TaqMan® PreAmp Master Mix Kit

Protocol

Applied Biosystems

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Contents

Preface

This preface covers:

Safety

Safety Alert Words

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

Definitions

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

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

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

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

Chemical Hazard Warning

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

Chemical Safety Guidelines

To minimize the hazards of chemicals:

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

About MSDSs

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

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

Obtaining MSDSs

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

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field of the MSDS Search page:
   a. Type in the chemical name, part number, or other information that you expect to appear in the MSDS of interest.
   b. Select the language of your choice.
   c. Click **Search**.
3. To view, download, or print the document of interest:

a. Right-click the document title.

b. Select:

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

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

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

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

c. Enter the required information.

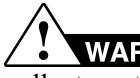
d. Click **View/Deliver Selected Documents Now**.

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

Chemical Waste Hazards

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

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



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

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

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

Waste Disposal

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

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

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

Biological Hazard Safety

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

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/nara/cfr/waisidx_01/ 29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

http://www.cdc.gov

How to Obtain More Information

Related Documentation

Related Documentation

See the following related documents for more information on the topics in this guide:

- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Absolute Quantification Getting Started Guide* (PN 4347825)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Relative Quantification Getting Started Guide* (PN 4347824)
- *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684)

- *Real-Time PCR Systems Chemistry Guide: Applied Biosystems 7900HT Fast Real-Time PCR Systems and 7300/7500/7500 Fast Real-Time PCR Systems* (PN 4348358)
- *User Bulletin #2: Relative Quantitation of Gene Expression* (PN 4303859)
- *Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide* (PN 4364016)

Note: For additional documentation, see "How to Obtain Support" on page x.

Send Us Your Comments

Applied Biosystems welcomes your comments and suggestions for improving its user documents. You can e-mail your comments to:

techpubs@appliedbiosystems.com

IMPORTANT! The e-mail address above is only for submitting comments and suggestions relating to documentation. To order documents, download PDF files, or for help with a technical question, go to **http://www.appliedbiosystems.com**, then click the link for **Support**. (See "How to Obtain Support" below).

How to Obtain Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Preamplification Overview

This protocol covers:

Purpose of the Kit

The TaqMan® PreAmp Master Mix Kit (PN 4384267) is intended for use with very small quantities of cDNA (1 to 250 ng). This kit allows you to increase the quantity of specific cDNA targets for gene expression analysis using TaqMan® Gene Expression Assays. Starting material is increased prior to PCR and the resulting preamplification product is then used for PCR.

