase functions as both a thermoreactive reverse transcriptase and a thermostable DNA polymerase. The r*Tth* DNA Polymerase reverse transcribes RNA efficiently in the presence of Mn^{2+} and at elevated temperatures (≤60 °C). It also provides the 5´ to 3´ nuclease activity necessary for the cleavage of the fluorogenic probe.

TaqMan EZ RT-PCR Kit

Reagents supplied with this kit make it possible to perform RT-PCR and the subsequent 5´ nuclease assay in a single tube/single buffer system. This kit also contains an amplicon contamination control system with AmpErase® UNG, which is optional.

The TaqMan EZ RT-PCR Kit contains two modules:

- TaqMan EZ RT-PCR Core Reagents
- TaqMan GAPDH Control Reagents

The TaqMan EZ RT-PCR Core Reagents Kit contains reagents to perform the reverse transcription of RNA to cDNA and the subsequent 5´ nuclease assay. The TaqMan GAPDH Control Reagents module contains the primers, probe, and Control RNA template for fluorogenic detection of the human GAPDH transcript.

The TaqMan EZ RT-PCR Kit may be used for Real Time Detection or Plate Read (end point) Detection of an RNA transcript. Detection may be performed using a Sequence Detection System available from Applied Biosystems.

GAPDH Fluorogenic Probe

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe is supplied to demonstrate performance. The probe detects the RNA transcript of the human GAPDH gene (Ercolani *et al.*, 1988), a constitutively expressed housekeeping gene.

The GAPDH probe supplied with the TaqMan GAPDH Control Reagents uses a JOE reporter dye to detect amplification of the Control RNA template included in the kit. Custom probes are designed for detection of a specific target. Probes can be designed to anneal to either strand of template.

Materials and Equipment

Kit Components

The TaqMan EZ RT-PCR Kit (P/N N808-0235) contains enough PCR reagents for up to 200 reactions (50 µL each), including 100 GAPDH Control reactions.

The kit is composed of two modules described in the tables below

TaqMan EZ RT-PCR Core Reagents (P/N N808-0236)

Reagent	Volume	Description
r*Tth* DNA Polymerase	400 µL	Two tubes containing 2.5U/µL of rT*th* DNA Polymerase in 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA,1 mM DTT, 50% glycerol, 0.5% (w/v) Tween 20.
AmpErase UNG	100 µL	One tube containing 1 U/µL uracil N-glycosylase in 30 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 5% glycerol (v/v), 0.5% (w/v) Tween 20.
deoxy ATP	320 µL	One tube containing 10 mM deoxyadenosine triphosphate
deoxy CTP	320 µL	One tube containing 10 mM deoxycystosine triphosphate
deoxy GTP	320 µL	One tube containing 10 mM deoxyguanosine triphosphate
deoxy UTP	320 µL	One tube containing 20 mM deoxyuridine triphosphate
5X TaqMan EZ Buffer	2 mL	Two tubes containing 250 mM Bicine, 575 mM potassium acetate, 0.05 mM EDTA, 300 nM Passive Reference 1, 40% (w/v) glycerol, pH 8.2
Manganese Acetate solution	2 mL	Two tubes containing 25 mM Mn $(OAc)_2$

TaqMan GAPDH Control Reagents (P/N 402869)

Reagent	Volume	Description
GAPDH Forward Primer	100 µL	One tube containing a 10 µM solution of primer in TE buffer.
GAPDH Reverse Primer	100 µL	One tube containing a 10 µM solution of primer in TE buffer.
GAPDH Probe	100 µL	One tube containing a 5 µM solution of a fluorogenic probe in TE buffer.
Control RNA (Human)	100 µL	One tube containing 50 ng/µL of total Human RNA in 10 mM Tris-HCl, pH 7.0, 100 mM NaCl, 1 mM EDTA

Materials Required but Not Supplied

The following items are required when using TaqMan EZ RT-PCR Kit, but are not supplied. See the table for source information.

