Assays-on-Demand™ Gene Expression Products

Protocol

Applied Biosystems

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Product Overview

Product Description

Assays-on-Demand™ products for Gene Expression are biologically informative, preformulated gene expression assays that provide rapid, reliable results on human RefSeq transcripts.

If the assay you need is not currently available, check the Web site at a later time or use our Assays-by-Design℠ service.

The Assays-by-Design service is an assay development service that designs, synthesizes, formulates, and delivers analytically quality-controlled primer and probe sets for single nucleotide polymorphism (SNP) genotyping and gene expression assays based on sequence information submitted by the customer. To place an order, contact your Applied Biosystems representative.

Purpose

Assays-on-Demand™ Gene Expression Products are designed for the detection and quantification of specific nucleic acid sequences. These products provide researchers with optimized, ready-to-use 5′ nuclease assays for human transcripts. Gene expression quantification is performed in a two-step reverse transcription-polymerase chain reaction (RT-PCR) in which the PCR step is coupled with a 5′ fluorogenic nuclease assay.

Product Properties

The following are properties of Assays-on-Demand Gene Expression Products:

- All Assays-on-Demand Gene Expression Products are designed and optimized to work with the TaqMan® Universal PCR Master Mix, with or without AmpErase® UNG.
- A variety of targets and endogenous controls can be assayed in singleplex reactions in the same 384-well or 96-well reaction plate with universal thermal cycling parameters.
- The Assays-on-Demand Gene Expression Products are designed to amplify target cDNA without amplifying genomic DNA (for multi-exon genes denoted M in the assay name). Assays denoted S in the assay name are designed within an exon and, by definition, will detect genomic DNA.

The latest information on specific product uses is available on the Applied Biosystems Web site:

http://www.appliedbiosystems.com

Available Products

Assays-on-Demand Gene Expression Products (P/N 4331182) consist of target assays and endogenous control assays to be used in a 5′ nuclease assay to amplify and detect expression of specific nucleic acid sequences.

Visit our Web site to view the available products:

http://www.appliedbiosystems.com

For more information about ordering Assays-on-Demand Gene Expression Products, see "Ordering Assays" on page 11.

About Target Assays

Target assays from the Assays-on-Demand Gene Expression Products possess the following characteristics:

- A variety of Assays-on-Demand Gene Expression Products is available for detecting transcripts corresponding to many biological pathways.
- Many of the target assays are specifically designed to detect and quantify cDNA sequences without detecting genomic DNA.
- Target assays contain TaqMan® MGB probes (6-FAM™ dye-labeled) combined with primers at non-limiting concentrations.

About Endogenous Control Assays

Endogenous control assays from the Assays-on-Demand Gene Expression Products possess the following characteristics:

- The controls are specifically designed to amplify cDNA sequences unless otherwise noted.
- Endogenous control assays contain TaqMan MGB probes (6-FAM dye-labeled) combined with primers at non-limiting concentrations. These are designed to be used as external endogenous controls in a singleplex assay.
- Available endogenous controls will include the following:
  - Eukaryotic 18S ribosomal RNA (18S rRNA)
  - Human large ribosomal protein (RPLP0)
  - Human β-actin (ACTB)
  - Human cyclophilin A (PPIA)
  - Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
  - Human phosphoglycerokinase 1 (PGK1)
  - Human β_2-microglobulin (β2M)
  - Human β-glucoronidase (GUSB)
  - Human hypoxanthine ribosyltransferase 1 (HPRT1)
  - Human transcription factor IID/TATA-box binding protein (TBP)
  - Human transferrin receptor (CD71) (TFRC)