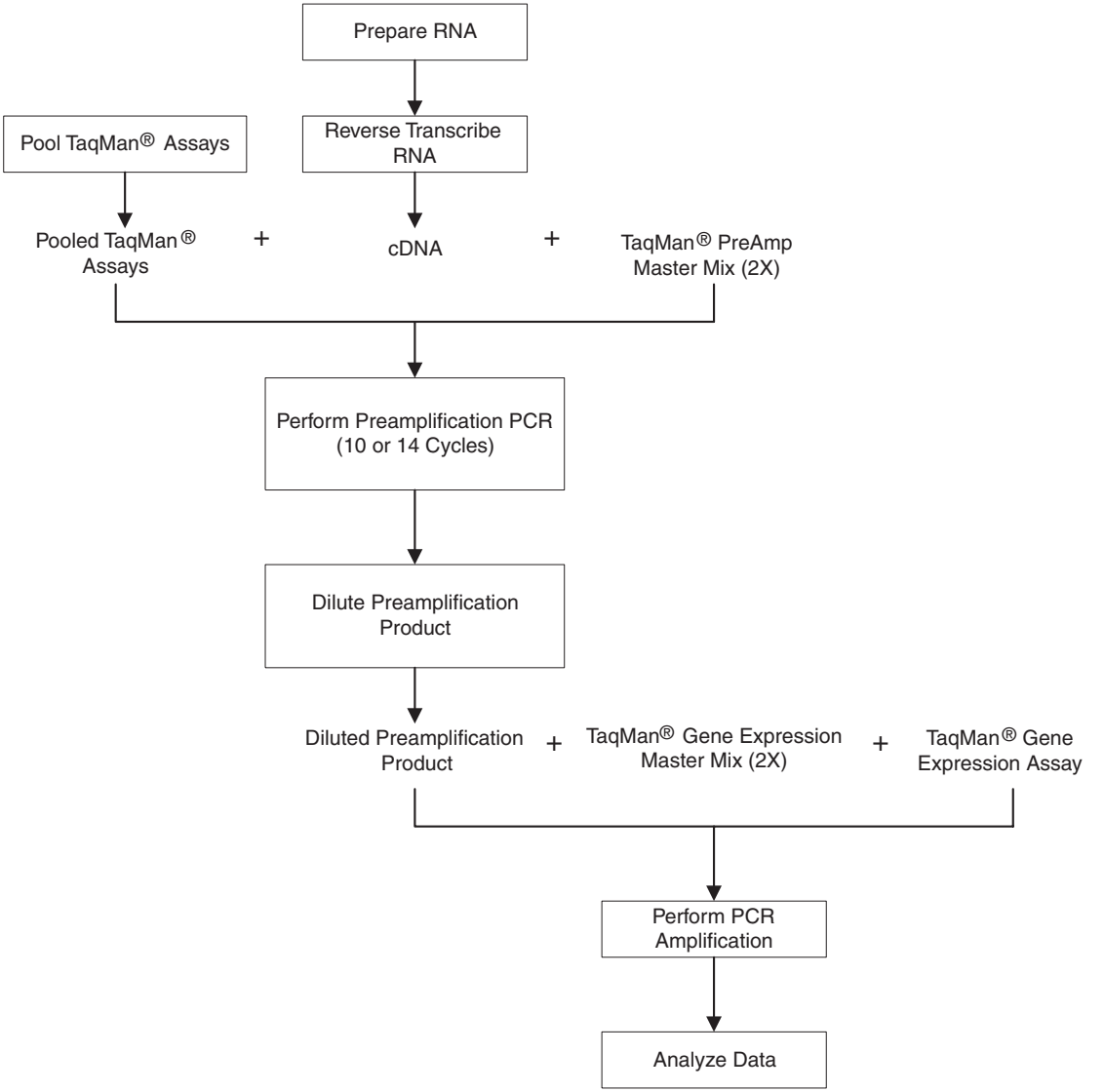
The TaqMan PreAmp Master Mix Kit enables multiplex preamplification of up to 100 targets at a time and provides unbiased amplification of specific amplicons for analysis with TaqMan Gene Expression Assays.

Process Overview

Up to 100 TaqMan Gene Expression Assays can be pooled together for preamplification. Combine the pooled assays with your cDNA and the PreAmp Master Mix, then perform preamplification PCR.

Workflow

The workflow below shows the entire preamplification process.



Required Materials

Kit Contents

Table 1-1 lists the components in the TaqMan® PreAmp Master Mix Kit (PN 4384267). Store the master mixes at 4 °C.

Table 1-1 TaqMan PreAmp Master Mix Kit contents

Kit Component	Quantity
TaqMan PreAmp Master Mix (2×)	1 mL (sufficient for 40 reactions)
TaqMan® Gene Expression Master Mix	5 mL
TaqMan® PreAmp Master Mix Kit Quick Reference Card (PN 4384556)	1

Obtaining This Protocol

To obtain a pdf of this protocol:

1. Go to **http://www.appliedbiosystems.com**, then click the click for **Support**.
2. Click **Product & Service Literature**.
3. On the Documents on Demand search page, enter **4384557**, then click **Search**.
4. Select one or more checkboxes to indicate how you want to obtain the document:
   - **Download** – To download the document from the Applied Biosystems web site
   - **Email** – To receive the pdf in an email
5. Click **View/Deliver Selected Items Now**.
6. If you selected to receive the pdf by email, enter required information, then click **View/Deliver Selected Items Now**.

Materials Needed

Table 1-2 Materials for pooling TaqMan assays

Item	Source
Equipment	
Centrifuge, quick spin	Major Laboratory Supplier (MLS)
Pipettors: 1- to 20-µL range; 20- to 200-µL range; 100- to 1000-µL range	MLS
Vortexer	MLS
Consumables	
Microcentrifuge tubes, 1.5-mL	MLS
Pipette tips, sterile, filtered: 1- to 20-µL range; 20- to 200-µL range; 100- to 1000-µL range	MLS
Reagents	
TaqMan® Gene Expression Assays	Applied Biosystems 4331182, 4351372
Tris-EDTA (TE) Buffer, 1×	MLS‡

‡. For the Material Safety Data Sheet (MSDS) of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Table 1-3 Materials for preamplification

