TaqMan® Human Cytokine Card

Protocol

Applied Biosystems

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1 Introduction

Getting Started Quickly

If familiar with the theory behind TaqMan® Human Cytokine Card chemistry or ABI PRISM® 7700 Sequence Detection System data collection, read the sections of this protocol listed below. They contain the minimum amount of reading required to conduct TaqMan Human Cytokine Card experiments.

- Chapter 1: Introduction
  - "Designing TaqMan Human Cytokine Card Experiments" on pages 1-7 to 1-8
  - "Preventing Contamination" on page 1-9
  - "Materials and Equipment" on pages 1-10 to 1-11
- Chapter 2: Reverse Transcription
- Chapter 3: PCR
- Chapter 4: Data Analysis
- Chapter 5: Interpreting Results

If unfamiliar with the concepts behind the cytokine card chemistry or ABI PRISM 7700 data collection, read the following sections in addition to the ones listed above.

- Chapter 1: Introduction
  - "" on pages 1-1 to 1-6
- Appendix A: Theory of Operation

Product Overview

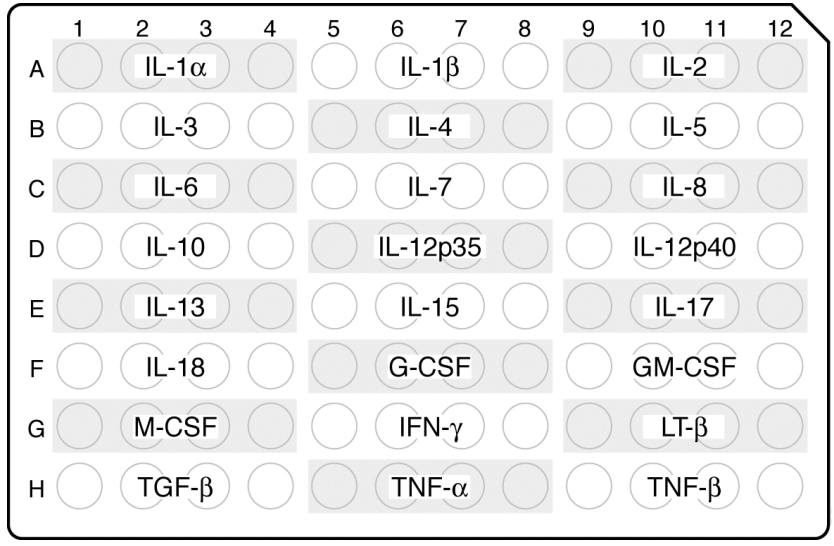
The TaqMan Human Cytokine Card is a research tool for profiling human cytokine gene expression using the Comparative C_T Method of relative quantification. The card evaluates a single cDNA sample generated from human total RNA in a two-step RT-PCR experiment. The card functions as the reaction vessel for the PCR/sequence detection step. The wells of the card contain the fluorogenic 5´ nuclease assays that detect the amplification of 24 cytokine targets. Relative

levels of cytokine gene expression are determined from the fluorescence data generated during PCR using the ABI PRISM® 7700 Relative Quantification Software.

TaqMan Human Cytokine Card Design

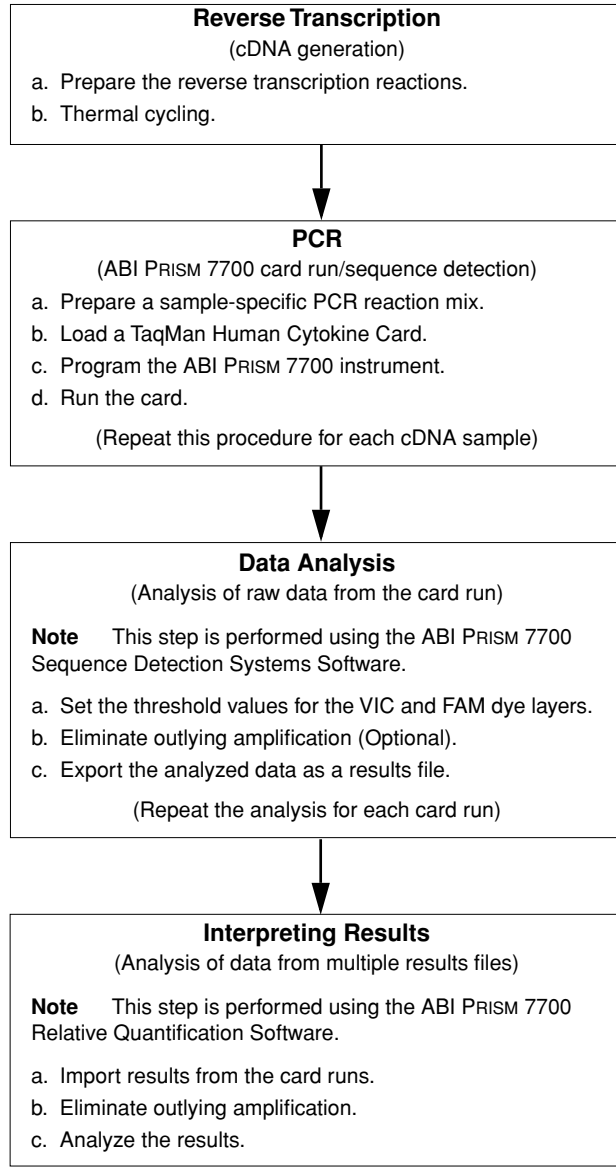
The TaqMan Human Cytokine Card consists of a specially developed 96-well consumable divided into 24 sets of replicates, one set for each cytokine assay. Each well contains TaqMan® MGB probes and primers for one human cytokine mRNA target. A 20X 18S rRNA endogenous control of TaqMan® MGB probe and primers is supplied for multiplex assays.

The figure below illustrates the configuration of the cytokine gene expression assays on the card.



Procedure Flowchart

The following diagram provides an overview of this protocol.



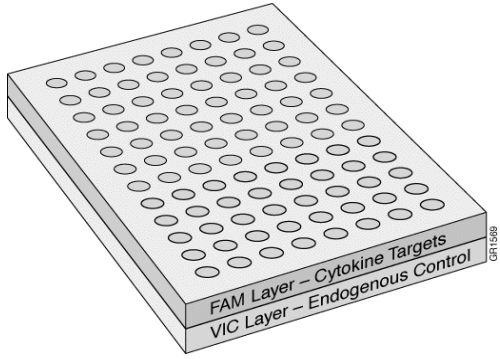
Virtual Dye Layers

The TaqMan Human Cytokine Card permits the amplification of cytokine and endogenous control cDNA using a multiplexed fluorogenic 5´ nuclease assay. The assay consists of two reactions, each a complete PCR system with corresponding probe and primers.

The fluorogenic probes of the multiplexed assay function as follows:

- Probes labeled with the FAM dye detect the amplification of 24 cytokine cDNA targets.
- Probes labeled with the VIC™ dye detect the amplification of cDNA generated from the 18S rRNA endogenous control.

The following conceptual figure illustrates the virtual dye layer configuration of a TaqMan Human Cytokine Card.



Genomic DNA Contamination

TaqMan Cytokine Assays

TaqMan probes and primers for the 24 cytokine target assays span exon junctions to minimize the contribution of contaminating genomic DNA. Performance tests demonstrate that TaqMan assays can be run with samples containing up to 10,000 copies of genomic DNA without detection of contaminants.

TaqMan 18S rRNA Endogenous Control Assay

The 18S rRNA endogenous control assay is not RNA specific and consequently is affected by genomic DNA contamination. However, because of the extremely high expression level of rRNA, even gross contamination has a negligible effect on the relative quantification values obtained from the card.

Competition Between Multiplexed Reactions

Because cellular expression of 18S rRNA is several magnitudes greater than typical cytokine mRNA expression, domination by the 18S reaction is a concern. To minimize the competition between the reactions, the 18S endogenous control assay is primer limited to prevent it from competing with the amplification of the cytokine target sequences.

For more information about controlling competition between reactions and the primer limitation concept, see the following publications:

- *ABI PRISM 7700 Sequence Detection System User Bulletin #2: Relative Quantitation of Gene Expression* (P/N 4303859)
- *ABI PRISM 7700 Sequence Detection System User Bulletin #5: Multiplex PCR with* TaqMan *VIC Probes* (P/N 4306236).

Quality Control

Functional verification of the preloaded probes and primers within the TaqMan Human Cytokine Card is performed as part of the Applied Biosystems manufacturing quality control process. In this process, the performance of each cytokine target assay is verified using plasmids that contain the cytokine's specific cDNA target sequence.

System Performance Guarantee

TaqMan Human Control Total RNA is available from Applied Biosystems for demonstrating the performance of the TaqMan Human Cytokine Card. If the control total RNA is run in the card using the conditions below, the average C_T value for the 18S endogenous control will be fewer than 12 cycles and the ΔC_T values for five cytokine targets will be as follows:

Target	ΔC_T[a]
IL-10	Below 20
Lymphotoxin-B	Below 20
TGF-B	Below 17
TNF-a	Below 20
TNF-B	Below 17

a. ΔC_T = Median C_T (FAM C_T – VIC C_T) for a group of replicates

Note The targets above were chosen because they are significantly expressed in the control sample.

To achieve the above results, follow the protocol in Chapter 2, "Reverse Transcription" to perform an RT conversion using 2 µg of control total RNA in 100 µL of reaction volume. After the RNA is converted to cDNA, make a sample-specific PCR reaction mix using 150 µL Universal Master Mix, 118 µL water, 30 µL of 20X 18S Primer and Probe Mix, and 2 µL cDNA. Fill and run a TaqMan Human Cytokine Card with the reaction mix according to the procedure in this manual (Chapter 3, "PCR."). Conduct the data analysis as described in Appendix B, "Demonstrating Performance with Control RNA."

Designing TaqMan Human Cytokine Card Experiments

About the Comparative C_T Method of Relative Quantification

Relative gene expression values can be obtained from ABI PRISM 7700 run data using the Comparative C_T Method for relative quantification. In the Comparative C_T Method, quantity is expressed relative to a calibrator sample that is used as the basis for comparative results. Therefore, the calibrator is the 1X sample and all other quantities are expressed as an *n*-fold difference relative to the calibrator.

For more information on the Comparative C_T Method of Relative Quantification, see the following publications:

- *ABI PRISM 7700 Sequence Detection System User Bulletin #2: Relative Quantitation of Gene Expression*
- *ABI PRISM 7700 Relative Quantification Software User's Manual*

Significance of the Calibrator Sample

All TaqMan Human Cytokine Card relative quantification experiments require data from a calibrator sample. During analysis, the ABI PRISM 7700 Relative Quantification Software calculates the levels of cytokine gene expression in samples relative to the level of expression in the calibrator. Thus, the calibrator sample is an integral part of the relative quantification calculation because it serves as the basis for the comparative results.

Examples of possible calibrator samples include:

- A zero timepoint sample in a timecourse experiment
- An untreated sample (versus treated samples)
- A resting sample (versus activated samples)

Note For more information on the use of a calibrator sample in relative quantification, see *ABI PRISM 7700 Sequence Detection System User Bulletin #2: Relative Quantitation of Gene Expression*.

Design Guidelines

Observe the following guidelines when designing TaqMan Human Cytokine Card experiments:

- Load each card with sample-specific PCR reaction mix (cDNA sample + TaqMan® Universal PCR Master Mix + 20X 18S Primer and Probe Mix) made from a single sample.

  Individual cards are not designed to evaluate multiple cDNA samples.

- Install the Sequence Detection Systems (SDS) Software version 1.7.1 or later to all instruments devoted to running TaqMan Human Cytokine Cards.

- Run TaqMan Human Cytokine Cards within 30 minutes of loading them with sample-specific PCR reaction mix.

  To ensure the highest degree of reproducibility, Applied Biosystems recommends scheduling card runs so that each card is run within 30 minutes of the time it is loaded. When a loaded card sits for an extended period of time, the probes and primers within the wells of the card begin to diffuse into the adjoining channels. This diffusion of critical reagents can diminish the potential signal generated during the PCR, and can therefore affect the results of the experiment.

- Run all TaqMan Human Cytokine Cards from the same comparative experiment on the same ABI PRISM 7700 instrument.

  Running all cards from the same experiment on one ABI PRISM 7700 instrument ensures a high degree of reproducibility and consistency.

- When analyzing results with the SDS software:
  - Set FAM dye layer threshold values identically for all cards in the same comparative experiment.
  - Set VIC dye layer threshold values identically for all cards in the same comparative experiment.

  To compare the data from different card runs, the threshold values for each card must match exactly. The ABI PRISM 7700 Relative Quantification Software cannot analyze data from card runs that have different threshold values within the respective dye layers.

Preventing Contamination

Contamination and the 5´ Nuclease Assay

PCR using the 5´ nuclease assay requires special laboratory practices to avoid false positive amplifications (Kwok and Higuchi, 1989). The assay's high-throughput, repetitive nature can potentially amplify a single DNA molecule (Saiki *et al.*, 1985; Mullis and Faloona, 1987).

Using 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. The gene was 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

Please 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 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 with 10% bleach solution.