User-Supplied Materials

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)	
Sequence Detection primers ♦ Min 4000 pmol purified for sequence detection ♦ Min 40,000 pmol purified for sequence detection ♦ Min 130,000 pmol purified for sequence detection	Applied Biosystems ♦ P/N 4304970 ♦ P/N 4304971 ♦ P/N 4304972
TaqMan® MGB Probe ♦ 5000 to 6000 pmoles ♦ 15,000 to 25,000 pmoles ♦ 50,000 to 100,000 pmoles	Applied Biosystems ♦ P/N 4316034 ♦ P/N 4316033 ♦ P/N 4316032
TaqMan® TAMRA Probe ♦ 5000 to 6000 pmoles ♦ 15,000 to 25,000 pmoles ♦ 50,000 to 100,000 pmoles	Applied Biosystems ♦ P/N 450025 ♦ P/N 450024 ♦ P/N 450003
MicroAmp® Optical 96-Well Reaction Plate and Optical Caps	Applied Biosystems (P/N 403012)
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems (P/N N801-0560)
ABI PRISM™ 384-Well Clear Optical Reaction Plate with Barcode	Applied Biosystems (P/N 4309849)
Note The MicroAmp Optical 96-Well Reaction Plate may be sealed with: ♦ MicroAmp Optical Caps or ♦ ABI PRISM™ Optical Adhesive Cover	

User-Supplied Materials *(continued)*

Item	Source
MicroAmp® Optical Caps	Applied Biosystems (P/N 4323032)
MicroAmp® 96-well Tray/Retainer Set (10sets)	Applied Biosystems (P/N 403081)
ABI PRISM Optical Adhesive Cover Starter Pack containing 20 optical adhesive covers, one applicator, and one compression pad. **Note** The MicroAmp Optical 96-well Reaction Plate may be sealed with MicroAmp Optical caps or ABI PRISM Optical Adhesive Cover	Applied Biosystems (P/N 4313663)
MicroAmp® Optical Tubes	Applied Biosystems (P/N N801-0933)
Sequence Detection Systems Spectral Calibration Kit (for 7700 instrument only)	Applied Biosystems (P/N 4305822)
Sequence Detection Systems 384-Well Spectral Calibration Kit	Applied Biosystems (P/N 4323977)
ABI PRISM® 7900 Sequence Detection Systems 96-Well Spectral Calibration Kit	Applied Biosystems (P/N 4328639)
ABI PRISM® 7000 Sequence Detection Systems Spectral Calibration Kit	Applied Biosystems (P/N 4328895)
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	FMC BioProducts (P/N 54928)
Pipette tips, with filter plugs	MLS
Pipettors	MLS
Polypropylene tubes	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Vortexer	MLS

Storage and Stability

Upon receipt, store the TaqMan EZ RT-PCR Kit at –15 to –25 °C in a constant-temperature freezer. Store the product away from light. This product is light sensitive. 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.

Ordering MSDSs

You can order free additional copies of MSDSs for chemicals manufactured or distributed by Applied Biosystems using the contact information below.

To order documents by automated telephone service:

Step	Action
1	From the U.S. or Canada, dial **1.800.487.6809**.
2	Follow the voice instructions to order documents (for delivery by fax). **Note** There is a limit of five documents per fax request.

To order documents by telephone:

In the U.S.	Dial **1.800.345.5224**, and press **1**.
In Canada	Dial **1.800.668.6913**, and press **1** for English or **2** for French.

To view, download, or order documents through the Applied Biosystems Web site:

Step	Action
1	Go to **http://www.appliedbiosystems.com**
2	Click **SERVICES & SUPPORT** at the top of the page, click **Documents on Demand**, then click **MSDS.**
3	Click **MSDS Index**, search through the list for the chemical of interest to you, then click on the MSDS document number for that chemical to open a PDF version of the MSDS.

For chemicals not manufactured or distributed by Applied Biosystems, call the chemical manufacturer.

Preventing Contamination

Hot Start PCR

To improve PCR specificity and sensitivity by controlling mispriming events, the Hot Start technique was introduced (Faloona *et al.*, 1990). Hot Start PCR is a simple modification of the original PCR process in which the amplification reaction is started at an elevated temperature. This was initially performed manually, by adding an essential component of the reaction to the reaction mixture only after that mixture had been heated to an elevated temperature. However, this approach was often cumbersome and time consuming, especially when using large numbers of samples.