Item	Source
Thermal Cycler (Select One)	
Applied Biosystems 9800 Fast Thermal Cycler	Applied Biosystems 4352604
GeneAmp® PCR System 9700	Applied Biosystems N8050200

Table 1-3 Materials for preamplification *(continued)*

Item	Source
Equipment	
Centrifuge	MLS
Pipettors: 1- to 20-µL range; 20- to 200-µL range; 100- to 1000-µL range	MLS
PCR Consumables (Select One)	
MicroAmp™ Optical 96-Well Reaction Plates and: • MicroAmp™ Clear Adhesive Films (100 films) • MicroAmp™ Optical Film Compression Pad (5 pads)	Applied Biosystems 4306737 4306311 4312639
Microcentrifuge tubes, 0.2-mL	MLS
Consumables	
Microcentrifuge tubes, 1.5-mL	MLS
Pipette tips, sterile, filtered: 1- to 20-µL range; 20- to 200-µL range; 100- to 1000-µL range	MLS
Reagents	
Ice	–
Nuclease-free water	MLS‡

‡. For the Material Safety Data Sheet (MSDS) of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Table 1-4 Materials for PCR amplification

Item	Source
Real-Time PCR System (Select One)	
Applied Biosystems 7900HT Fast Real-Time PCR System	Applied Biosystems 4329001
Applied Biosystems 7500 Real-Time PCR System	Applied Biosystems 4351104, 4351105
Applied Biosystems 7300 Real-Time PCR System	Applied Biosystems 4351101
Equipment	
Centrifuge, refrigerated	MLS
Pipettors: 1- to 20-µL range; 20- to 200-µL range; 100- to 1000-µL range	MLS
Plates (Select One Type)	
MicroAmp™ Optical 384-Well Reaction Plate with Barcode (50 plates)	Applied Biosystems 4309849
MicroAmp™ Optical 96-Well Reaction Plate with Barcode (20 plates)	Applied Biosystems 4306737
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (20 plates)	Applied Biosystems 4346906
Consumables	
MicroAmp™ Optical Adhesive Film (100 covers)	Applied Biosystems 4311971
Microcentrifuge tubes, 1.5-mL	MLS
Pipette-tips, sterile, filtered: 1- to 20-µL range; 20- to 200-µL range; 100- to 1000-µL range	MLS

Table 1-4 Materials for PCR amplification *(continued)*

Item	Source
Reagents	
TaqMan® Gene Expression Assays	Applied Biosystems 4331182, 4351372
TaqMan® Gene Expression Master Mix:	Applied Biosystems
• Mini-Pack, one 1-mL tube (40 × 50-µL reactions)	4370048
• 1-Pack, one 5-mL bottle (200 × 50-µL reactions)	4369016
• 2-Pack, two 5-mL bottles (400 × 50-µL reactions)	4369514
• 5-Pack, five 5-mL bottles (1000 × 50-µL reactions)	4369510
• 10-Pack, ten 5-mL bottles (2000 × 50-µL reactions)	4369542
• Bulk Pack, one 50-mL bottle (2000 × 50-µL reactions)	4370074
Nuclease-free water	MLS‡
Tris-EDTA (TE) Buffer, 1×	MLS

‡. For the Material Safety Data Sheet (MSDS) of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Pooling the TaqMan Assays

In this procedure you prepare a 0.2× pooled TaqMan assay mix for running the preamplification reaction.

See Table 1-2 on page 4 for required materials to pool TaqMan assays.

Recommended TaqMan Assays

Applied Biosystems recommends the following:

- Pool TaqMan assays with a $C_T \leq 35$ when using 0.3 ng/µL cDNA.
- Do not include the 18S TaqMan assay in the pool because it is so highly expressed.
- Check the uniformity of preamplification (see page 20).

Pooling the TaqMan Assays

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

To pool the TaqMan assays:

1.	In a microcentrifuge tube, combine equal volumes of each 20× TaqMan® Gene Expression Assay, up to a total of 100 assays. For example, to pool 50 TaqMan assays, combine 10 µL of each TaqMan assay.
2.	Dilute the pooled TaqMan assays using 1× TE buffer so that each assay is at a final concentration of 0.2×. For the above example, add 500 µL of 1× TE buffer to the pooled TaqMan assays for a total volume of 1 mL. **Note:** Applied Biosystems recommends 1× TE Buffer to maintain stability during long-term storage.