Materials and Equipment

TaqMan Card Upgrade Package and Components

The TaqMan® Human Cytokine Card Upgrade Package and components are available as follows:

Components	Part Number
TaqMan Card Upgrade Package[a]	4311899
ABI PRISM® 7700 Relative Quantification Software	4313010
ABI PRISM® Card Adaptor	—
ABI PRISM® Card Filling Station	—
TaqMan Human Control Total RNA (50 ng/1 µL)	4307281
TaqMan® Human Cytokine Cards (10 cards)	4330448
TaqMan® Human Cytokine Card Protocol	4307577
TaqMan® Universal PCR Master Mix[b]	4304437
Vacuum tubing and gauge	—

a. Includes all the components listed above and a service installation visit.

b. The TaqMan Universal PCR Master Mix is 2X in concentration and contains sufficient reagent to perform 33 cards (150 µL each). The mix is optimized for TaqMan reactions and contains AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference, and optimized buffer components.

Storage Guidelines

The table below lists the storage conditions for the kit materials.

Component	Storage Conditions
TaqMan Human Cytokine Cards	2–8 °C, dark
20X 18S Primer and Probe Mix	–15 to –25 °C
TaqMan Universal PCR Master Mix	2–8 °C, dark
TaqMan Human Control Total RNA	–15 to –25 °C
TaqMan Human Control cDNA	–15 to –25 °C

IMPORTANT Do not remove TaqMan Human Cytokine Cards from the packaging until ready to load them with reaction mix. Excessive exposure to light can damage the probes.

Ordering Applied Biosystems Kits and Reagents

To order additional kits and reagents, please contact Applied Biosystems at one of the regional sales offices listed on the back of this protocol. Have the part number of the kit or reagent of interest available when ordering.

Reagents and Equipment Not Included

In addition to the reagents supplied in the TaqMan Human Cytokine Card Upgrade Package, other items are required for this protocol. Unless otherwise noted, many of the instruments and materials listed below are available from major laboratory suppliers (MLS).

User-Supplied Instruments

Instruments	Source
ABI PRISM® 7700 Sequence Detection System	Applied Biosystems[a]
Microcentrifuge	MLS
Welch® DUOSEAL Series Two-Stage, Belt-Drive Vacuum Pump[b]	VWR Catalog (P/N 54973-075)
Vacuum Trap, Kontes®	VWR Catalog (P/N KT926300-0021)

a. Contact your local Applied Biosystems Sales Office for the instrument best suited to your needs. See the back cover of this protocol for office locations.

b. Substitute vacuum pumps must be oil-based and capable of pulling a minimum vacuum of 2.5×10^{-3} Torr (600 microns).

User-Supplied Materials

Materials	Source
MicroAmp® Reaction Tubes with Caps, 0.2-mL	Applied Biosystems (P/N N801-0612)
Gloves, disposable, powder-free	MLS
Microcentrifuge tubes, sterile 1.5-mL	MLS
Pipettors, positive-displacement or air-displacement	MLS
Pipette tips, aerosol resistant	MLS
Polypropylene tubes	MLS
Water, RNase-free, distilled, deionized	MLS

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.

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.

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**, or from outside the U.S. and Canada, dial **1.858.712.0317**.
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	♦ To order in English, dial **1.800.668.6913** and press **1**, then **2**, then **1** ♦ To order in French, dial **1.800.668.6913** and press **2**, then **2**, then **1**
From any other country	See the specific region under “To Contact Technical Support by Telephone or Fax (Outside North America)” .

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 of the MSDS.

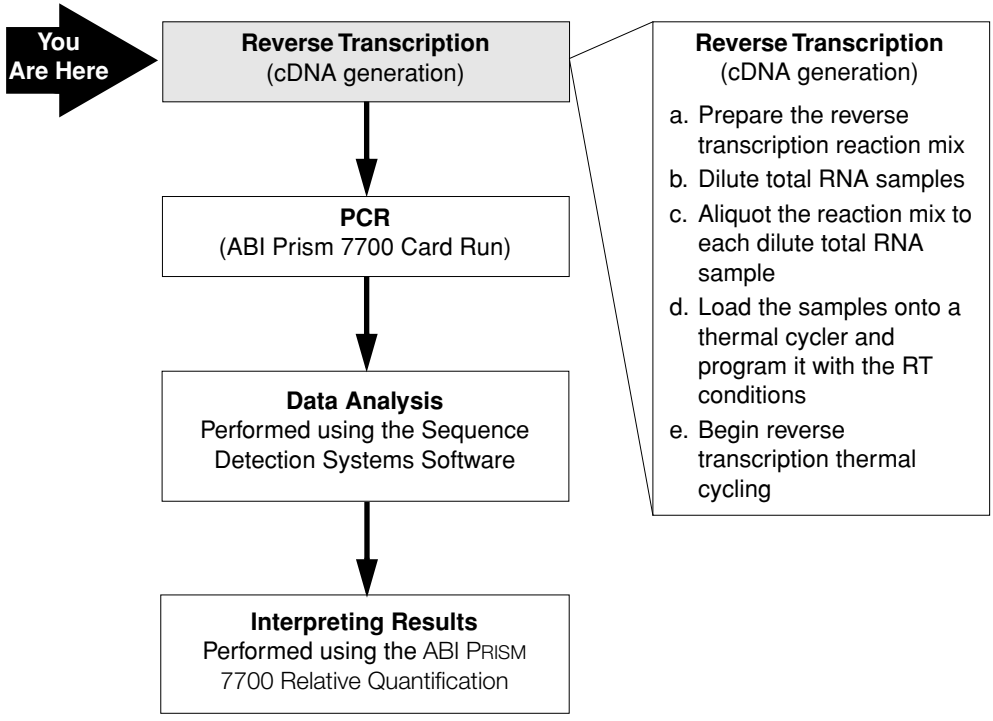
For chemicals not manufactured or distributed by Applied Biosystems, call the chemical manufacturer.

Reverse Transcription 2

Overview

Synthesis of cDNA from total RNA samples is the first step in the two-step RT-PCR cytokine gene expression quantification experiment. In this step, random hexamers from the TaqMan® Reverse Transcription Reagents prime total RNA samples for reverse transcription using MultiScribe™ Reverse Transcriptase.

Where You Are in the Procedure



Preparing the RNA Template

Recommended Template

Use only human total RNA samples to generate cDNA for the TaqMan® Human Cytokine Card.

The following table lists the known template incompatibilities:

Template	Explanation
Poly A+	The 18S rRNA endogenous control assay cannot accurately evaluate cDNA generated from poly A+ RNA samples because most of the rRNA has been removed from them.
Non-human	Except for 18S rRNA, all assays on the TaqMan Human Cytokine Card are human-specific.

Template Quality

The quality of your results is directly related to the purity of your RNA template. Therefore, use only well-purified samples with the TaqMan Human Cytokine Card. Because ribonuclease and genomic DNA contamination are common problems in gene expression studies, purify your samples accordingly to ensure the best results.

Note TaqMan Human Cytokine assays have been experimentally proven not to detect up to 10,000 copies of contaminating genomic DNA per card.

Template Quantity

If possible, use spectrophotometric analysis to determine the concentrations of purified total RNA samples before reverse transcription. The table below lists the recommended range of initial template quantities for the reverse transcription (RT) step.

Initial Template	Quantity of total RNA (per 100-µL RT reaction)
Human Total RNA	60 ng–2 µg

Performing Reverse Transcription

Guidelines

Follow the guidelines below to ensure optimal RT performance:

- The TaqMan Human Cytokine Card is designed to assay cDNA generated from only human total RNA samples.

  Poly A^+ RNA samples are not recommended for cytokine card experiments, because most rRNA has been removed from them.

- A 100-μL RT reaction will efficiently convert a maximum of 2 μg total RNA to cDNA. Perform multiple RT reactions in multiple wells if using more than 2 μg total RNA.

- Use only random hexamers to reverse transcribe the total RNA samples for cytokine gene expression assays.

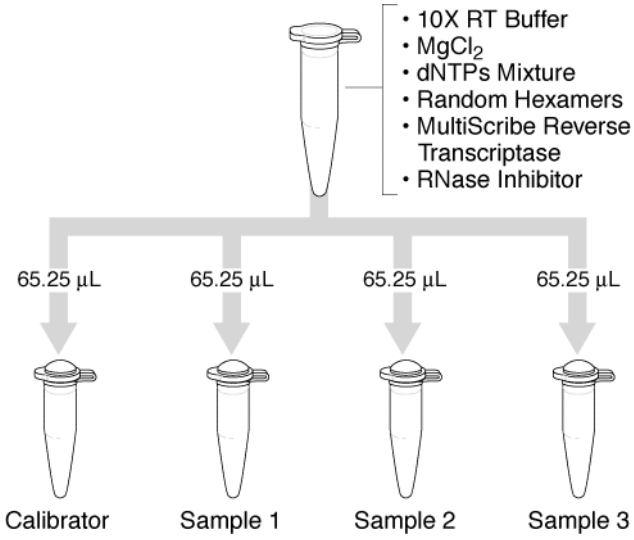
Preparing the Reactions

The following procedure describes the preparation of three different test samples and a calibrator sample for reverse transcription. Scale the recommended volumes accordingly for the number of samples needed using the TaqMan Reverse Transcription Reagents (P/N N808-0234).

To prepare the reverse transcription reactions:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>In a 1.5-mL microcentrifuge tube, prepare a reaction mix for all total RNA samples to be reverse transcribed.
<table>
<tr><th rowspan="2">Component</th><th colspan="2">Volume (µL)[a]</th><th rowspan="2">Final Value</th></tr>
<tr><th>Per Sample</th><th>Reaction Mix (x4)</th></tr>
<tr><td>10X RT Buffer</td><td>10.0</td><td>40.0</td><td>1X</td></tr>
<tr><td>25 mM $MgCl_2$</td><td>22.0</td><td>88.0</td><td>5.5 mM</td></tr>
<tr><td>deoxyNTPs Mixture</td><td>20.0</td><td>80.0</td><td>500 µM per dNTP</td></tr>
<tr><td>Random Hexamers</td><td>5.0</td><td>20.0</td><td>2.5 µM</td></tr>
<tr><td>RNase Inhibitor</td><td>2.0</td><td>8.0</td><td>0.4 U/µL</td></tr>
<tr><td>MultiScribe Reverse Transcriptase (50 U/µL)</td><td>6.25</td><td>25.0</td><td>3.125 U/µL</td></tr>
<tr><td>Total</td><td>65.25</td><td>261.0</td><td>—</td></tr>
</table>
a. If changing the reaction volume, make sure the final proportions are consistent with the recommended values above.</td></tr>
<tr><td>2</td><td>Label four 1.5-mL microcentrifuge tubes for the three test samples and the calibrator sample.</td></tr>
<tr><td>3</td><td>Transfer 60 ng–2 µg (up to 34.75 µL) of each total RNA sample to the corresponding microcentrifuge tube.</td></tr>
<tr><td>4</td><td>If necessary, dilute each total RNA sample to a volume of 34.75 µL with RNase-free, deionized water.</td></tr>
<tr><td>5</td><td>Cap the tubes and gently tap each to mix the diluted samples.</td></tr>
<tr><td>6</td><td>Briefly centrifuge the tubes to eliminate air bubbles in the mixture.</td></tr>
<tr><td>7</td><td>Label four 0.2-mL MicroAmp® Reaction Tubes for the three total RNA test samples and the calibrator sample.</td></tr>
</table>

To prepare the reverse transcription reactions: *(continued)*

Step	Action
8	Pipet 65.25 µL of the reaction mix (from step 1) to each MicroAmp Reaction Tube (from step 7). 
9	Transfer 34.75 µL of each dilute total RNA sample to the corresponding MicroAmp Reaction Tube.
10	Cap the reaction tubes and gently tap each to mix the reactions.
11	Briefly centrifuge the tubes to force the solution to the bottom and to eliminate air bubbles from the mixture.

Thermal Cycling

To conduct reverse transcription thermal cycling:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Load the reactions into a thermal cycler.</td></tr>
<tr><td>2</td><td>Program your thermal cycler with the following conditions:

<table>
<tr><th rowspan="2">Step</th><th>Hexamer Incubation[a]</th><th>Reverse Transcription</th><th>Reverse Transcriptase Inactivation</th></tr>
<tr><td>HOLD</td><td>HOLD</td><td>HOLD</td></tr>
<tr><td>Temp.</td><td>25 °C</td><td>37 °C</td><td>95 °C</td></tr>
<tr><td>Time</td><td>10 min</td><td>60 min</td><td>5 min</td></tr>
<tr><td>Volume</td><td colspan="3">100 µL</td></tr>
</table>
a. When using random hexamers for first-strand cDNA synthesis, a primer incubation step (25 °C for 10 min) is necessary to maximize primer-RNA template binding.</td></tr>
<tr><td>3</td><td>Begin reverse transcription.