Amplicon Contamination

Because of the enormous amplification possible with PCR, amplicon carryover can result in sample contamination. Contamination could also come from samples with high DNA levels or from positive control templates.

AmpErase UNG treatment can prevent the reamplification of carryover PCR products. This method involves substituting dUTP for dTTP in the PCR Reagent Mix and treating the mix with the enzyme uracil N-glycosylase (UNG, EC 3.2.2) prior to amplification (Longo *et al.*, 1990).

When dUTP replaces dTTP as a dNTP substrate in PCR, AmpErase UNG treatment can remove up to 200,000 copies of a previously amplified product per 50 µL reaction.

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

False Positives

Special laboratory practices are necessary in order to avoid false positive amplifications (Higuchi, *et al.*, 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 from samples with high DNA levels or from 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 prior to 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-) prior to 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 *et al.*, 1989).

Although the protocol and reagents described here are capable of degrading or eliminating large numbers of carried over PCR products, we encourage users to continue using the specific devices and suggestions described in this protocol booklet and in Kwok (1990) and Higuchi(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 further 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 depends 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 attempt to use AmpErase UNG in subsequent amplification of dU-containing PCR template, such as in nested-PCR protocols. The UNG will degrade the dU-containing PCR product, preventing further amplification.

Additional Considerations

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 further 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 dimers 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 is not a substrate for AmpErase UNG (Delort *et al.*, 1985) and thus the primers will not be degraded. Biotin-dUMP derivatives are not substrates for AmpErase UNG.

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

General PCR Practices

Certain laboratory practices are necessary in order to avoid false positive amplifications (Kwok and Higuchi, 1989). This is because the PCR process is capable of amplifying single DNA molecules (Saiki *et al.*, 1985; Mullis *et al.*, 1987; Saiki *et al.*, 1988).

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever contamination is possible.

- Maintain separate areas and dedicated equipment and supplies for sample preparation, for PCR setup, and for PCR amplification and 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 capped as much as possible.
- Clean lab benches and equipment periodically with 10% bleach solution.

Fluorescent Contaminants

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

Preparing GAPDH Control Reactions 2

Overview

About This Chapter

This chapter describes the preparation of GAPDH control reactions.

In This Chapter

The following topics are discussed in this chapter:

Preparing GAPDH Control Reactions

Overview

This procedure is optimized for the GAPDH Control Reagents. This example requires that six GAPDH reactions and three No Template Control reactions run concurrently. The No Template Control is the complete PCR formulation without the target RNA. Changes in any of the components other than the target RNA in the PCR formulation require a separate No Template Control. The plate setup can be altered to accommodate a different number of reactions.

Preparation of Reagents

Prior to use, thaw all reagents and keep the tubes on ice. When the reagents are thawed, mix each tube component by vortexing. Using a microcentrifuge, briefly spin down the tube contents. Protect the fluorescent dye labeled probe from excessive exposure to light. When finished with the kit, return it to the –15 to –25 °C freezer.

Preparing Reagents Mix

Prepare a Reagent Mix containing all the kit components except the target RNA. The Reagent Mix is used for the GAPDH reactions and the No Template Controls.

Preparing a Reagent Mix of RT-PCR components is recommended in order to increase the accuracy of the results. The use of a Reagent Mix reduces the number of reagent transfers and minimizes volume loss due to pipetting. To account for loss of liquid during pipetting, the volume of reaction mix should reflect volumes that are 10% greater than what is needed.