Preparing cDNA from RNA

About the cDNA Reverse Transcription Kit

Applied Biosystems recommends the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (PN 4368814) for preparing cDNA. The kit contains all necessary materials and reagents for the reverse transcription of total RNA to single-stranded cDNA.

IMPORTANT! Applied Biosystems has designed and developed TaqMan Gene Expression Assays for use with samples reverse transcribed from total RNA using the High Capacity cDNA Reverse Transcription Kit. Other protocols have not been tested for use with TaqMan Gene Expression Assays.

General Process

Use the High Capacity cDNA Reverse Transcription Kit to synthesize single-stranded cDNA from total RNA samples. The process involves:

1. Preparing the RT master mix
2. Preparing the cDNA reaction plate
3. Performing reverse transcription

Note: Refer to the *High-Capacity cDNA Reverse Transcription Kit Protocol* (PN 4375575) for additional guidelines and instructions. The protocol is not shipped with the kit. Download the protocol from the Applied Biosystems Documents on Demand web site at

http://www.docs.appliedbiosystems.com/search.taf

RNA Template Guidelines

For optimal performance of the High Capacity cDNA Reverse Transcription Kit, TaqMan PreAmp Master Mix, and TaqMan Gene Expression Assays, Applied Biosystems recommends using RNA with the following characteristics:

- Greater than 60 µL of sample
- Between 0.002 and 0.2 µg/µL in concentration of RNA
- Less than 0.005% of genomic DNA by weight
- Free of inhibitors of reverse transcription and PCR
- Dissolved in PCR-compatible buffer
- RNA integrity number ≥7, as measured by the Agilent Bioanalyzer

- Free of RNase activity

  Note: If you suspect that the RNA contains RNase activity, add RNase inhibitor to the reverse transcription reaction at a final concentration of 1.0 U/µL. It is not necessary to add RNase inhibitor to the reverse transcription reaction if the RNA was purified using the ABI PRISM® 6100 Nucleic Acid PrepStation and Applied Biosystems nucleic acid purification reagents.

- Nondenatured

  Note: It is not necessary to denature the RNA. Denaturation of the RNA may reduce the yield of cDNA for some gene targets.

Reagent and Sample Preparation Guidelines

Follow the guidelines to ensure optimal performance of the High Capacity cDNA Reverse Transcription Kit and of the TaqMan Gene Expression Assays:

- Use nuclease-free pipette tips and reagents to minimize degradation of the RNA.
- Observe standard laboratory practices when handling RNA.

Running the Preamplification Reaction

In this step, you perform multiplex preamplification of up to 100 specific cDNA targets to increase the quantity of the desired cDNA targets for gene expression analysis using TaqMan Gene Expression Assays.

See Table 1-3 on page 4 for required materials to run the preamplification reaction.

Before You Begin

Before performing preamplification with limited biological samples, Applied Biosystems recommends checking whether all amplicons are amplified uniformly without bias. See "Appendix A: Checking Preamplification Uniformity (Optional)" on page 20.

Reagent Preparation Guidelines

Following these guidelines ensures optimal performance:

- Keep all TaqMan Gene Expression Assays protected from light, in the freezer, until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
- Prior to use:
  - Homogenize the TaqMan PreAmp Master Mix by gently swirling the tube.
  - Thaw any frozen cDNA samples by placing them on ice. When thawed, mix the samples by vortexing and then centrifuge the tubes briefly.
  - Thaw the Gene Expression Assays by placing them on ice. When thawed, mix the assays by vortexing and then centrifuge the tubes briefly.

Determining Preamplification Conditions

First determine whether to perform 10 or 14 preamplification cycles:

- **10 preamplification cycles** – More suitable for reactions using lower numbers of pooled assays; requires a shorter run time; produces material sufficient for fifty 20-µL or twenty 50-µL PCR amplification reactions
- **14 preamplification cycles** – More suitable for reactions using higher numbers of pooled assays; more suitable when samples are extremely limited; produces material sufficient for two hundred 20-µL or eighty 50-µL PCR amplification reactions

Use the table below to determine the remaining preamplification conditions, based on the number of preamplification cycles.