Protocol Overview

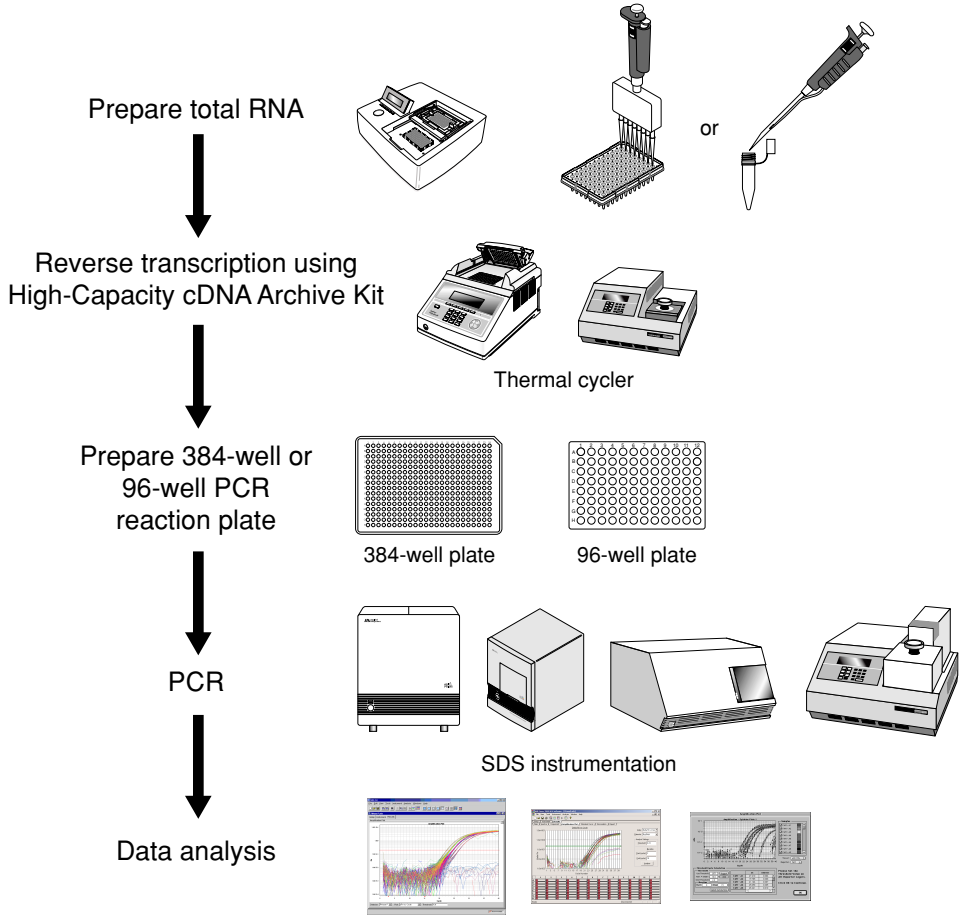
About This Protocol

This protocol provides the following:

- Background information about gene expression assays
- A list of materials and equipment that can be used to perform gene expression assays using Assays-on-Demand Gene Expression Products
- Guidelines for synthesizing cDNA from total RNA using the High Capacity cDNA Archive Kit (P/N 4322171)
- Instructions for amplifying cDNA using Assays-on-Demand Gene Expression Products on an Applied Biosystems Sequence Detection System (SDS)
- General guidelines for data analysis

Procedure Flowchart

The following diagram provides a simplified overview of the procedure for using Assays-on-Demand Gene Expression Products.



About Gene Expression Assays

Overview

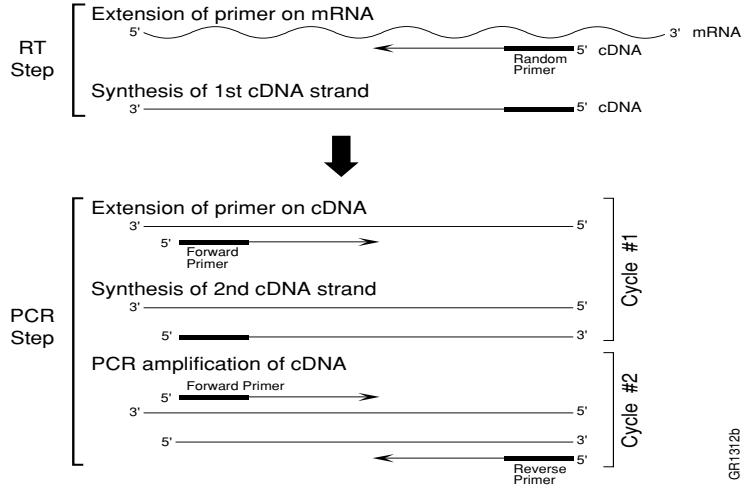
The gene expression assays contain:

- Two primers for amplifying the sequence of interest
- One TaqMan MGB probe (6-FAM dye-labeled) for detecting the sequence of interest

Two-Step RT-PCR

The gene expression assays using Assays-on-Demand Gene Expression Products are performed in a two-step RT-PCR. The figure below illustrates the assay steps.

Note: The figure below does not show hybridization of the TaqMan MGB probe (6-FAM dye-labeled).



In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using random primers from the High Capacity cDNA Archive Kit. In the PCR step, PCR products are synthesized from cDNA samples using the TaqMan Universal PCR Master Mix (with or without AmpErase UNG).