IMPORTANT After thermal cycling, store all cDNA samples at –15 to –25 °C and proceed to Chapter 3, “PCR.”</td></tr>
</table>

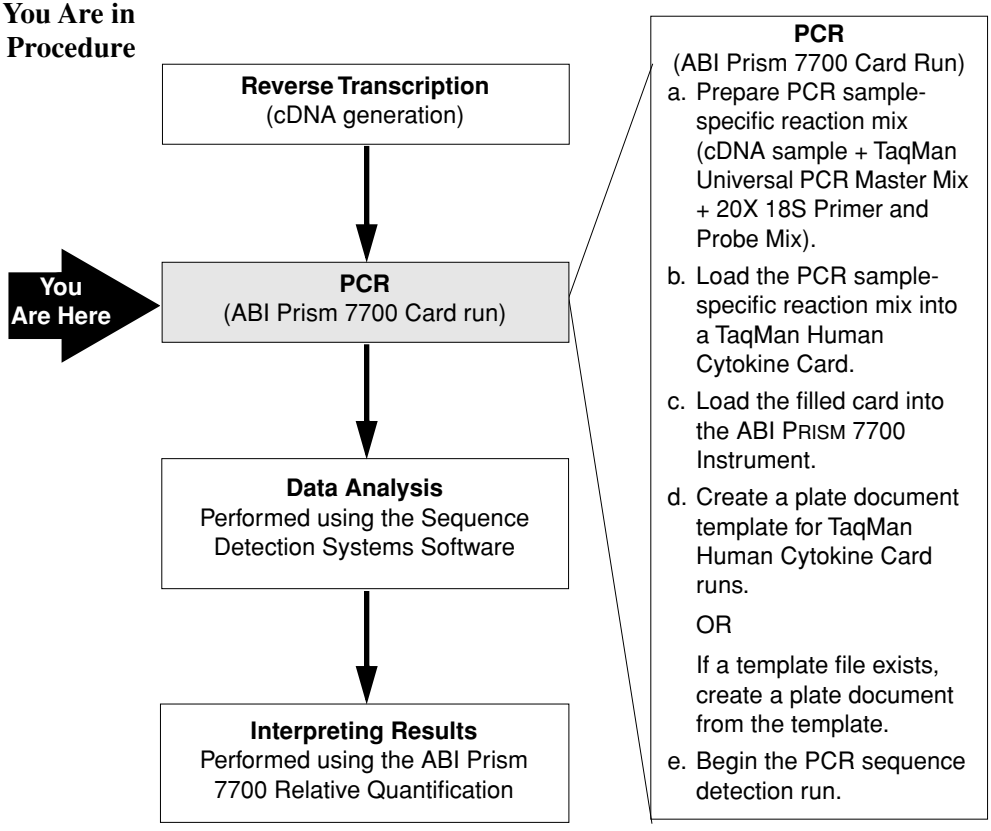
PCR

3

Overview

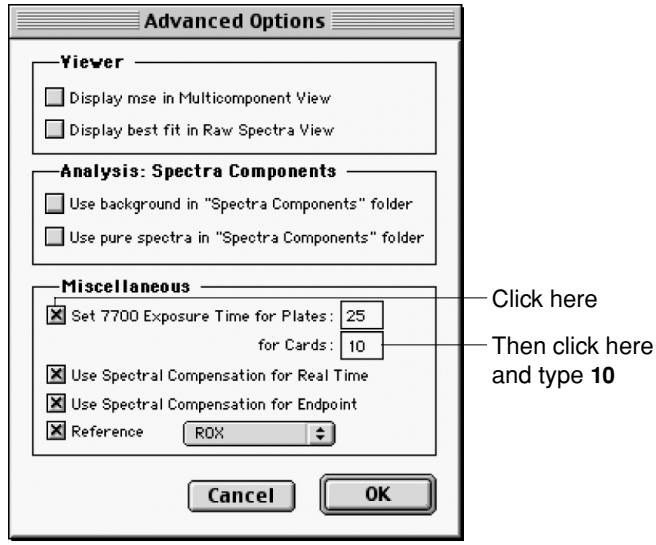
Amplification of cDNA is the second step in the TaqMan® Human Cytokine Card two-step RT-PCR experiment. In this step, AmpliTaq® Gold DNA polymerase amplifies cDNA synthesized from the original total RNA sample. Because each cytokine card can evaluate only one cDNA sample, you must repeat this step for each sample in the analysis.

Where You Are in the Procedure



Adjusting the Exposure Time for Card Runs

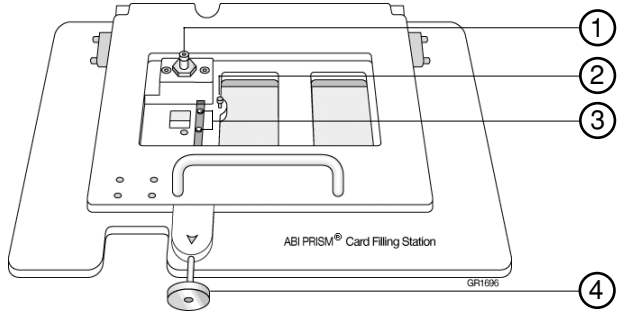
ABI PRISM® cards typically return a stronger fluorescent signal than standard MicroAmp® Optical Plates. Consequently, the CCD camera exposure time must be decreased to compensate for the higher signal intensity.

Step	Action
1	Launch the Sequence Detection Systems (SDS) software.
2	Select **Advanced options** from the **Diagnostics** submenu off of the **Instrument** menu.
3	From the **Miscellaneous** group box, activate the manual setting: a. Click the **Set 7700 Exposure Time** checkbox. b. Click the **Set 7700 Exposure Time for Cards** text field and type **10**. 
4	Click **OK**.

About the ABI PRISM Filling Station

Description

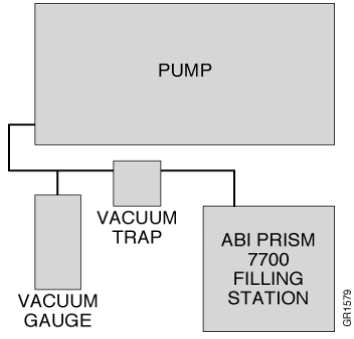
Samples are loaded individually into TaqMan Human Cytokine Cards for PCR thermal cycling using a specialized tool called the ABI PRISM® Card Filling Station. The Filling Station coordinates loading by first exposing the channels of the reaction card to a vacuum and then opening the evacuated card to a sample-specific PCR reaction mix.

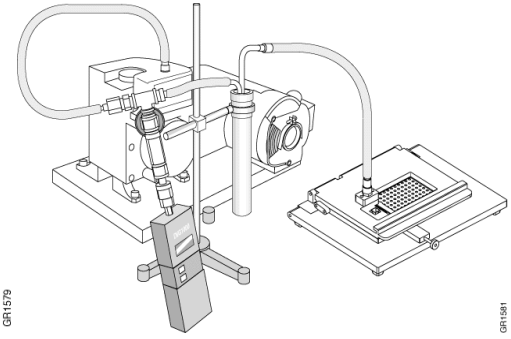


Number	Component	Description
1	Vacuum Attachment	The attachment for connecting the Filling Station to a vacuum pump.
2	Sealing Screw	**IMPORTANT** Do not adjust. The Filling Station screws are extremely sensitive.
3	Actuator Screws	
4	Actuator	The switch for exposing the card to the PCR sample or the vacuum.

Laboratory Setup

The ABI PRISM Filling Station operates in combination with a vacuum pump, a vacuum trap, and a gauge. The figures below illustrate the arrangement of the equipment as seen in the laboratory.





Sample Preparation

Guidelines

Follow the guidelines below to ensure optimal PCR performance:

- Load each TaqMan Human Cytokine Card with sample-specific PCR reaction mix made with a single cDNA sample.

  Cytokine cards are not designed to evaluate multiple cDNA samples simultaneously.

- Run each TaqMan Human Cytokine Card within 30 minutes of loading it.

  When loaded cards sit, the TaqMan® probes and primers within the wells of the card begin to diffuse into the adjoining channels. The diffusion of critical reagents diminishes the potential signal that can be generated during PCR and can therefore affect the results of the experiment.

- Run all TaqMan Human Cytokine Cards for a single experiment on the same ABI PRISM 7700 instrument.

  Running all cards on the same instrument ensures a high degree of reproducibility and consistency in the resulting data.

- Load 300 µL of sample-specific PCR reaction mix per card to ensure adequate filling.

  Smaller volumes may result in insufficiently filled cards.

- Do not attempt to refill partially-filled cards.

  Upon contact, the sample-specific PCR reaction mix resuspends the dried TaqMan probes and primers within the wells of the card. When the partially-filled card is evacuated, these reagents are carried away with the solution.

Recommended Quantity

The following table lists the recommended range of human total RNA converted to cDNA for the PCR.

Sample	Sample Quantity per Card (300-µL sample-specific reaction mix)[a]
Total RNA converted to cDNA	60 ng–2 µg

a. 300 µL sample-specific PCR reaction mix = (150 µL cDNA sample + 20X 18S Primer and Probe Mix + H_20)(150 µL TaqMan® Universal PCR Master Mix)

Preparing a Sample-Specific PCR Reaction Mix

IMPORTANT Load only one sample-specific PCR reaction mix (cDNA sample + 20X 18S Primer and Probe Mix + TaqMan Universal PCR Master Mix) per TaqMan Human Cytokine Card. Applied Biosystems recommends running your calibrator sample first.

Note This procedure is optimized for TaqMan PCR Universal Master Mix.

To prepare a sample-specific PCR reaction mix:

Step	Action
1	Label a 1.5-mL microcentrifuge tube.
2	Retrieve a cDNA sample from the freezer (see step 3 on page 2–6). If frozen, thaw the sample by rolling it between your fingers.
3	Transfer the recommended quantity of cDNA sample (up to 150 µL – endogenous control) to the labeled microcentrifuge tube. **IMPORTANT** To avoid cross contamination of the reverse transcription products, slowly and carefully remove the cap from the thermal cycling tube.
4	Dilute the sample to 120 µL with RNase-free, deionized water.
5	Add 150 µL of TaqMan PCR Universal Master Mix (2X) to the microcentrifuge tube containing the dilute cDNA sample. **⚠ CAUTION CHEMICAL HAZARD. TaqMan Universal PCR Master Mix** may cause eye and skin irritation. It may cause discomfort if swallowed or inhaled. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
6	Add 30 µL of 20X 18S Primer and Probe Mix.
7	Cap the microcentrifuge tube and mix the solution by gentle inversion.
8	Centrifuge the tube to eliminate air bubbles from the mixture.

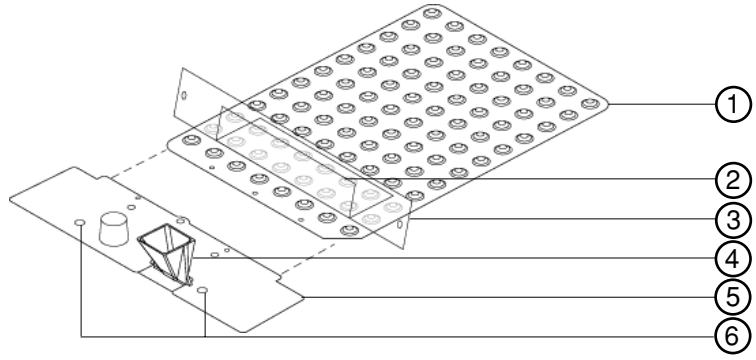
To prepare a sample-specific PCR reaction mix: *(continued)*

Step	Action
9	Place the sample-specific PCR reaction mix on ice and prepare a TaqMan Human Cytokine Card as described on page 3-9.

Loading the TaqMan Human Cytokine Card

About ABI PRISM Cards

The following figure shows an exploded view of an ABI PRISM® card:



Number	Component	Description
1	Reaction card	Acts as the vessel for the PCR. The consumable consists of a series of 96 interconnected wells pre-loaded with dried TaqMan probes and primers.
2	Adhesive flap	Used to seal the reaction card after it has been filled with sample-specific PCR reaction mix
3	Adhesive backing	
4	Fill reservoir	A reservoir for the sample-specific PCR reaction mix before it enters the card
5	Fill consumable	A disposable component that channels fluid from the fill reservoir into the reaction card
6	Alignment holes	Aid in aligning the card within the ABI PRISM Filling Station

Guidelines for Loading Cards

Follow the guidelines below to ensure proper loading of the card.

- Allow a card to adjust to room temperature before loading it.

  Low temperatures will prevent efficient fluid transfer through the microchannels of the fill consumable.

- Do not remove a card from its packaging until you are ready to load it with sample-specific PCR reaction mix.

  Excessive exposure to light damages the fluorescent probes.

- Do not twist or bend the soft fill consumable.

  The seal between the reaction card and the fill consumable is crucial to the loading procedure. If broken, the seal may leak and result in an inadequately filled card.

- Use caution when opening and closing the ABI PRISM Filling Station.

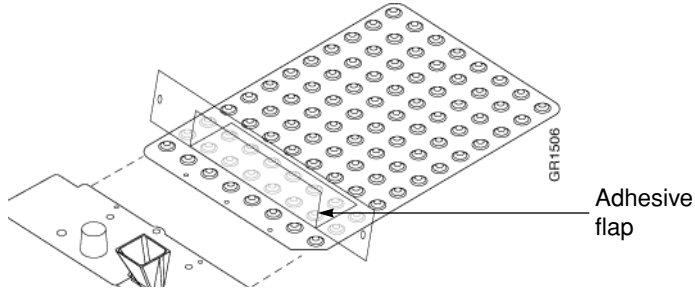
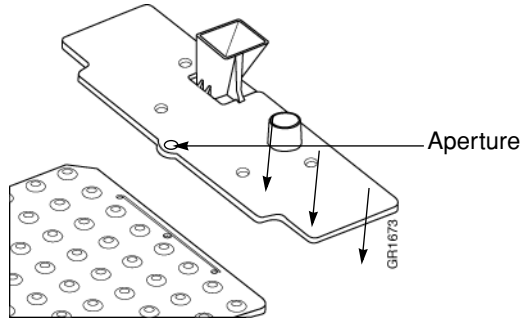
  The station lid is not designed to remain in an open position.