Number of Preamplification Cycles	Dilution Factor of Preamplification Products With 1× TE Buffer	Final Volume of Diluted Preamplification Product
10	1:5	250 µL
14	1:20	1 mL

Running the Preamplification Reaction

To set up and run the preamplification reaction:

1. Prepare each preamplification reaction in a 0.2-mL or 1.5-mL microcentrifuge tube, depending on the total volume:

Component	Volume‡ (µL/Reaction)	Final Concentration
TaqMan PreAmp Master Mix (2×)	25.0	1×
Pooled assay mix (0.2×)	12.5	0.05× (each assay)
1–250 ng cDNA sample + nuclease-free water	12.5	0.02–5.0 ng/µL
Total	50.0	—

‡. To increase the number of reactions, use whole multiples of the specified volumes. Aliquot only 50 µL per reaction in the 96-well plate.

2. Cap the microcentrifuge tube or seal the 96-well plate with a MicroAmp Clear Adhesive Film.
3. Mix the reactions by gently inverting the tube or plate, then centrifuge briefly.
4. (Optional) If using a 96-well plate, place a MicroAmp Optical Film Compression Pad on top of it.

To set up and run the preamplification reaction: ***(continued)***

<table>
<tr><td>5.</td><td>Load the plate or tubes into the thermal cycler.</td></tr>
<tr><td>6.</td><td>Set up the thermal cycling conditions:
<table>
<tr><td rowspan="2"></td><td rowspan="2">Enzyme Activation</td><td colspan="2">Preamplification PCR</td></tr>
<tr><td colspan="2">HOLD</td><td colspan="2">CYCLE (10 or 14 cycles)</td></tr>
<tr><td></td><td></td><td>Denature</td><td>Anneal/ Extend</td></tr>
<tr><td>Temp</td><td>95 °C</td><td>95 °C</td><td>60 °C</td></tr>
<tr><td>Time</td><td>10 min</td><td>15 sec</td><td>4 min</td></tr>
</table></td></tr>
<tr><td>7.</td><td>Start the run.</td></tr>
<tr><td>8.</td><td>Upon completion, immediately remove the plate from the thermal cycler and place it on ice.</td></tr>
<tr><td>9.</td><td>Go to “Performing PCR Amplification” on page 14, or you may store aliquots of the preamplification product at –20 °C.

Note: If you choose to store the preamplification product at –20 °C, store it in aliquots to minimize freeze-thaw cycles.</td></tr>
</table>

Performing PCR Amplification

In this step, the DNA polymerase [from the TaqMan Gene Expression PCR Master Mix (2✕)] amplifies the preamplified target cDNA, using sequence-specific primers and TaqMan MGB probe (from the TaqMan Gene Expression Assay Mix).

See Table 1-4 on page 6 for required materials to perform PCR amplification.

PCR Process

Performing the PCR step requires the following procedures:

1. Setting up the plate document (see below)
2. Preparing the reaction plate
3. Running the plate

Setting Up the Plate Document

Refer to the appropriate instrument user guide for instructions on how to set up the plate document.

Reagent Preparation Guidelines

Following these guidelines ensures optimal PCR performance:

- Keep all TaqMan Gene Expression Assays protected from light, in the freezer, until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
- Prior to use:
  - Mix the PCR master mix thoroughly by swirling the bottle.
  - Resuspend the TaqMan Gene Expression Assay mix by vortexing and then centrifuge the tube briefly.
  - Thaw any frozen preamplified cDNA samples by placing them on ice. When thawed, resuspend the samples by vortexing and then centrifuge the tubes briefly.
- Prepare the PCR reaction mix before transferring to the reaction plate for thermal cycling and data analysis.

PCR Reaction Mix Components

The recommended reaction sizes vary depending on the reaction plate used. Prepare the plate so that each PCR reaction contains the components listed in the following table.

Component	Volume (µL) / Reaction	
	20-µL Reactions (384-Well and Fast Plates)	**50-µL Reactions (96-Well Plates)**
TaqMan Gene Expression Assay (20X)	1.0	2.5
Diluted preamplified cDNA products (diluted 1:5 or 1:20)‡	5.0	12.5
TaqMan Gene Expression Master Mix (2X)	10.0	25.0
Nuclease-free water	4.0	10.0
Total Volume	20.0	50.0

‡. If you performed 14 preamplification cycles instead of 10, increase the dilution factor to 1:20 to account for the higher product yield (step 1 on page 16).

Preparing the PCR Reaction Plate

Applied Biosystems recommends performing four replicates of each reaction.