Reagent Mix Preparation

Component	Volume for One Sample (µL)	Final Concentration	Volume for 12 Samples (µL)
RNase-free water	21.5	–	258
5X TaqMan EZ Buffer	10	1X	120
Manganese acetate (25 mM)	6	3 mM	72
dATP (10 mM)	I.5	300 µM	18
dCTP (10 mM)	I.5	300 µM	18
dGTP (10 mM)	I.5	300 µM	18

Reagent Mix Preparation *(continued)*

Component	Volume for One Sample (µL)	Final Concentration	Volume for 12 Samples (µL)
dUTP (20 mM)	l.5	600 µM	18
GAPDH Forward Primer (10 µM)	1	200 nM	12
GAPDH Reverse Primer (10 µM)	1	100 nM	12
GAPDH Probe (5 µM)	1	100 nM	12
r*Tth* DNA Polymerase (2.5 U/µL)	2.0	0.1 U/µL	24
AmpErase UNG (1 U/µL)	0.5	0.01 U/µL	6
Total Mix	49	–	588

GAPDH Control Assay

Perform the GAPDH Control Assay as described below.

To perform the GAPDH Control assay:

Step	Action
1	Prepare the Reagent Mix. Mix gently by inversion. **⚠ CAUTION CHEMICAL HAZARD. AmpErase(R) uracil N-glycosylase** may cause eye and skin irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2	Label one microcentrifuge tube GAPDH Control and one microcentrifuge tube No Template Control (NTC).
3	Aliquot 343 µL (seven volumes) of Reagent Mix into the GAPDH Control microcentrifuge tube.
4	Aliquot 196 µL (four volumes) of Reagent Mix into the NTC microcentrifuge tube.
5	a. To the NTC tube add 4 µL of 1X TE Buffer. b. Cap the tube immediately to prevent DNA contamination. Mix gently by inversion.
6	a. To the GAPDH Control tube, add 7 µL of Control RNA (Human). b. Cap the tube immediately and mix gently by inversion.

To perform the GAPDH Control assay: *(continued)*

Step	Action
7	Aliquot 50 µL of the Reagent Mix into the appropriate number of reaction tubes (six GAPDH Controls, three No Template Controls).
8	Cap the tubes and transfer to the appropriate instrument for PCR amplification.
9	Perform RT-PCR.

PCR Amplification

Thermal Cycling Parameters

The following thermal cycler parameters are optimized for GAPDH detection. See instrument manuals for details on operation.

Note The conditions that are optimal for the GAPDH Control Reagents may not be suitable for each researcher's experimental application.

Thermal Cycler	Times and Temperatures				
	Initial Step UNG Treatment	RT	Deactivation of UNG	Each of 40 Cycles	
				Melt	Anneal/ Extend
7900HT SDS, 7700 SDS, or 7000 SDS -or- GeneAmp 5700 -or- GeneAmp PCR System 9600	HOLD	HOLD	HOLD	CYCLE	
	2 min 50 °C	30 min 60 °C	5 min 95 °C	20 sec 94 °C	1 min 62 °C

Reagent and Cycle Guidelines

3

Overview

About This Chapter

This chapter describes reagent optimization guidelines and cycle optimization guidelines.

In This Chapter

The following topics are discussed in this chapter:

Designing Primers and Probes

Probes

Probes can be designed using Primer Express software as described in the *Primer Express User Bulletin* (P/N 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.
- Both probes should be on the same strand.
- 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 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.
- The T_m of each primer should be 58 to 60 °C.
- The five nucleotides at the 3´ end of each primer should have no more than two G and/or C bases.

Reagent Optimization Guidelines

Overview

To detect a transcript of interest using fluorescence, a set of primers and a custom fluorescent probe must be designed. This probe can be added directly to an established, optimized PCR protocol, or it may be necessary to optimize reactions for each primer-template pair. This can be achieved by varying the concentrations of the following system components:

- RNA template
- Manganese ion
- dNTP

The effect of these variations can be evaluated by comparing the magnitude of ΔR_n for endpoint analysis or the C_T (threshold cycle) for Real-Time Detection after each change.

RNA Template

Start with enough copies of the RNA template to be sure of obtaining a signal by 25 to 30 cycles. Preferably, begin with 10,000 copies of template and less than 100 ng total RNA per 50 µL reaction. Low concentrations of target RNA may require up to 35 or more cycles to produce sufficient product for analysis. High levels of total RNA(>l µg) may cause inhibition.

The Control RNA provided in the TaqMan GAPDH Control Reagents is formulated at 50 ng/µL. The copy number for the GAPDH transcript has been found to be approximately 100 copies per picogram.