⚠ WARNING PHYSICAL HAZARD. If left open, the ABI PRISM Filling Station lid may unpredictably close and cause an injury.

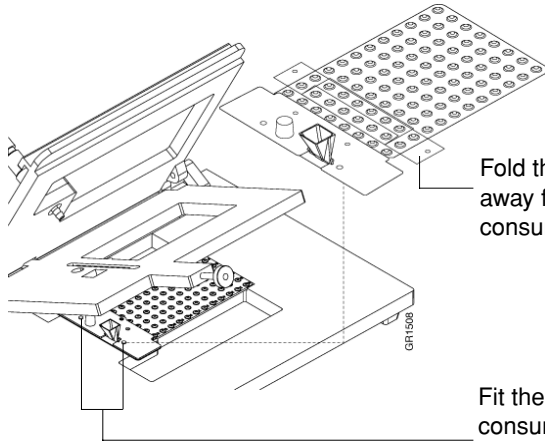
Preparing a Card

⚠ WARNING PHYSICAL HAZARD. If left open, the ABI PRISM Filling Station lid may unpredictably close and cause an injury. The lid is not designed to remain in an open position.

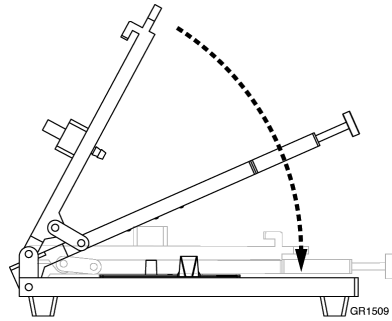
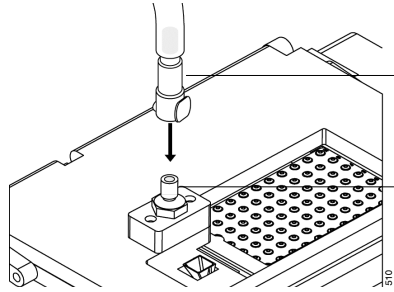
To prepare the TaqMan Human Cytokine Card for loading:

Step	Action
1	Carefully remove a TaqMan Human Cytokine Card from its packaging.
2	Fold the adhesive flap attached to the card back, in order to accommodate the fill consumable. 
3	Attach a fill consumable to the card. a. Remove the white adhesive backing from the fill consumable. b. Align the protruding aperture and two pins on the edge of the fill consumable to the holes in the card (see below). c. Once aligned, press gently on across both sides to secure the fill consumable in place. 

To prepare the TaqMan Human Cytokine Card for loading: *(continued)*

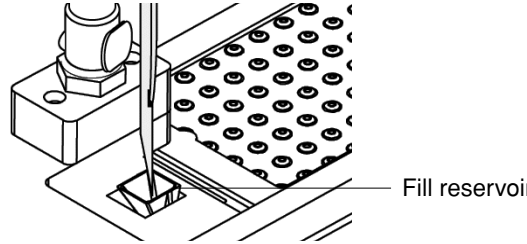
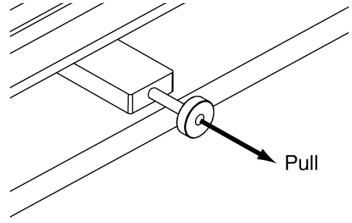
Step	Action
4	Carefully load the card into the ABI PRISM Card Filling Station. a. Orient the card so that the pins on the station align with the holes in the soft fill consumable as indicated in the figure below. b. Once the pins are correctly aligned, press down firmly on the top of the fill consumable to ensure a good fit. **IMPORTANT** Do not press down on the junction between the fill consumable and cytokine card. **IMPORTANT** The adhesive flap must be folded away from the fill consumable for proper operation of the Filling Station. The following illustration demonstrates the correct technique for loading a card into the Filling Station. 

To prepare the TaqMan Human Cytokine Card for loading: *(continued)*

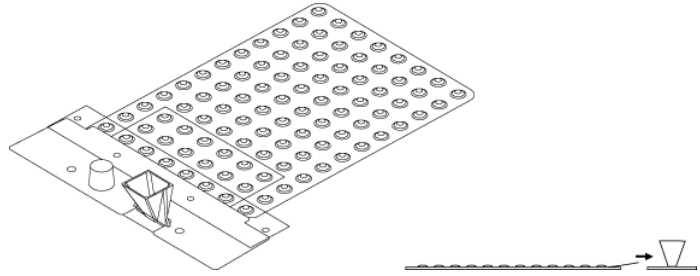
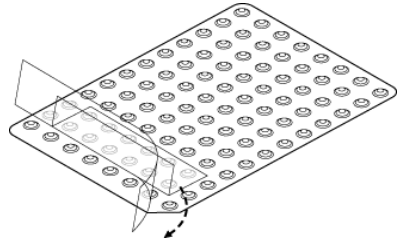
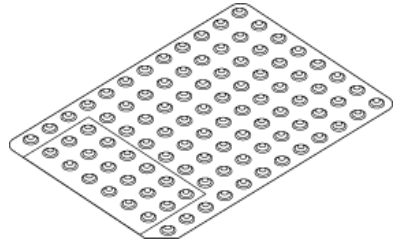
Step	Action
5	Close the ABI PRISM Card Filling Station lid. **IMPORTANT** Press firmly on the top of the ABI PRISM Card Filling Station to ensure that it is completely closed. 
6	If necessary, connect the vacuum hose to the attachment on the ABI PRISM Filling Station lid. The end of the vacuum hose contains a quick-release valve that "clicks" when locked into place. **Note** Leave the vacuum hose attached to the lid at all times except during maintenance or transportation of the station. 
7	Turn on the vacuum pump. Allow the vacuum pump to evacuate the card until the gauge on the hose stabilizes at or less than **600** microns. **IMPORTANT** Do not fill the card at a vacuum greater than 600 microns. Above that reading, the vacuum is not strong enough to adequately fill the card and may result in the loss of both the cDNA sample and the card. Do not attempt to refill partially filled cards.

Loading and Sealing a Card

To fill and seal the card for thermal cycling:

Step	Action
1	After the vacuum reaches **600** microns, pipet 300 µL of the sample-specific PCR reaction mix (cDNA sample + TaqMan Universal PCR Master Mix + 20X 18S Primer and Probe Mix) from page 3-5 into the fill reservoir of the Filling Station. Use the pipet tip to dislodge any bubbles that appear at the bottom of the fill reservoir. 
2	In one motion, firmly pull the Filling Station actuator to its maximum extension. **Note** Pulling the actuator may be fairly difficult and require some physical strength. The TaqMan Human Cytokine Card fills with sample-specific PCR reaction mix. **IMPORTANT** Do not attempt to force the actuator back into the closed position. It retracts automatically when the lid is opened. 
3	Complete the following as quickly as possible: a. Turn off the vacuum pump. b. Open the Filling Station lid. **⚠ WARNING PHYSICAL HAZARD. If left open, the ABI PRISM Filling Station lid may unpredictably close and cause an injury. The lid is not designed to remain in an open position.**
4	Remove the card from the Filling Station.

To fill and seal the card for thermal cycling: *(continued)*

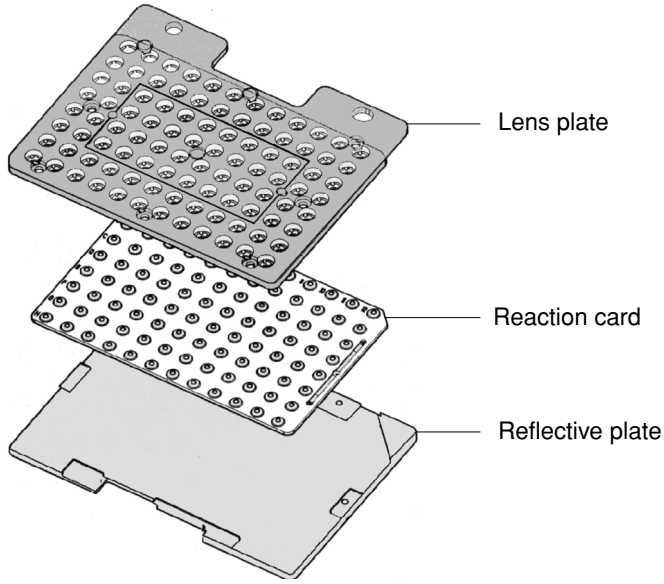
Step	Action
5	Detach and discard the fill consumable, then remove any adhesive remaining on the surface of the card. **IMPORTANT** Remove all of the adhesive from the card. The adhesive may interfere with the card seal and allow the sample-specific PCR reaction mix to leak during thermal cycling. 
6	Bend back the adhesive flap, and peel off the plastic backing. 
7	Fold the adhesive flap over the front edge of the card and press firmly on the flap to ensure an adequate seal.  The card is now filled and ready to be loaded into the ABI PRISM 7700 Sequence Detection System.

Loading a Card into an ABI PRISM 7700 Sequence Detection System

About the ABI PRISM Card Adaptor Design

The unique design of the TaqMan Human Cytokine Card requires the use of an ABI PRISM Card Adaptor for use on the ABI PRISM® 7700 Sequence Detection Systems (SDS) instrument. The card adaptor is a specialized device that ensures adequate heat transfer and fluorescent data collection occur during the PCR.

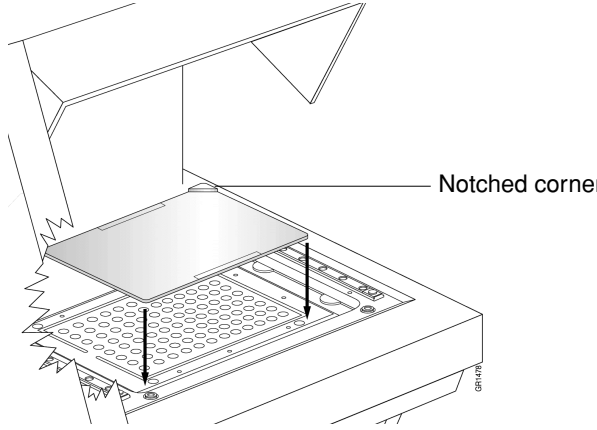
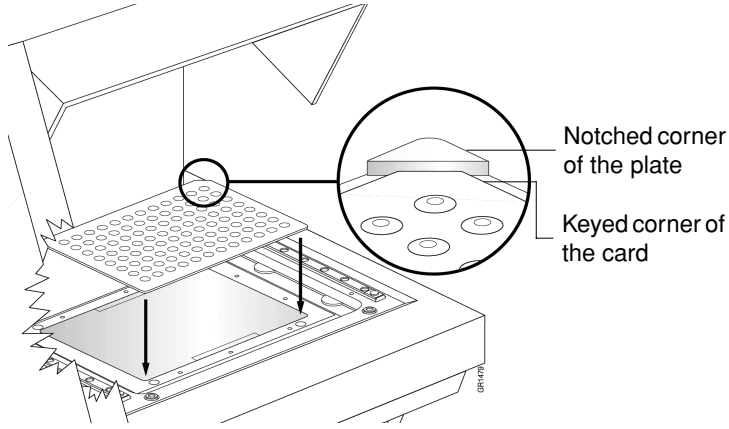
The following figure illustrates the components of the adaptor:



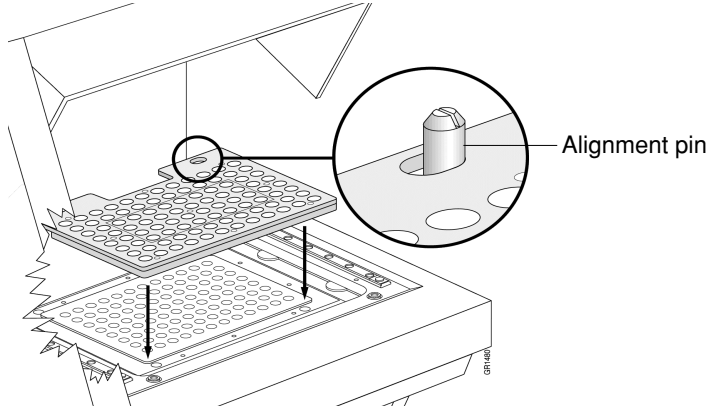
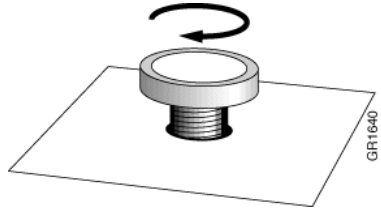
Component	Description
Lens plate	Houses 96 lenses that direct the focal point of the argon ion laser into the wells of the reaction card. The top of the lens plate contains a label to indicate the correct orientation of the plate.
Reaction card	Contains a cDNA sample and the necessary reagents for the PCR.
Reflective plate	An aluminum plate that ensures efficient conduction and uniform heat transfer to all wells of the reaction card. The plate contains a notched corner to aid in correctly orienting the card for thermal cycling.