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

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

To prepare the PCR reaction plate:

1. Dilute the preamplification products using 1× TE Buffer. The dilution factor varies according to the number of preamplification cycles you performed:

Number of Preamplification Cycles	Dilution Factor
10	1:5
14	1:20

Note: Applied Biosystems recommends 1× TE Buffer for stability during long-term storage.

To prepare the PCR reaction plate: ***(continued)***

2. Prepare the PCR reaction mix for each sample (in quadruplicate) separately:

Component	Volume (µL) for Four Reactions‡	
	20-µL Reactions (384-Well Setup)	50-µL Reactions (96-Well Setup)
TaqMan Gene Expression Assay (20×)	5.0	12.5
Preamplified cDNA products (diluted 1:5 or 1:20)§	25.0	62.5
TaqMan Gene Expression Master Mix (2×)	50.0	125.0
Nuclease-free water	20.0	50.0
Total Volume	100.0	250.0

‡. An additional reaction is included in the calculations to provide excess volume for the loss that occurs during reagent transfers.

§. If you are performing 14 cycles, increase the dilution factor to account for the higher product yield (step 1 on page 16).

3. Mix the solutions by gently pipetting up and down, then cap the tubes.
4. Centrifuge the tubes briefly to spin down the contents and eliminate air bubbles from the solutions.
5. Transfer the appropriate volume of each reaction mixture to wells of an optical plate.
6. Cover the plate with an optical adhesive cover or with optical flat caps.
7. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions.

Running the Plate

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

To run the plate on the 7900HT, 7500, or 7300 system:

1. Place the reaction plate in the instrument.
2. Use the default thermal cycling conditions (standard mode):

Step	UDG Activation‡	AmpliTaq Gold® Enzyme Activation	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temp	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min

‡. The 2-min, 50 °C step is required for optimal UDG activity.

3. Set the reaction volume according to the volume used.
4. Start the run.

Analyzing Results

Overview

Data analysis varies depending on the instrument. Refer to the appropriate instrument user guide for instructions on how to analyze your data.

General Process

The general process for analyzing the data from gene expression assays involves the following procedures:

1. Viewing the amplification plots for the entire plate
2. Setting the baseline and threshold values
3. Use the comparative C_T method to analyze your data

Resources for Data Analysis

Refer to the following documents for more information about analyzing your data:

- The appropriate instrument user guide
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Absolute Quantification Getting Started Guide* (PN 4347825)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Relative Quantification Getting Started Guide* (PN 4347824)
- *Real-Time PCR Systems Chemistry Guide: Applied Biosystems 7900HT Fast Real-Time PCR Systems and 7300/7500/7500 Fast Real-Time PCR Systems* (PN 4348358)
- *User Bulletin #2: Relative Quantitation of Gene Expression* (PN 4303859)
- *Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR* – Support document available on the Applied Biosystems support web site. To obtain, see "How to Obtain Support" on page x.

Appendix A: Checking Preamplification Uniformity (Optional)

Applied Biosystems recommends checking whether all amplicons are amplified uniformly without bias before you perform preamplification with your limited biological samples. Checking preamplification uniformity involves performing a relative quantitation experiment with your selected TaqMan Gene Expression Assays to compare amplification of cDNA to amplification of preamplified cDNA.

Recommended Template

Applied Biosystems recommends checking preamplification uniformity using cDNA that is non-limiting, such as a control sample, and that was prepared using the High Capacity cDNA Reverse Transcription Kit.

Preparing Preamplified cDNA

1. Pool your TaqMan Gene Expression Assays as described in "Pooling the TaqMan Assays" on page 8.

   Note: Make sure to include an endogenous uniformity reference gene in your pool. For human gene expression assays, Applied Biosystems recommends using CDKN1B (Assay ID HS00153277_m1) because of its consistent gene expression profile.

2. Using your non-limited cDNA sample, prepare preamplified cDNA according to "Running the Preamplification Reaction" on page 11.

Preparing the Uniformity Reaction Plate

For each TaqMan Gene Expression Assay that you want to include in your pool, set up two sets of separate reactions using the procedures in "Preparing the PCR Reaction Plate" on page 16:

- **cDNA**: Instead of using diluted preamplified cDNA, use 0.3 ng/µL of the non-limited cDNA sample that is not preamplified
- **Preamplified cDNA**: Use diluted preamplified non-limited cDNA

Running the Uniformity Plate

Perform a relative quantitation run on a real-time PCR system according to "Running the Plate" on page 18.