Manganese Ion Concentration

The optimum manganese ion concentration must be determined empirically by testing concentrations from 2 to 5 mM manganese acetate for each primer/probe set. Too little or too much $Mn(OAc)_2$ could reduce amplification efficiency or result in amplification of non-target sequences. If the samples contain EDTA, citrate, or other chelators, raise the $Mn(OAc)_2$ concentration in the reaction mix proportionately. Manganese acetate concentrations should also be adjusted in parallel with significant changes in the concentrations of template or dNTPs to keep free manganese ion constant.

dNTP Concentration

Keep concentrations of dNTPs in the reaction mix balanced. If the concentration of any one of these is significantly different from the rest, the r*Tth* DNA Polymerase may misincorporate, slow down, and

terminate prematurely (Perkin Elmer, 1996; Innis *et al.*, 1988). Substitution of dUTP for dTTP for control of PCR product carryover, however, will require higher concentrations of dUTP (typically twice that of any other dNTP) for the best amplification (Kwok, 1990; Orrego, 1990).

Cycle Optimization Guidelines

Hold Cycles

The thermal cycling procedure consists of a series of holds followed by repetitive cycles.

For the TaqMan EZ RT-PCR Kit, the recommended holds are:

- 50 °C for 2 minutes, contamination control with AmpErase UNG
- 60 °C for 30 minutes, reverse transcription with r*Tth* DNA Polymerase
- 95 °C for 5 minutes, deactivation of UNG

The only step of these holds that may need optimization is the 60 °C reverse transcription step. The efficiency of this step may be dependent upon target size and secondary structure.

Denaturation Temperature

The purpose of the denaturation step (95 °C) is to separate the complementary strands of nucleic acid.

The half life of the r*Tth* DNA Polymerase activity (approximately 20 minutes at 95 °C) suggests 95 °C as the maximum practical denaturation temperature. Glycerol effectively lowers the T_m of primers and the required denaturation temperature of PCR product by 2.5 to 3.0 °C (Gelfand and White,1990).

Using the kit components, the final concentration of glycerol in the reaction is 10%. At 94 °C in the presence of 10% glycerol, therefore, the effective denaturation temperature is 96.5 to 97.0 °C.

Annealing Temperature

The primer annealing/extension step (55 to 70 °C, depending upon the primer T_m) allows hybridization of the primers to the single-stranded DNA, initiation of polymerization, and completion of primer extension.

Higher annealing temperatures (>55 °C) generally result in much more specific product (Williams, 1989; Rychlik *et al.*, 1990). The optimum annealing temperature can be determined empirically by testing at increments of 5 °C or smaller, until the maximum in fluorescent signal is reached. At these temperatures, r*Tth* DNA Polymerase has significant activity, so extension of primed templates is occurring.

IMPORTANT Residual AmpErase UNG is active below 55 °C, so the annealing temperature should be kept at or above this temperature. There may be a small amount of UNG that was not inactivated. If you want to do any post-PCR steps, make sure that you use samples right away. Otherwise, store the reactions at –20 °C and thaw right before using them.

Data Analysis and Interpretation

4

Overview

About This Chapter

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

In This Chapter

The following topics are discussed in this chapter:

Data Analysis

Detection Systems

The TaqMan EZ RT-PCR Kit is designed for Real Time Detection or Plate Read Detection. Contact your local Applied Biosystems sales representative for information regarding Sequence Detection Systems.

Real Time Detection monitors fluorescence during each PCR cycle. Plate Read (end point) Detection collects one fluorescence scan per tube, after PCR is completed.

Features of Run Types on Sequence Detection Application

Run Type	Fluorescence Detection	Analysis Results
Real Time	At each PCR cycle	Quantitation of template
Plate Read	Only end-point	Detection of template

Interpreting the Results

Normalization

Passive Reference 1 (ROX) is a dye included in the 5X TaqMan EZ Buffer. It does not participate in the 5´ nuclease assay. Passive Reference 1 provides an internal reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations resulting from factors such as 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 Value

Normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of Passive Reference 1 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 (No Template Control), as in Plate Read (end point).