About the Probes

The TaqMan MGB probes contain:

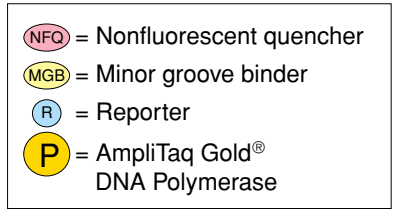
- A reporter dye (6-FAM) linked to the 5′ end of the probe
- A minor groove binder (MGB)

  This modification increases the melting temperature (T_m) without increasing probe length (Afonina *et al.,* 1997; Kutyavin *et al.,* 1997), which allows the design of shorter probes.
- A nonfluorescent quencher (NFQ) at the 3′ end of the probe

  Because the quencher does not fluoresce, Applied Biosystems sequence detection systems can measure reporter dye contributions more accurately.

5′ Nuclease Assay Process

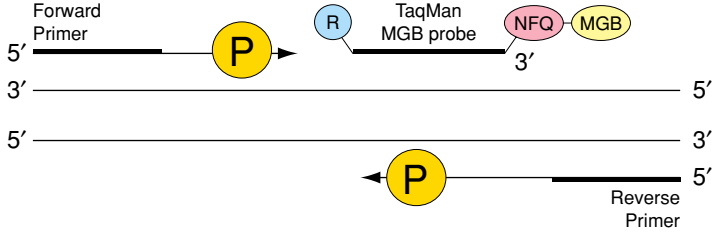
The 5′ nuclease assay process takes place during PCR amplification. The legend for the illustrations is shown below.



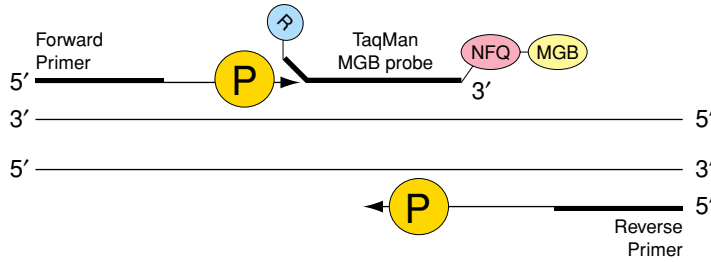
Polymerization

During PCR, the TaqMan MGB probe (6-FAM dye-labeled) anneals specifically to a complementary sequence between the forward and reverse primer sites.

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).

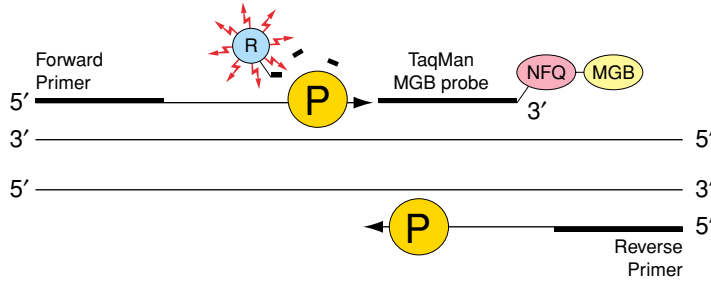


Strand Displacement



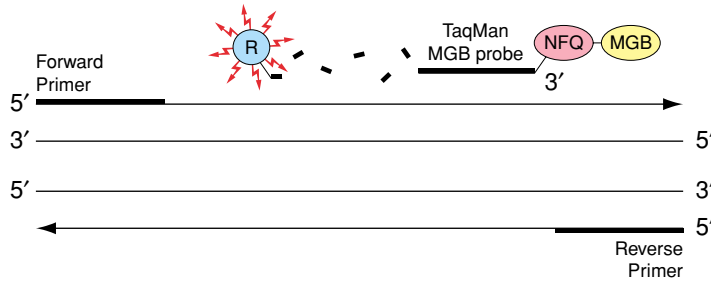
Cleavage

AmpliTaq Gold DNA polymerase cleaves only probes that are hybridized to the target. Cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter. The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, any nonspecific amplification is not detected.



Polymerization Completed

Polymerization of the strand continues, but the 3′ end of the probe is blocked to prevent extension of the probe during PCR.



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

Definitions of Terms

This protocol assumes these definitions:

- **Endogenous control**—RNA or DNA that is present in each experimental sample as isolated

  An endogenous control assay is performed in a separate well from the target assay in a singleplex reaction.
- **Target**—The genetic sequence of interest

Materials and Equipment

Available Products

The Assays-on-Demand Gene Expression Products are available as shown in the table below.