Loading a Card for Sequence Detection

To load the card into the ABI PRISM 7700 Instrument:

Step	Action
1	Slide the sample block cover back, exposing the sample block.
2	Place the reflective plate on top of the sample block so that the notched edge is located in the far right corner. 
3	Place the filled TaqMan Human Cytokine Card on top of the reflective plate so that the keyed corner of the card aligns with the notched edge of the plate. 

To load the card into the ABI PRISM 7700 Instrument: *(continued)*

Step	Action
4	Place the lens plate on top of the card so that the holes in the plate fit over the alignment pins on the sample block. **IMPORTANT** Make sure that the lens plate is oriented so that the face with the **This Side Up** label is visible when the plate is in place. 
5	Slide the cover over the sample block and tighten the lid. **Note** Because the dimensions of the card sandwich differ from those of a standard plate, the white alignment line on the tightening knob may not turn to the same position as with a standard plate when tight.  **IMPORTANT** Tighten the cover as much as possible to ensure that the card is adequately sealed and uniformly heated.

Running TaqMan Human Cytokine Cards

About SDS Plate Documents

Every TaqMan Human Cytokine Card run on the ABI PRISM 7700 Sequence Detection System requires the creation of a card-specific plate document within the instrument software. The 7700 instrument uses the plate document to organize and store the fluorescence data gathered during the PCR.

Plate documents contain the following information:

- Dye layer setup
- Target/sample configurations
- Thermal cycling parameters
- Data collection parameters

Using a Template

A template file alleviates the need for repetitious construction of TaqMan Human Cytokine Card plate documents. Template files are identical to plate document files except that they do not contain fluorescence data from a sequence detection systems run. Once created, an unlimited number of identical SDS plate documents can be created from the template. Because comparative analysis involves multiple runs and SDS plate documents for cards are identical, it is more efficient to create and use a template file than to create a plate document for each card run.

Setting the Thermal Cycling Parameters

To configure the PCR thermal profile for the reverse transcription:

Step	Action
1	Launch the Sequence Detection Systems software. **Note** If a plate document automatically appears on your screen, select **Close** from the **File** menu to close it.
2	From the **File** menu, select **New ⌘–N**.
3	Configure a new plate document with the following attributes. **From menu…** / **Select…** Plate Type / Single Reporter Plate Format / The Card Run / Real Time **Note** TaqMan Human Cytokine Cards can only be used with SDS software version 1.7.1 or later. If the options above do not appear in the **New Plate** dialog box, update your instrument software.
4	Click the **Thermal Cycler Conditions** button.
5	Configure the thermal cycling profile with the following conditions:

Stage	**1**	**2**	**3**	
Description	UNG Activation[a]	AmpliTaq Gold Activation[b]	PCR	
	HOLD	HOLD	CYCLE (35 cycles)	
			Denature	Anneal/ Extend
Temperature	50 °C	**99 °C**[c]	**99 °C**[c]	60 °C
Time	2 min	10 min	15 sec	1 min

a. Required for optimal AmpErase UNG enzyme activity.

b. Required for AmpliTaq Gold DNA Polymerase activation.

c. A setting of 99 °C is necessary for this step because of the ABI PRISM card's unique thermokinetic properties.

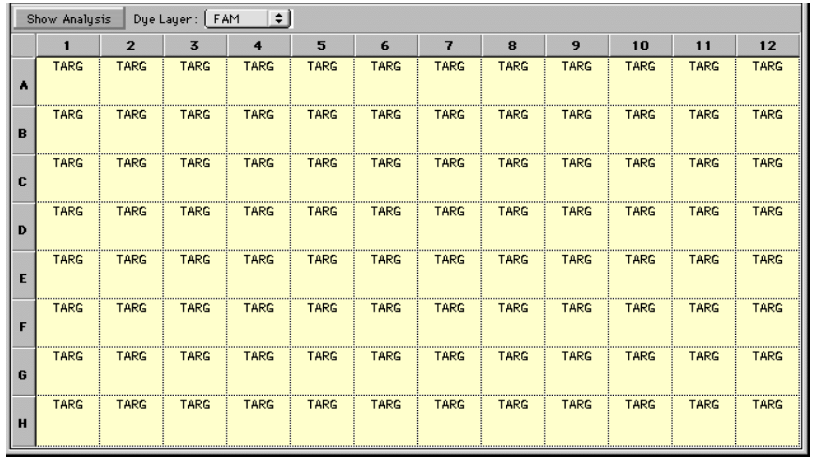
6	Click the **Sample Volume** text field and type **100** µL.

Configuring the Dye Layers

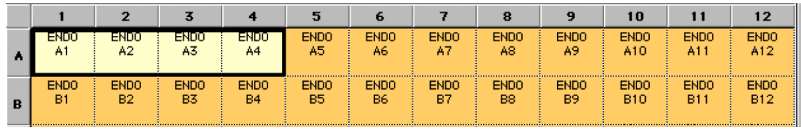
Note If the “TARG - RelQ Target” or the “ENDO - RelQ Endogenous Control” do not appear in the Sample Type pop-up menus, configure the sample types as explained on page 6-11.

To configure the FAM and VIC dye layers:

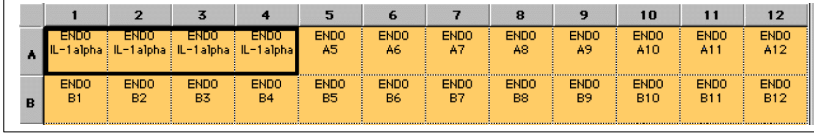
Step	Action
1	Select **FAM** from the **Dye Layer** pop-up menu.
2	Select all wells of the plate document.
3	Select **TARG - RelQ Target** from the **Sample Type** pop-up menu. The SDS software labels all selected wells as **TARG**.



4	Select **VIC** from the **Dye Layer** pop-up menu.
5	Select all wells of the plate document.
6	Select **ENDO – RelQ Endogenous Control** from the **Sample Type** pop-up menu.
7	Select cells A1–A4.



8	Click the **Sample Name** text field and type **IL-1alpha**.



To configure the FAM and VIC dye layers: *(continued)*

Step	Action
9	Repeat steps step 7–8 for each target cytokine so that the plate document mirrors the assay configuration of the TaqMan Human Cytokine Card.

TaqMan Human Cytokine Card Assay Configuration

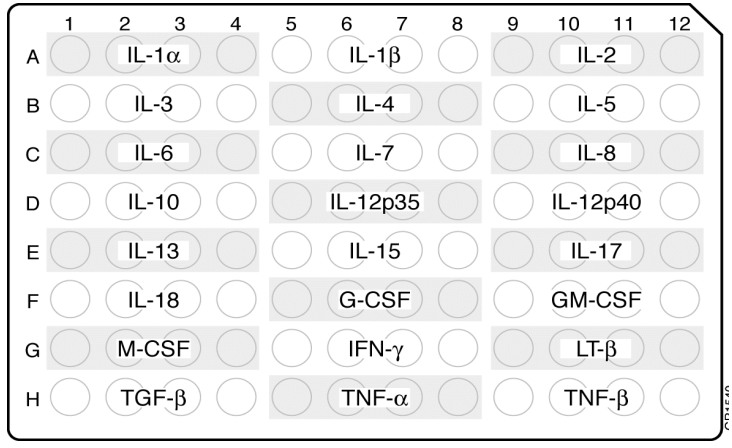
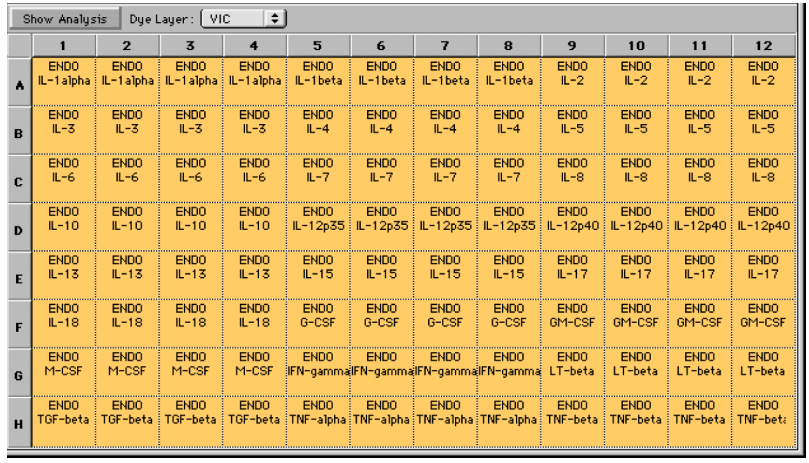
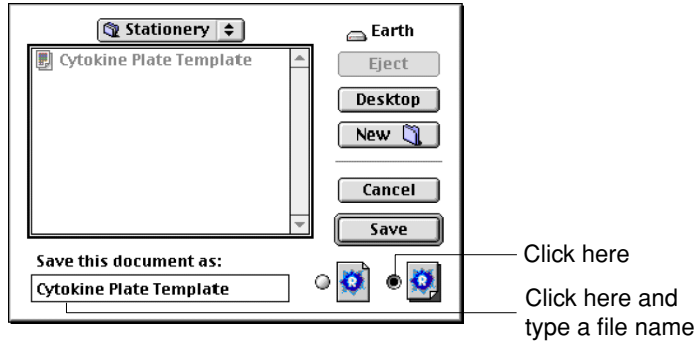


Plate Document Configuration



Saving the Plate Document as a Template

To save the plate document as a template file:

Step	Action
1	Select **Save As** from the **File** menu. The **Save As** dialog box appears.
2	Complete the following actions: a. Click the **Stationery Pad** radio button. b. Click the **Save this document as** text field and type a name for the template file. 
3	Select a location for the software to place the template file.
4	Click **OK**. The software saves the template file.
5	Select **Close** from the **File** menu. When prompted to save the document, select **Don't Save**. The software closes the template document.

Running the Card

To run the card using the template file:

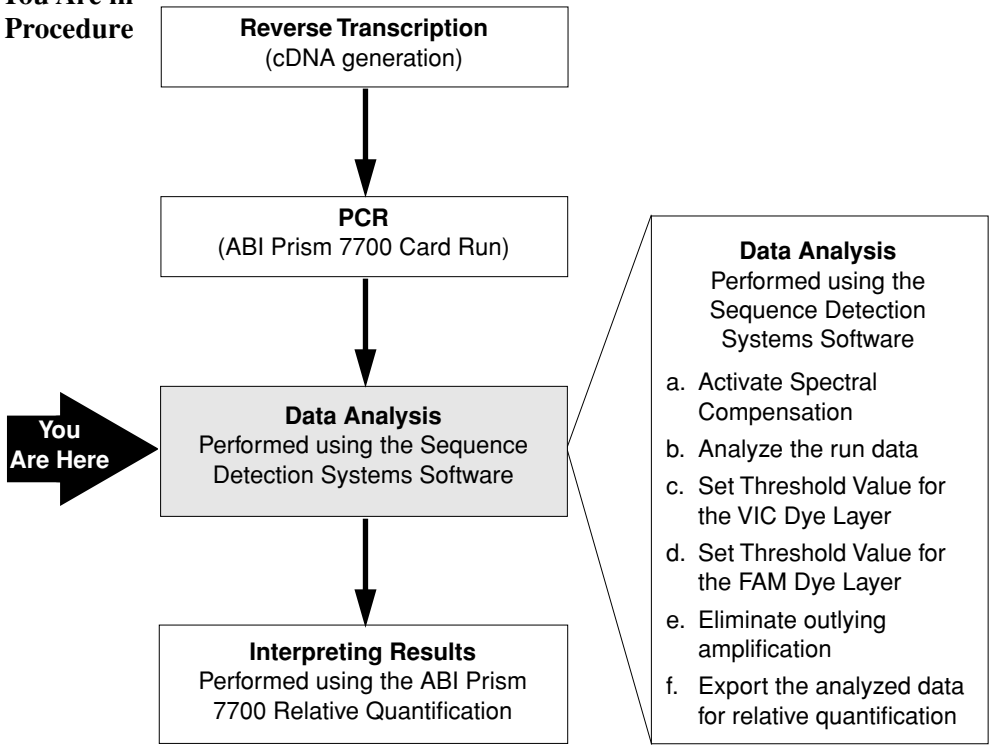
Step	Action
1	Select **Open** from the **File** menu.
2	Using the browser, select the **Cytokine Card Template** file.
3	Click **Open**. The SDS software creates a plate document with attributes identical to that of the template file.
4	Click the **Show Analysis** button to toggle to the **Analysis View**.
5	Click **Run** to begin thermal cycling. **Note** If a card is run immediately after a plate, the instrument may pause momentarily before ramping. The unique thermokinetic properties of the TaqMan Human Cytokine Card require that the instrument cool the heated cover to 65 °C before initiating thermal cycling. When the first card run is complete; prepare, load, and run the next sample-specific PCR reaction mix in the comparative experiment. Repeat the PCR step for each of the remaining cDNA samples and then proceed to Chapter 4, “Data Analysis.”

4 Data Analysis

Overview

Before calculating relative quantification values from the results of the card run, the raw data must be analyzed and exported to a results file. The analysis procedure consists of setting threshold values for the FAM and VIC™ dye layers and eliminating outlying amplification.