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+ =	Emission Intensity of Reporter / Emission Intensity of Passive Reference	PCR with template
R_n- =	Emission Intensity of Reporter / Emission Intensity of Passive Reference	PCR without template or early cycles of a Real Time reaction

Real-Time Detection

The threshold cycle or C_T value is the cycle at which a statistically significant increase in ΔR_n is first detected.

On the graph of R_n versus cycle number (Figure 4-1), the threshold cycle occurs when the Sequence Detection Application begins to detect the increase in signal associated with an exponential growth of PCR product.

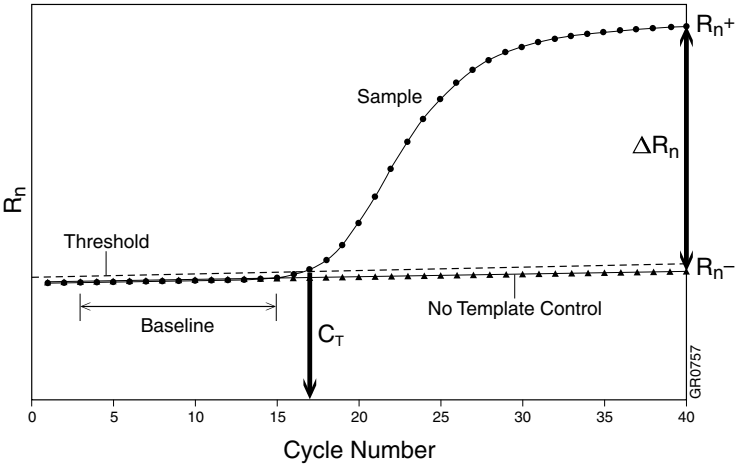


Figure 4-1 Amplification plot, R_n versus cycle number

Plate Read Analysis

Plate Read analysis relies on end point data to calculate ΔR_n. To ensure statistically high confidence levels using either the GAPDH probe or a custom probe, run the protocol with at least three No Template Controls per microplate.

A positive result is defined as ΔR_n values greater than the threshold ΔR_n. The threshold ΔR_n is calculated by multiplying the standard deviation of three R_n- values by an appropriate multiplier. The value for the appropriate multiplier is selected from a table of Student's t-distribution values and depends upon the desired confidence level (Beyer, 1984). For 99% confidence levels, the multiplier is 6.965.

When more than three No Template Controls are run, the multiplier for the standard deviation decreases. Refer to a table of Student's t-distribution values for the appropriate multiplier (Beyer, 1984).

To evaluate reproducibility, calculate the coefficient of variation on replicate samples. Inconsistent results (coefficients of variation exceeding 10%) may be caused by pipetting errors and incomplete mixing of DNA solutions.

Determining Threshold ΔR_n Value

To determine threshold ΔR_n:

- On one microplate, measure both Reporter and Passive Reference 1 fluorescence, and determine the normalized Reporter for each No Template Control tube (R_n-).
- Determine the mean and standard deviation of R_n-.
- When running three No Template Controls, multiply the R_n- standard deviation by 6.965 to determine the threshold ΔR_n- for the system.

For example, if the mean R_n- for a system is 0.5 with a standard deviation of 0.02, the threshold ΔR_n, is 6.965 x 0.02 or 0.14. Any ΔR_n>0.14 is a positive result and indicates the sample contains target.

System Performance Guarantee

Positive Result

When using 50 ng of Control RNA (Human), the GAPDH primers and probe in a 50 µL reaction, a positive result will be achieved on a Sequence Detection Instrument under the conditions recommended in this protocol.

A positive result on a Real-Time instrument is defined as a series of 36 replicates that give a mean C_T value with a standard deviation ≤ 0.16.

A positive result on an end-point instrument 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.

System Demonstrated Performance

The detection range has been demonstrated to be between 2 pg and 200 ng of total RNA control. This is equal to approximately 200 to 2 x 10^7 GAPDH copies respectively. Appropriate C_T values for 50 ng of Control RNA per reaction should be in the range of 15 to 22 cycles.