Product	Part Number	Contents	Volume	Description
Target Assay	4331182	20X Target Assay Mix	250 µL	One tube containing sequence-specific primers and TaqMan MGB probe (6-FAM dye-labeled)
Endogenous Control Assay		20X Endogenous Control Assay Mix		

Assay Naming

Gene expression assays from the Assays-on-Demand Gene Expression Products are named using the following format:

Hsnnnnnnnn_XY

This 13-digit identifier includes the following components:

- Hs—corresponds to the species (for example, Homo sapien)
- nnnnnnnn–corresponds to the eight digits identifying the assay
- X—corresponds to the exons
  - m for assays over an exon-exon boundary
  - s for assays within an exon or to a single-exon gene
- Y—corresponds to the assay revision number

Ordering Assays

Visit our Web site to order the available assays:

www.appliedbiosystems.com

Use the part number (P/N 4331182) and the 13-digit assay name (described above) to locate and order the assay you need.

Storage and Stability

Follow the guidelines below for storing Assays-on-Demand Gene Expression Products:

- Store the assay mixes at –15 to –25 °C
- Minimize freeze-thaw cycles
- Keep all Assays-on-Demand Gene Expression Products protected from direct exposure to light. Excessive exposure to light may affect the fluorescent probes.

Equipment and Materials Not Included

The following tables include required and optional equipment and materials for using Assays-on-Demand Gene Expression Products. Unless otherwise noted, many of the items listed are available from major laboratory suppliers (MLS).

Instruments from Applied Biosystems

Instruments	Source
ABI PRISM® 7900HT Sequence Detection System	Contact your local Applied Biosystems sales office.
ABI PRISM® 7000 Sequence Detection System	
ABI PRISM® 7700 Sequence Detection System	
GeneAmp® 5700 Sequence Detection System	
ABI PRISM™ 6700 Automated Nucleic Acid Workstation	
ABI PRISM™ 6100 Nucleic Acid PrepStation	
GeneAmp® PCR System 9700 thermal cycler	
GeneAmp® PCR System 9600 thermal cycler	

User-Supplied Materials

Materials	Source
6700 ABI PRISM™ 96-Well Optical Reaction Plate With Barcode (code 128)	Applied Biosystems (P/N 4306737)
ABI PRISM™ 96-Well Optical Reaction Plate With Barcode (code 128)	Applied Biosystems (P/N 4326659)
ABI PRISM™ 384-Well Clear Optical Reaction Plate With Barcode (code 128)	Applied Biosystems (P/N 4309849)
ABI PRISM™ Optical Adhesive Covers	Applied Biosystems (P/N 4311971)
ABI PRISM™ Optical Caps, 8 caps/strip	Applied Biosystems (P/N 4323032)
ABI PRISM™ Cap Installing Tool	Applied Biosystems (P/N 4330015)
High-Capacity cDNA Archive Kit	Applied Biosystems (P/N 4322171)
MicroAmp® Multi Removal Tool	Applied Biosystems (P/N 4313950)
Reagent Tubes With Caps, 10-mL	Applied Biosystems (P/N 4305932)
TaqMan Universal PCR Master Mix	Applied Biosystems (P/N 4304437)

User-Supplied Materials *(continued)*

Materials	Source
TaqMan Universal PCR Master Mix, No AmpErase® UNG	Applied Biosystems (P/N 4324018)
Accessories for tubes of assay mixes • Decapper for single caps (P/N 54000) • Decapper for eight caps (P/N 54001) • TPE cap cluster for simultaneously capping of 96 individual polypropylene tubes, 50 capmats/bag (P/N 53001)	Micronic BV* PO Box 604 8200 AP Lelystad Netherlands Telephone: +31(0)320.277077 Fax: +31(1)320.277088
Centrifuge with plate adapter	MLS
Disposable gloves	MLS
Microcentrifuge	MLS
Microsoft® Excel or equivalent spreadsheet and analysis software	Software suppliers
Pipet tips, aerosol resistant	MLS
Pipettors: • Positive-displacement • Air-displacement • Multichannel	MLS
Polypropylene tubes	MLS
RNase inhibitor	Applied Biosystems (P/N 8080119)
RNase-free, sterile-filtered water	MLS
Vortexer	MLS

*Other vendors supply similar products

Applied Biosystems Documents

Documents	Part Number
High-Capacity cDNA Archive Kit Protocol	4322169
TaqMan Cytokine Gene Expression Plate I Protocol	4306744
TaqMan Universal PCR Master Mix Protocol	4304449
User Bulletin #2: Relative Quantitation of Gene Expression	4303859

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, accurate chemistry kit use, or safe use of a chemical.