Analyzing the Uniformity Results

Use a relative quantitation study to analyze your results and to determine $\Delta\Delta C_T$ values between the cDNA plate (cDNA that is not preamplified) and the preamplified cDNA plate. Refer to the appropriate relative quantitation document:

- *Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide* (PN 4364016)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantitation Getting Started Guide* (PN 4347824).

To check for preamplification uniformity:

1.	Calculate the average C_T values for each assay. **Note:** Set the cDNA plate as the calibrator under analysis settings.
2.	Calculate ΔC_T for cDNA by subtracting the average C_T value of the uniformity reference gene from the average C_T value of each gene: $\Delta C_{T\,(cDNA)} = \text{avg } C_{T\,(Target\ X)} - \text{avg } C_{T\,(Uniformity\ ref\ gene)}$ The purpose is to normalize each individual target to the desired uniformity reference gene when using cDNA that is not preamplified.
3.	Calculate ΔC_T for multiplex preamplification by subtracting the average C_T value of the uniformity reference gene from the average C_T value of each gene: $\Delta C_{T\,(Preamp)} = \text{avg } C_{T\,(Target\ X)} - \text{avg } C_{T\,(Uniformity\ ref\ gene)}$ The purpose is to normalize each individual target to the desired uniformity reference gene when using preamplified cDNA.
4.	Calculate $\Delta\Delta C_T$ between cDNA and preamplified cDNA by subtracting the ΔC_T value for cDNA (step 2) from the ΔC_T for multiplex preamplification (step 3): $\Delta\Delta C_T = \Delta C_{T\,(Preamp)} - \Delta C_{T\,(cDNA)}$ A $\Delta\Delta C_T$ value close to zero indicates preamplification uniformity. Typically, 90% of targets produce $\Delta\Delta C_T$ values within ±1.5.

Example Figure 1 shows an example of preamplification uniformity results for 10 assays. Note that all 10 assays in the example show preamplification uniformity ($\Delta\Delta C_T$ within ±1.5, or Log_{10} of $\Delta\Delta C_T$ within ±0.452).

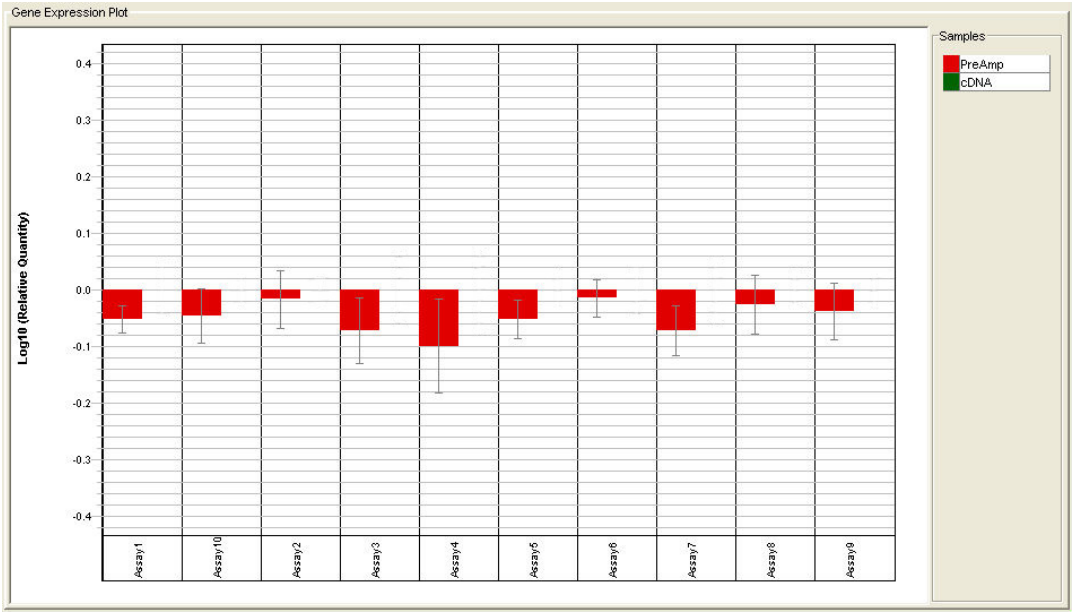


Figure 1 Log_{10} of fold difference between preamplified cDNA and non-preamplified cDNA

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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

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