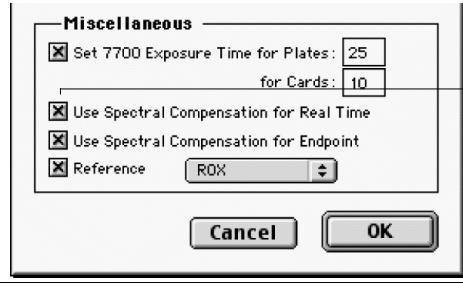
Where You Are in the Procedure



Activating Spectral Compensation

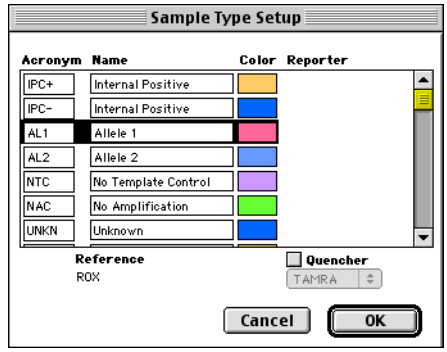
For all real-time runs, set the spectral compensation to ON during analysis of multiplex PCR assays. Activating spectral compensation provides improved spectral resolution for multi-reporter applications.

To activate spectral compensation:

Step	Action
1	Select **Advanced Options** from the **Diagnostics** submenu off of the **Instrument** menu. The **Advanced Options** dialog box opens.
2	Click the **Use Spectral Compensation for Real Time** check box from the **Miscellaneous** box. 
3	Click **OK**.

Non-Fluorescent Quencher

To setup for a non-fluorescent quencher at the 3′ end of the probe:

Step	Action
1	From the **Sample Type** pop-up menu, select **Sample Type Setup** The **Sample Type Setup** dialog box appears. 
2	Complete the **Sample Type Setup** dialog box: a. Uncheck the box next to **Quencher**, when using TaqMan® MGB probes. b. Click **OK**. The dialog box closes, and the plate read window becomes active.

Setting the Baseline Values

Automatic Baseline Calculation

The baseline values for TaqMan® Human Cytokine Card runs cannot be set manually. Version 1.7.1 and later of the ABI PRISM® 7700 Sequence Detection System (SDS) software automatically calculates and sets baselines for card runs.

IMPORTANT You must still set the baseline manually for plates run on the 7700 instrument. The automatic baseline calling feature of SDS software version 1.7.1 is exclusive to card runs.

Setting the Threshold Values

Threshold Requirements for Relative Quantification

Because TaqMan Human Cytokine Cards are used for comparative analysis, all cards in a comparison must:

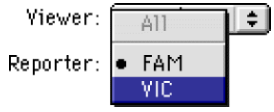
- Share identical threshold settings for the VIC™ dye layer
- Share identical threshold settings for the FAM dye layer

The relative quantification calculation relies on a comparison of threshold cycle (C_T) values from separate ABI PRISM 7700 Sequence Detection System runs. Because the threshold value affects the C_Ts calculated by the SDS software, the setting within each individual dye layer must be identical for all files in the comparison.

Note The ABI PRISM® 7700 Relative Quantification Software will not import multiple SDS results files that have different threshold settings.

Displaying Results on an Amplification Plot

To display the results on an amplification plot:

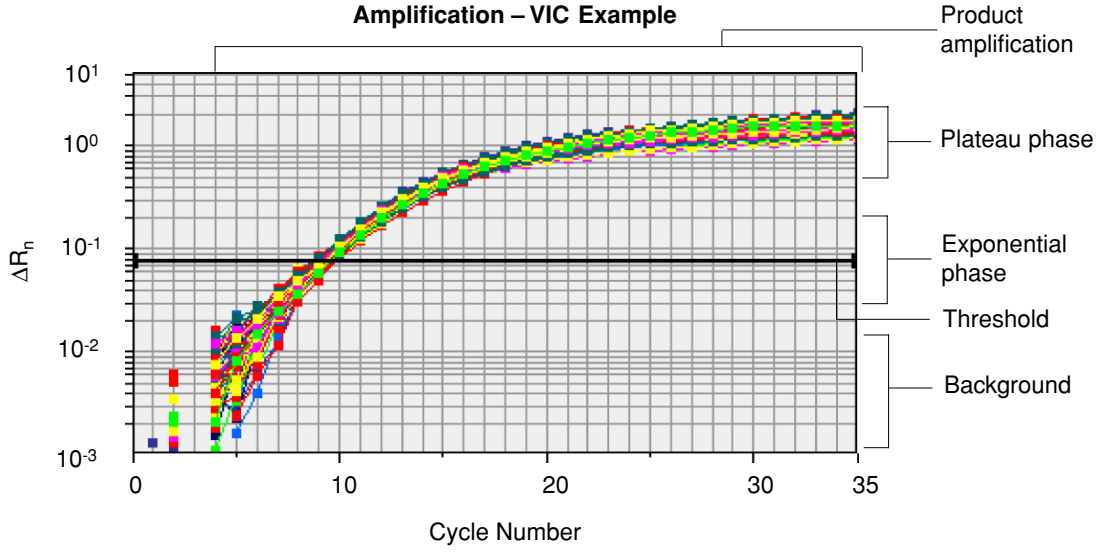
Step	Action
1	Select **Analyze (⌘-L)** from the **Analysis** menu. The SDS software analyzes the raw data and displays the results in an amplification plot (log ΔR_n vs. Cycle). If the software does not display the Amplification Plot, select **Amplification Plot (⌘-G)** from the **Analysis** menu.
2	Select **VIC** from the **Reporter** pop-up menu.  The VIC Endogenous Control Assay Amplification Plot appears.

Threshold Value Basics

Before exporting the data for relative quantification, the threshold value of each TaqMan Human Cytokine Card run must be adjusted for quantification. For any analysis of TaqMan Human Cytokine Cards, accurate quantification depends on the uniformity of the threshold values. If the threshold values are not adjusted properly or if they differ between cards in a comparative experiment, the resulting data will be invalid for relative quantification.

The threshold value must be determined once for each relative quantification experiment, typically with the results from the first card run. Subsequent runs within the same experiment must use the value determined from the calibrator card. Occasionally, the threshold value for an analysis group must be readjusted to compensate for a later run. In those cases, the readjustment is made to the outlying card and then applied to the others.

The threshold value of the calibrator card run can be determined by viewing the amplification plots for the card using the SDS software. The figure below shows a typical semi-logarithmic VIC amplification plot with the correct threshold setting.



Guidelines for setting the threshold value are explained on the following page.

Guidelines for Setting Thresholds

To set the threshold correctly:

- Set the threshold value within the exponential phase of the semi-logarithmic scale amplification plot.

  The exponential phase occurs within the range of data points that increase linearly when graphed on a semi-log plot.

- Set identical FAM thresholds for all cards in the same comparison.

  The ABI PRISM 7700 Relative Quantification Software cannot analyze data from card runs that have different FAM threshold values.

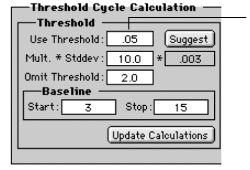
- Set identical VIC thresholds for all cards in the same comparison.

  The ABI PRISM 7700 Relative Quantification Software cannot analyze data from card runs that have different VIC threshold values.

Setting the Threshold for the VIC Dye Layer

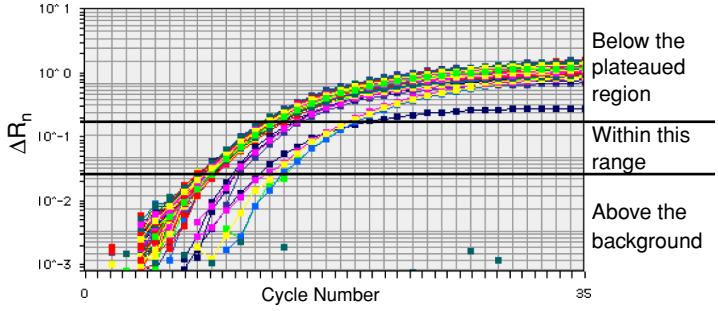
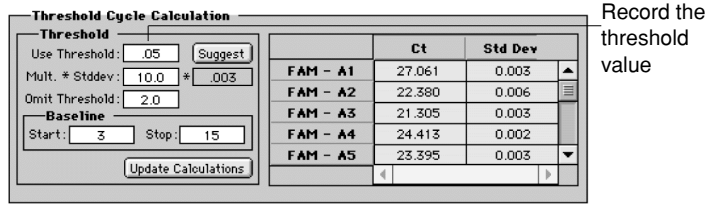
IMPORTANT The baseline values for TaqMan Human Cytokine Card runs cannot be set manually. Version 1.7.1 and later of the SDS software automatically calculates and sets the baseline for card runs.

To set the threshold value for the VIC dye layer:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Select from the following:
<table>
<tr><th>If setting the threshold for…</th><th>Then...</th></tr>
<tr><td>the first card in an experimental series</td><td>follow steps 2–5 of this procedure.</td></tr>
<tr><td>all subsequent cards</td><td>a. Click the Use Threshold text field of the Threshold box.
 

Click here and type the value for the threshold
b. Type the threshold value for the current dye layer (determined in step 4 of this procedure from the first card run in the series).
c. Click Update Calculations.
The SDS software updates the C_T and standard deviation values.</td></tr>
</table></td></tr>
<tr><td>2</td><td>Identify the components of the amplification curve as shown in “Threshold Value Basics” on page 4-5.</td></tr>
</table>

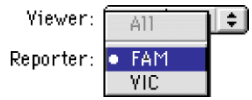
To set the threshold value for the VIC dye layer: *(continued)*

Step	Action
3	Click and drag the threshold line so that it is: ♦ Above the background noise ♦ Below the plateaued region ♦ Within the exponential phase of the amplification curve 
4	Record the threshold value. The software displays the threshold value in the **Use Threshold** text field within the **Threshold** box of the **Amplification Plot** window.  Apply the same threshold value to the VIC dye layer of all subsequent cards in the comparison.

Setting the Threshold for the FAM Dye Layer

Because the threshold value is only valid within a specific dye layer, the FAM and VIC threshold values must be set independently.

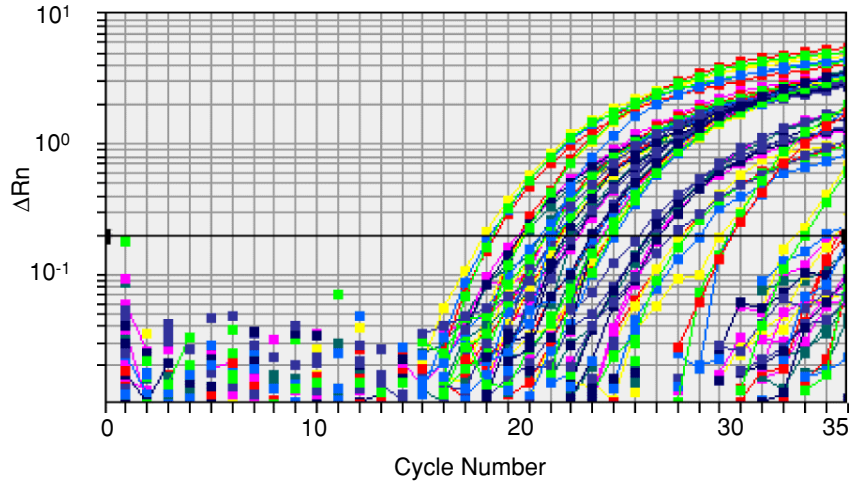
To set the threshold value for the FAM dye layer

Step	Action
1	Select **FAM** from the **Reporter** pop-up menu in the **Amplification Plot** dialog box.  The SDS software displays the FAM Amplification Plot.
2	Follow the procedure for "Setting the Threshold for the VIC Dye Layer" on pages 4-7 to 4–8.
3	Click **OK**.

Example Amplification Curve

The figure below shows a typical semi-logarithmic FAM amplification plot with the correct threshold setting.

Amplification – FAM Example



Eliminating Outlying Amplification

Overview

For any PCR, experimental error may cause some wells to amplify insufficiently or not at all. These wells typically produce C_T values that differ significantly from the average for the associated replicate wells. If included in the relative quantification calculations, these outliers can potentially result in erroneous relative gene expression measurements.

Modes of Outlier Removal

There are two methods for removing outlying data from the relative quantification calculation:

- Manual removal using the SDS software (optional)
- Manual removal using the Relative Quantification software (recommended)

The following section explains the manual mode for removing outlying data using the SDS software.

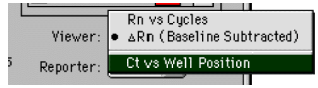
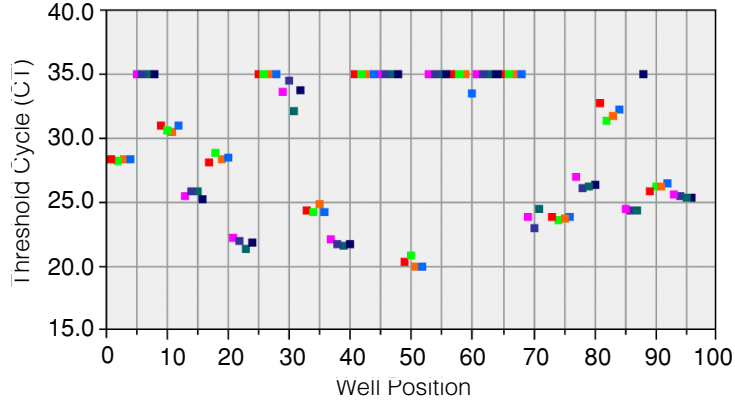
Note The latter method is explained in the *ABI PRISM 7700 Relative Quantification Software User's Manual*.