⚠ 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:

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:

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

Overview

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

AmpErase UNG

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

UNG acts on single- and double-stranded dU-containing DNA by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA (Longo *et al.*, 1990).

General PCR Practices

Follow these recommended procedures:

- 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 you suspect that they are contaminated.
- Maintain separate areas, dedicated equipment, and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes and reaction plates carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Use positive-displacement pipets or aerosol-resistant pipet tips.
- Clean lab benches and equipment periodically with freshly diluted 10% bleach solution.

Reverse Transcription

Overview

Synthesis of cDNA from total RNA samples is the first step in using Assays-on-Demand Gene Expression Products. Applied Biosystems recommends using the High-Capacity cDNA Archive Kit to obtain cDNA from total RNA samples.

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

General Process

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

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

Note: Refer to the *High-Capacity cDNA Archive Kit Protocol* (P/N 4322169) for additional guidelines and instructions.

RNA Template Guidelines

For optimal performance of the High-Capacity cDNA Archive Kit and of the Assays-on-Demand Gene Expression Products, 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
- 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 6700 Automated Nucleic Acid Workstation or the ABI PRISM 6100 Nucleic Acid PrepStation and Applied Biosystems nucleic acid purification reagents.

- Nondenatured

IMPORTANT! 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 below to ensure optimal performance of the High-Capacity cDNA Archive Kit and of the Assays-on-Demand Gene Expression Products.

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

Manual Method Overview

With the manual method, you perform the following:

1. Prepare 2X RT master mix.
2. Prepare the cDNA archive reaction plate manually.
3. Place the plate in the GeneAmp PCR System 9700 or 9600 thermal cycler for reverse transcription.

Preparing Reaction Master Mix

Prepare 2X RT master mix using the kit components before preparing the reaction plate.

To prepare 2X RT master mix:

1. Allow the kit components to thaw on ice.
2. Calculate the volume of components needed to prepare the cDNA archive reaction plate, using the table below.

Component	Volume (µL) / Reaction
10X Reverse Transcription Buffer	10
25X dNTPs	4
10X random primers	10
MultiScribe™ Reverse Transcriptase, 50 U/µL	5
Nuclease-free water	21
Total per Reaction	50

   Note: Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.

3. Pipette the required volumes of components into a 10-mL polypropylene tube.

   ⚠ WARNING CHEMICAL HAZARD. 10X RT Buffer may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate eyewear, clothing, and gloves.

4. Place the 2X RT master mix on ice until you prepare the cDNA archive reaction plate.

Preparing the cDNA Archive Reaction Plate

To prepare the cDNA archive reaction plate:

1.	Pipet 50 µL of 2X RT master mix into each well of a 96-well reaction plate.
2.	Pipet 50 µL of RNA sample into wells, pipetting up and down two times to mix.
3.	Cover the plate with caps.
4.	Centrifuge the plate briefly to spin down the contents and to eliminate any air bubbles.
5.	Place the plate on ice until you are ready to load the thermal cycler.

Selecting a Thermal Cycler

Use one of the following instruments for reverse transcription:

- GeneAmp PCR System 9700 thermal cycler
- GeneAmp PCR System 9600 thermal cycler

IMPORTANT! Because of the differences in ramp rates and thermal accuracy, you may need to adjust the settings if you choose to use other thermal cyclers.

Performing Reverse Transcription

To perform reverse transcription:

1. Program the thermal cycler conditions.

   IMPORTANT! These conditions are optimized for use with the Assays-on-Demand Gene Expression Products.

	Step 1	Step 2
Temperature	25 °C	37 °C
Time	10 min	120 min

   IMPORTANT! For most applications and when large amounts of cDNA are required, Applied Biosystems recommends 120 min at 37 °C for reverse transcription to achieve optimal conversion.

2. Set the reaction volume to **100 µL**.
3. Load the reaction plate into the thermal cycler.
4. Start the reverse transcription run.

PCR Amplification

Overview

Target amplification, using cDNA as the template, is the second step in using Assays-on-Demand Gene Expression Products. In this step, AmpliTaq Gold DNA polymerase from the TaqMan Universal PCR Master Mix (with or without AmpErase UNG) amplifies target cDNA synthesized from the RNA sample, using sequence-specific primers and TaqMan MGB probe (6-FAM dye-labeled) from the Target Assay Mix or from the Endogenous Control Assay Mix.