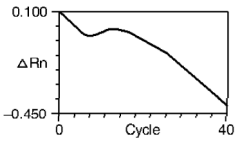
Troubleshooting

A

Troubleshooting

Observation	Possible Cause	Recommended Action
$\Delta R_n \leq$ No Template Control ΔR_n, and no amplification plot	Inappropriate reaction conditions	Troubleshoot RT-PCR optimization.
	Incorrect dye components chosen	Check dye component prior to data analysis.
	Reaction component omitted	Check that all the correct reagents were added.
	Incorrect primer or probe sequence	Resynthesize with appropriate sequence.
	Degraded template or no template added	Repeat with fresh template.
	Reaction inhibitor present	Repeat with purified template.
$\Delta R_n \leq$ No Template Control ΔR_n, and both reactions show an amplification plot	Amplicon contamination of reagents	Rerun reaction with AmpErase UNG.
	Template contamination of reagents	Check technique and equipment to confine contamination. Use fresh reagents.
		Run a No Enzyme Control and compare to a No Template Control.
Shifting R_n value during the early cycles of PCR (cycle 0 to 5)	Fluorescent emissions have not stabilized to new buffer conditions of reaction mix. This does not affect PCR or the final results	Reset default cycle to a higher value prior to analysis. Add probe to the buffer component and allow it to equilibrate at room temperature prior to Reagent Mix formulation.

Troubleshooting *(continued)*

Observation	Possible Cause	Recommended Action
Amplification plot dips downwards 	C_T value less than 15, amplification signal detected in early cycles	Reset upper value of baseline range to a lower value (two cycles lower than C_T value). Dilute the sample to increase the C_T value. See on-line tutorial: http://www.appliedbiosystems.com/support/tutorials/baseline
Multicomponent signal for ROX is not linear	Pure dye components are not correct	Rerun pure dye spectra.
	Incorrect dye components chosen	Choose correct dyes for data analysis.
Amplification plot is not within the log phase	PCR efficiency is poor	Re-optimize reaction conditions
C_T value is higher than expected	Less template added than expected	Increase sample amount
	Sample is degraded	Evaluate sample integrity.
C_T value is lower than expected	More sample added than expected	Reduce sample amount.
	Template or amplicon contamination	Review “Preventing Contamination” on page 1-13.
Standard deviation of C_T value >0.16	Variations in reaction performance	Repeat GAPDH Control reactions, paying particular attention to the protocol.
	Instrument variations	Check instrument performance for well-to-well reproducibility See instrument manuals.
	Inaccurate pipetting	Prepare a Reagent Mix. Use positive-displacement pipettors.

GAPDH Probe Characteristics

B

Overview

System Design

The mRNA target chosen was the transcript of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The mRNA amplicon target starts at base 1457 of the genomic sequence (HUMGAPDH in GenBank), spans two introns (intron number two from base 1482-3116, intron number three from base 3216 to 3306), and ends at base 3412. The mRNA amplicon size is 226 bases.

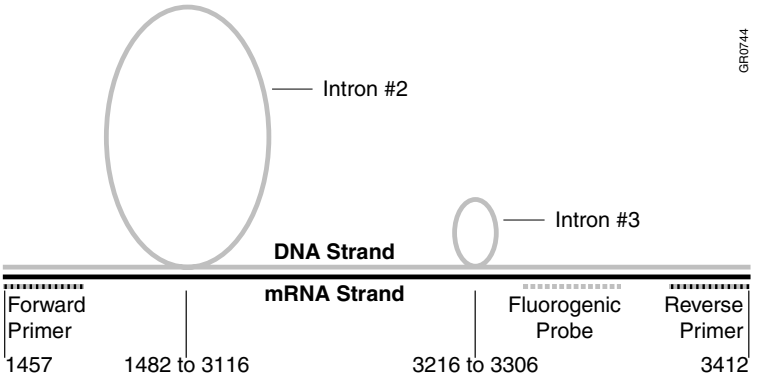


Figure B-1 Graphic representation of the genomic DNA sequence generated by the GAPDH mRNA target.

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D

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