Manual Removal of Outlying Data Using SDS (Optional)

Visualizing Outliers

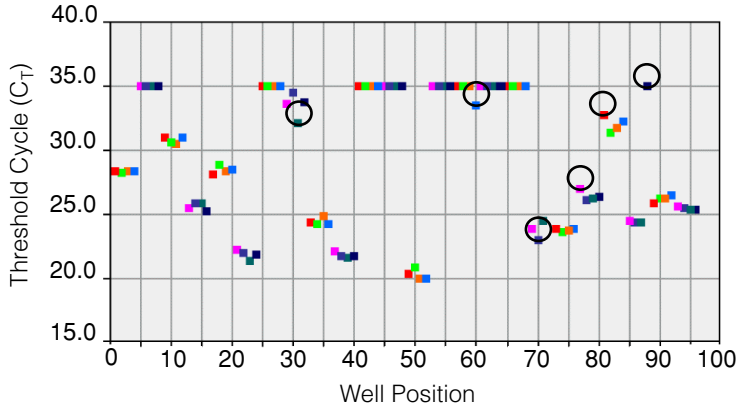
To ensure precise relative quantification, replicate groups must be carefully scrutinized for outlying wells. The C_T vs. Well Position view of the Amplification Plot allows you to examine each set of replicate wells for outliers.

To visualize the replicate groups for outlying amplification:

Step	Action
1	From the **Analysis** menu, select **Amplification Plot (⌘-G)**. The SDS software displays the results of the sequence detection run in an amplification plot of ΔR_n versus C_T.
2	From the **Viewer** pull-down menu, select **C_T vs. Well position**.  The SDS software displays results as a **C_T vs. Well position** plot. 
3	Select **FAM** from the **Reporter** pop-up menu. The SDS software displays the FAM **Well versus Threshold Cycle** display.

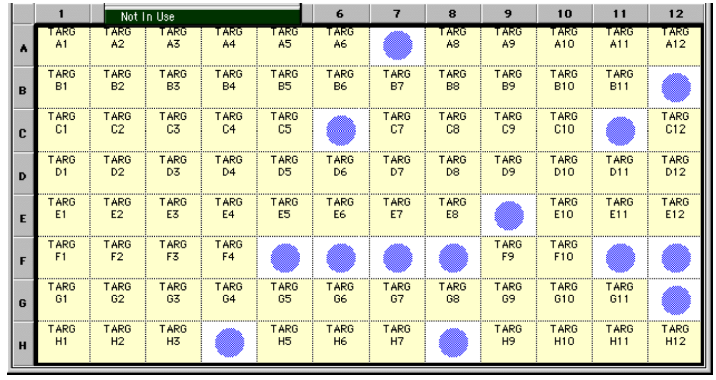
Identifying Outlying Replicate Wells

To identify outlying replicate wells:

Step	Action
1	Verify the uniformity of each set of replicate wells by comparing the groupings of C_T values. The figure below is an example **CT vs. Well** profile. The outlying wells have been circled to illustrate the point.  **Are outliers present?** / **Then...** Yes — record the well numbers of all outlying wells. No — go to "Exporting the Analyzed Run as a Results File" on page 4-14.
2	From the **Reporter** pop-up menu, select **VIC**. The SDS software displays the C_Ts for the endogenous control.
3	Repeat step 1 for the VIC dye layer.
4	Click **OK**.

Eliminating Wells from the Analysis

To eliminate a well with invalid data from the relative quantification calculation:

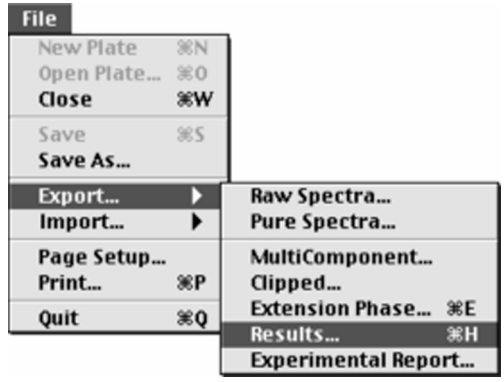
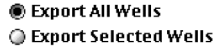
Step	Action
1	Hold down the **Shift** key and click each outlying well identified on the previous page. The SDS Software highlights each cell as it is selected.
2	From the **Sample Type** pull-down menu, select **Not In Use**.  The SDS software labels all the selected wells **Not In Use**. 
3	Repeat steps 1–2 for the VIC dye layer outlying wells.

Exporting the Analyzed Run as a Results File

Exporting a Results File

To analyze data from the TaqMan Human Cytokine Card, you must export the results of the card run to a results file. The SDS software can export results data from a sequence detection run in a tab-delimited format that is compatible with the ABI PRISM 7700 Relative Quantification Software.

To export the run data to an SDS results file:

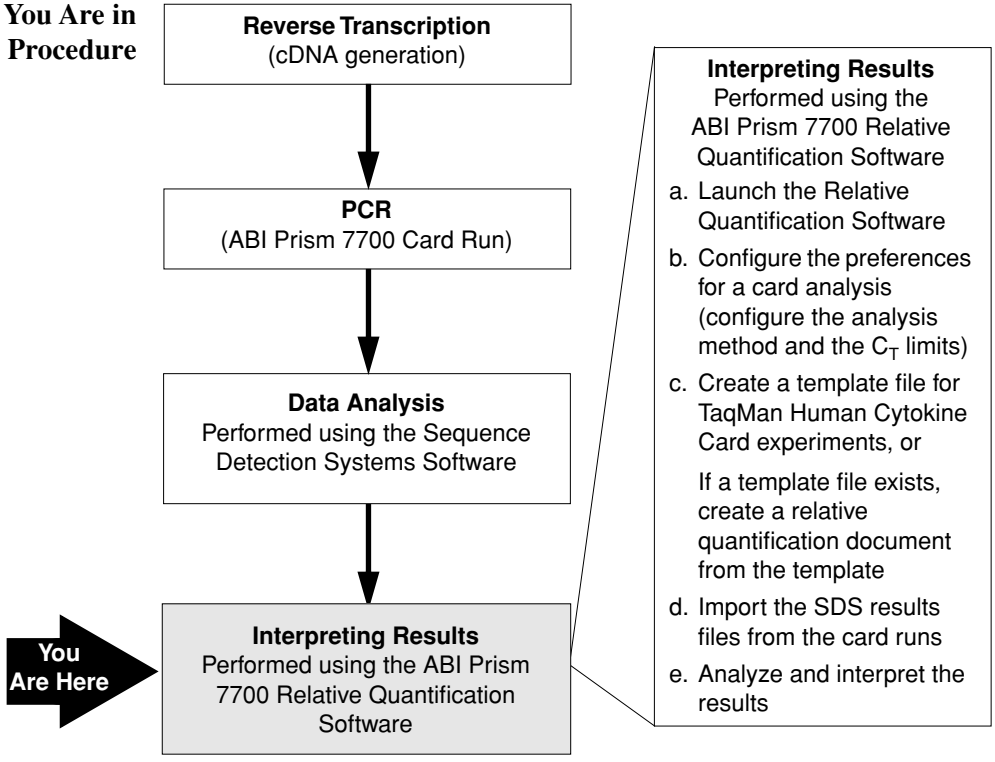
Step	Action
1	Select **Results (⌘-H)** from the **Export** submenu off of the **File** menu. 
2	Click the **Export result data as** text box and type a name for the exported file. 
3	Click the **Export All Wells** radio button. 
4	Click **Export**. The SDS software exports the data. The 7700 data file icon is shown below.  data.results
5	Close the SDS software.

5 Interpreting Results

Overview

The ABI PRISM® 7700 Relative Quantification Software calculates relative cytokine gene expression values from data in exported results files. To calculate relative quantification values from your exported data, follow the procedure for multiplex experiments as outlined in the *ABI PRISM 7700 Relative Quantification Software User's Manual* (P/N 4309937). This chapter contains information to help guide you through the analysis and to help you interpret the results.

Where You Are in the Procedure



Calculating Relative Cytokine Gene Expression

Rationale

The ABI PRISM 7700 Relative Quantification Software calculates relative cytokine gene expression levels from the threshold cycle (C_T) values obtained from TaqMan® Human Cytokine Card runs. The C_T is the cycle at which the first statistically significant increase in ΔR_n is detected during the PCR. Wells with greater initial template concentrations reach their C_T values at lower cycle numbers than wells containing lower template concentrations.

Because amplicons designed and optimized according to Applied Biosystems guidelines have amplification efficiencies approaching 100%, each PCR cycle corresponds to a twofold increase in product. Similarly, a change in C_T value of one cycle equates to a twofold difference in initial template concentration.

The relationship between C_T value and initial template concentration is the basis of the relative quantification calculation. The software calculates relative levels of gene expression by comparing the normalized cytokine assay C_Ts of experimental samples to a calibrator sample. The software displays the calibrator as a 1X sample and all other quantities as an *n*-fold difference relative to the calibrator.

Note For more information about Applied Biosystems guidelines for amplicon design, see the *TaqMan® PCR Universal Master Mix Protocol* (P/N 4304449).

References

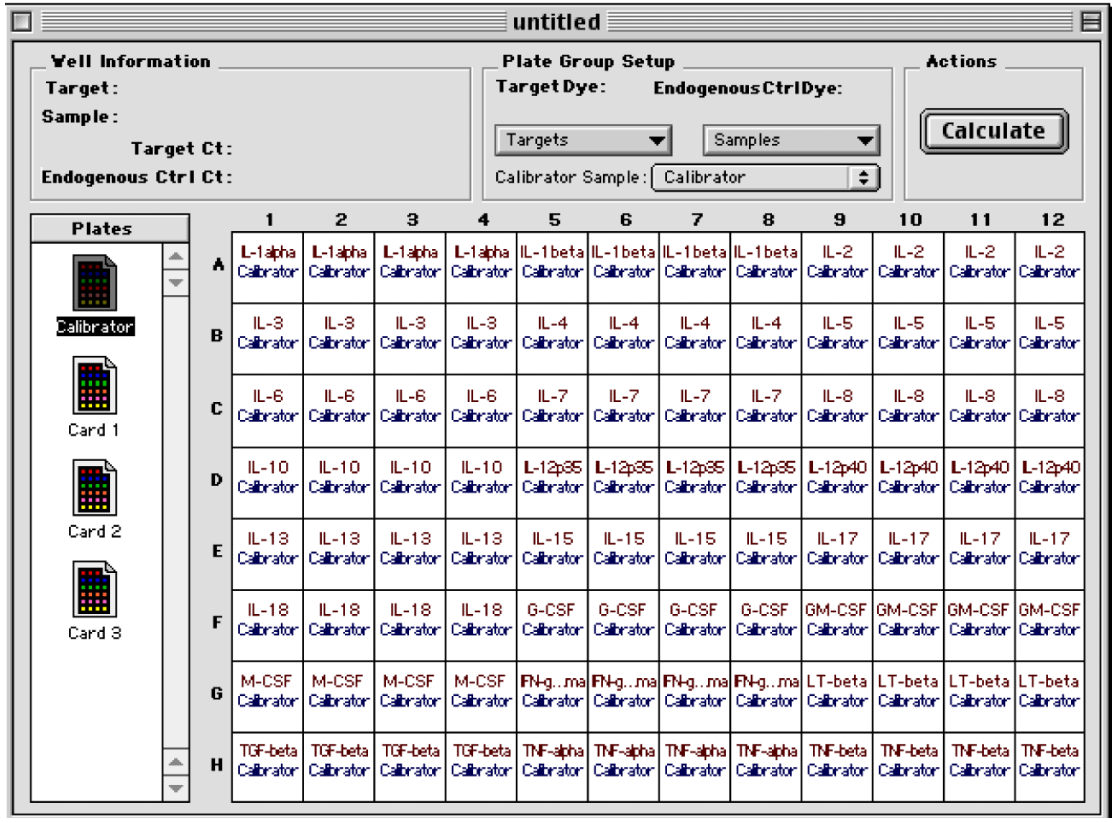
For more information on the topic of the Comparative C_T Method for relative quantification of gene expression, Applied Biosystems recommends the following resources:

- *ABI PRISM 7700 Sequence Detection Systems Software User Bulletin 2: Relative Quantification of Gene Expression* (P/N 4303859)
- *ABI PRISM 7700 Relative Quantification Software User's Manual* (P/N 4309937)

Creating a Template for Card Analyses

The *ABI PRISM 7700 Relative Quantification Software User's Manual* will instruct you to create a template file for TaqMan Human Cytokine Card analyses. The template file is time-saving device that will allow you to skip the laborious task of repetitive plate setup and configuration.

The following figure has been included to provide you with an example of a template document with the correct target configuration and layout.