IMPORTANT! The PCR step must be performed on an ABI PRISM Sequence Detection System. Thermal cyclers cannot be used because they lack the ability to detect and record the fluorescent signals generated by the cleavage of TaqMan probes.

PCR Process

Performing the PCR step for singleplex assays in 384-well or 96-well formats requires the following procedures:

1. Configuring the sequence detector plate document
2. Preparing the reaction plate
3. Running the plate

Configuring the Plate Document

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

cDNA Template Guidelines

For optimal performance of Assays-on-Demand Gene Expression Products, use 1 to 100 ng of RNA converted to cDNA per 50-µL reaction.

Reagent Preparation Guidelines

The following guidelines will ensure optimal PCR performance:

- Keep all Assays-on-Demand Gene Expression Products 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 TaqMan Universal PCR Master Mix (with or without AmpErase UNG) thoroughly by swirling the bottle.
  - Resuspend the assay mix by vortexing and then centrifuge the tube briefly.
  - Thaw any frozen 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 fluorescence analysis.

PCR Reaction Mix Components

Applied Biosystems recommends performing four replicates of each reaction. The recommended reaction size is 20 µL for a 384-well setup and 50 µL for a 96-well setup. Prepare the plate so that each PCR reaction contains the components as listed in the following table.

Component	Volume (µL) / Reaction	
	20-µL Reactions (384-Well Setup)	**50-µL Reactions (96-Well Setup)**
20X Target Assay Mix or 20X Endogenous Control Assay Mix	1.0	2.5
cDNA template (1 to 100 ng of RNA converted to cDNA) + RNase-free water	9.0	22.5
2X TaqMan Universal Master Mix (with or without AmpErase UNG)	10.0	25.0
Total Volume	20.0	50.0

⚠ WARNING CHEMICAL HAZARD. Target Assay Mix and Endogenous Control Assay Mix contain **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 Universal PCR 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.

Preparing the PCR Reaction Plate

Applied Biosystems recommends performing four replicates of each reaction. The recommended reaction size is 20 µL for a 384-well setup and 50 µL for a 96-well setup.

To prepare the reaction plate:

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

Component	Volume (µL) for Four 20-µL Reactions	Volume (µL) for Four 50-µL Reactions
20X Target Assay Mix or 20X Control Assay Mix	5.0	12.5
cDNA template (1 to 100 ng of RNA converted to cDNA) + RNase-free water	45.0	112.5
2X TaqMan Universal Master Mix (with or without AmpErase UNG)	50.0	125.0
Total Volume	100.0	250.0

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

   ⚠ WARNING CHEMICAL HAZARD. Target Assay Mix and Endogenous Control Assay Mix contain **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 Universal PCR 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.

2. Cap the tubes and mix the solutions by gentle inversion.

3. Centrifuge the tubes briefly to spin down the contents and eliminate any air bubbles from the solutions.

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

4.	Transfer 20 µL (for 384-well format) or 50 µL (for 96-well format) of each reaction mixture to wells of an optical reaction plate.
5.	Cover the plate with an optical adhesive cover or with optical caps.
6.	Centrifuge the plate briefly to spin down the contents and eliminate any 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:

1. Place the reaction plate in the sequence detector.

2. Use the default PCR thermal cycling conditions.

Step	AmpErase UNG Activation*	AmpliTaq Gold Enzyme 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

*The 2-min, 50 °C step is required for optimal AmpErase UNG activity when using TaqMan Universal PCR Master Mix (P/N 4304437). This step is not needed when using the TaqMan Universal PCR Master Mix, No AmpErase UNG (P/N 4324018).

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

Plate Format	Reaction Volume (µL)
384-well	20
96-well	50

4. Start the run.

Data Analysis

Overview

Data analysis varies depending on the product, assay, and 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. Using the relative standard curve method or the comparative C_T method to determine relative quantification

Resources for Data Analysis

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

- The appropriate instrument user guide
- *User Bulletin #2: Relative Quantitation of Gene Expression* (P/N 4303859)
- Data Analysis and Relative Quantification chapters in the *TaqMan Cytokine Gene Expression Plate I Protocol* (P/N 4306744)

  The protocol provides examples using multiplex reactions. Use the protocol as a guide for data analysis and relative quantification of singleplex reactions.

Note: These documents are available through the Internet (see "Services and Support" on page 31).

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Services and 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 for specific products.

Notes

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