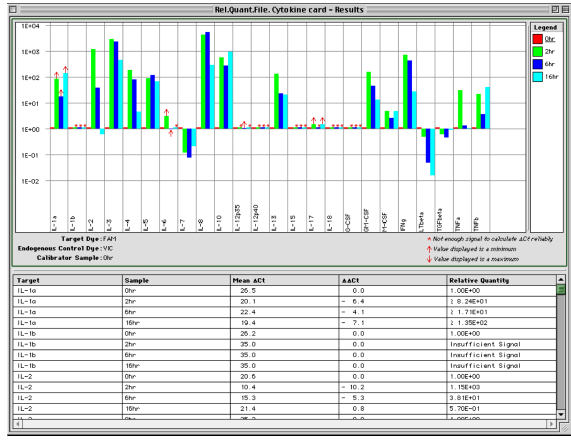


Interpreting Relative Quantification Results

About the Results Window

The ABI PRISM 7700 Relative Quantification Software analyzes the data from the exported results files and displays the analysis in the Results window. The figure below illustrates a typical results window generated from TaqMan Human Cytokine Card data.

Note The example profiles (shown below and on the following page) were created using run data from T cell total RNA samples kindly provided by Dr. R. de Waal Malefyt, DNAX Research Institute, Palo Alto, California, USA.



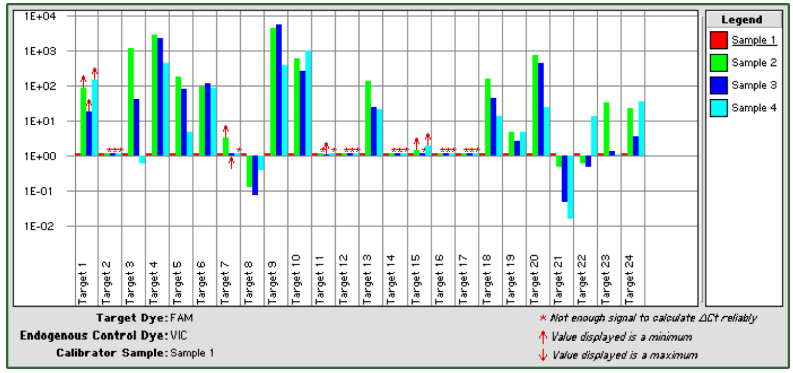
Target	Sample	Mean ΔCt	ΔΔCt	Relative Quantity
IL-1α	0hr	26.5	0.0	1.00E+00
IL-1α	2hr	20.1	- 6.4	↕ 8.24E+01
IL-1α	6hr	22.4	- 4.1	↕ 1.71E+01
IL-1α	16hr	19.4	- 7.1	↕ 1.35E+02
IL-1b	0hr	26.2	0.0	1.00E+00
IL-1b	2hr	35.0	0.0	Insufficient Signal
IL-1b	6hr	35.0	0.0	Insufficient Signal
IL-1b	16hr	35.0	0.0	Insufficient Signal
IL-2	0hr	20.6	0.0	1.00E+00
IL-2	2hr	10.4	- 10.2	1.15E+03
IL-2	6hr	15.3	- 5.3	3.81E+01
IL-2	16hr	21.4	0.8	5.70E-01

Element	Description
Gene Expression Profile	A graphic representation of the results of the relative quantification calculations displayed on a semi-logarithmic scale. The elements of the profile are described on the following page.
Results Table	A numerical representation of the sample data at various stages of the relative quantification calculation. **Note** See the *ABI PRISM 7700 Relative Quantification Software User's Manual* for information on the calculation.

About the Cytokine Gene Expression Profile

The ABI PRISM 7700 Relative Quantification Software displays the results of the cytokine assays as the normalized mRNA level in test samples relative to the normalized level of that mRNA in the corresponding calibrator sample.

The gene expression profile is a graphic representation of the results of the relative quantification calculations. The gene expression profile is located in the upper panel of the Results window. The following figure illustrates a typical TaqMan Human Cytokine Card profile.



X-Axis

The X-axis of the gene expression profile lists all of the cytokine targets involved in the analysis. Within each grouping, the software displays the relative quantity of the target in each sample.

Y-Axis

The Y-axis displays the relative quantities of the cytokine targets on a semi-logarithmic scale. The quantities shown are relative to the calibrator sample. Each increment corresponds to a ten-fold difference in gene expression.

Note Because the calibrator sample is compared to itself, the level of cDNA expression in the calibrator always appears as 1 (1E+00).

Sample Bars Each bar represents the level of target gene expression within a sample listed in the legend.

Symbol	Definition
↑	**Minimum target gene expression level** Displayed on all associated sample bars when: ♦ A calibrator sample produces an Average C_T ≥35 cycles ♦ A test sample produces an Average C_T <35 cycles At this level, the calibrator sample does not contain enough cytokine target cDNA for an accurate comparison. Therefore, the stated expression level is the minimum possible value for the associated test sample. **Note** The software displays ≥ in the cells of the results table that correspond to the values displayed in the bar graph.
↓	**Maximum target gene expression level** Displayed when: ♦ A test sample produces an Average C_T ≥35 cycles ♦ A calibrator sample produces an Average C_T <35 cycles At this level, the test sample does not contain enough cytokine target cDNA for an accurate comparison with the calibrator sample. Therefore, the stated expression level is the maximum possible value for the sample. **Note** The software displays ≤ in the cells of the results table that correspond to the values displayed in the bar graph.
*	**Expression levels cannot be determined from the given data** Displayed when both of the following conditions are met: ♦ A calibrator sample produces an Average C_T ≥35 cycles ♦ A test sample produces an Average C_T ≥35 cycles At these levels, the calibrator and test samples do not contain enough cytokine target cDNA for an accurate comparison. Because both signals are insufficient, the software cannot accurately evaluate the expression levels. **Note** The software displays **Insufficient Signal** in the corresponding cells of the results table.

6 Troubleshooting

Overview This chapter provides information on how to troubleshoot the following areas:

- Loading the card
- Data analysis
- Interpreting the results

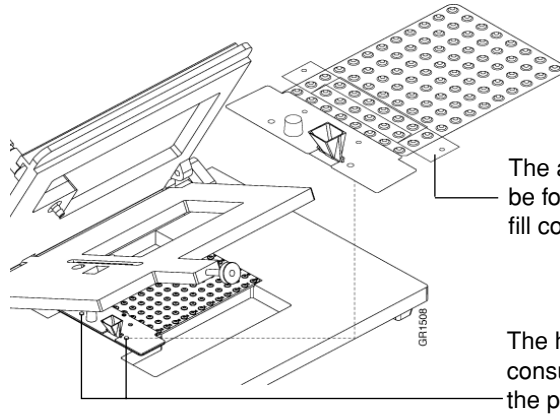
Loading the Card

Troubleshooting Card Loading

Observation	Possible Cause(s)	Recommended Action
Vacuum is not reaching the proper level (≤600 Microns)	♦ The ABI PRISM® Card Filling Station is not completely closed ♦ The tubing between the pump and the Filling Station contains a loose connection ♦ The battery in the vacuum gauge is low ♦ The vacuum pump requires maintenance ♦ The ABI PRISM® Human Cytokine Card is not aligned properly inside the Filling Station ♦ The fill consumable and the reaction card are not aligned	Conduct the following: a. Press down on the Filling Station lid to ensure that it is fully closed. b. Check the tubing and connections for leaks. c. Test and change the battery in the vacuum gauge if necessary. d. If the problem persists, verify that the cytokine card is positioned correctly within the Filling Station as explained on page 6-3. e. If the problem persists, verify the integrity of the connection between the reaction card and fill consumable as explained on page 6-4.
The sample does not enter the card when the actuator is pulled	The card and fill consumable are not aligned properly inside the Filling Station	Verify that the cytokine card is positioned correctly within the Filling Station as explained on page 6-3.
The sample partially enters the card when the plunger is pulled		
Large bubbles appear in the card immediately after filling **Note** Cards typically contain small bubbles after thermal cycling.		

Verifying the Position of the Card Within the Filling Station

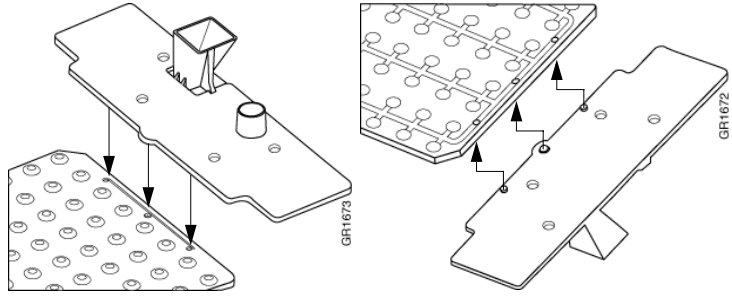
To verify that the card is positioned correctly within the Filling Station:

Step	Action
1	Turn off the vacuum pump.
2	Using a pipet, remove the sample from the reservoir and store it temporarily on ice.
3	Open the ABI PRISM Filling Station.
4	Inspect the card for the following:  The adhesive flap must be folded away from the fill consumable The holes in the card's fill consumable must align with the pegs in the bay of the ABI PRISM Filling Station
5	Reposition the cytokine card and re-attempt to load the card. **IMPORTANT** Do not attempt to refill partially-filled cards. Upon contact, the sample-specific PCR reaction mix resuspends the dried TaqMan® probes and primers within the wells of the card. When the partially-filled card is evacuated, these reagents are carried away with the solution.

Verifying the Consumable/Card Connection

The connection between the reaction card and the fill consumable is crucial for loading TaqMan Human Cytokine Cards.

To verify the integrity of the connection between the reaction card and the fill consumable:

Step	Action
1	Turn off the vacuum pump.
2	Using a pipet, temporarily remove the sample from the reservoir and store it on ice.
3	Open the Filling Station.
4	Remove the TaqMan Human Cytokine Card from the Filling Station.
5	Inspect the connection between the reaction card and the fill consumable. The consumable design includes two alignment pins and a small aperture through which the sample enters the card. When situated correctly, the pins and aperture align with three holes on the keyed end of the reaction card. The following figures illustrate this card/consumable relationship from opposing angles.  If the card components do not align as shown above, readjust the position of the fill consumable to restore the connection.
6	Load the TaqMan Human Cytokine Card into the ABI PRISM Filling Station and re-attempt the loading procedure.

Troubleshooting Data Analysis

Observation	Possible Cause	Recommended Action
In the Amplification Plot view, the fluorescent signals show little or no growth (Figure 6-1). AND In the Multicomponent View, all well signals slowly degrade to background by the end of the run (Figure 6-2).	The heated cover was not tightened adequately	Remember to tighten the heated cover completely for future runs.
	The O-ring on the lens plate that seals the card fill port is damaged	If the problem persists, replace the O-ring on the top plate of the card adaptor with the part included with the kit.
	The alignment pins on the 7700 Sample Block may be interfering with heat transfer	See "Poor to no amplification in the wells of the top row (wells A1–A12)" on page 6-8.

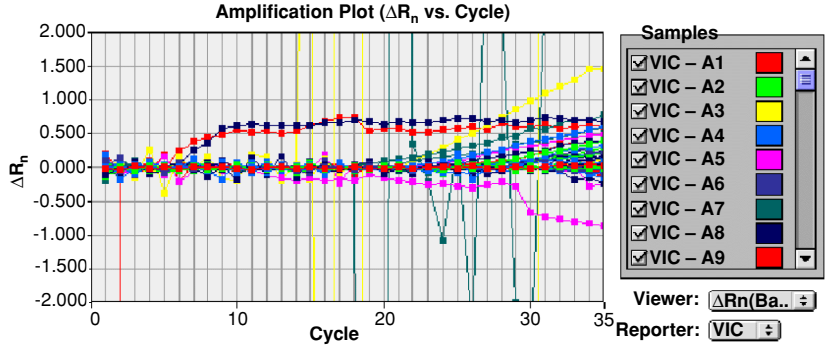


Figure 6-1 Little or No Growth in the Amplification Plot

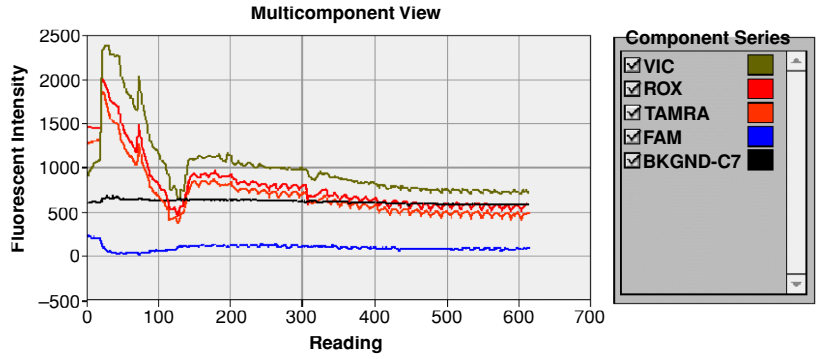


Figure 6-2 Degrading Multicomponent Signals

Troubleshooting Data Analysis *(continued)*

Observation	Possible Cause	Recommended Action
Growth curves from wells without amplification drift upward during the later cycles (15–35) (see Figure 6-3) AND/OR Growth curves on all wells slope downward during the early cycles correlating with a loss in precision of the data (see Figure 6-3)	The plate document file is in Standard Plate format	Create a new plate document for a card run and import the Labview® file from the problem run. See “Correcting for Plate Document Type” on page 6-8. **Note** The poor precision of the problem run cannot be corrected after the run is complete.

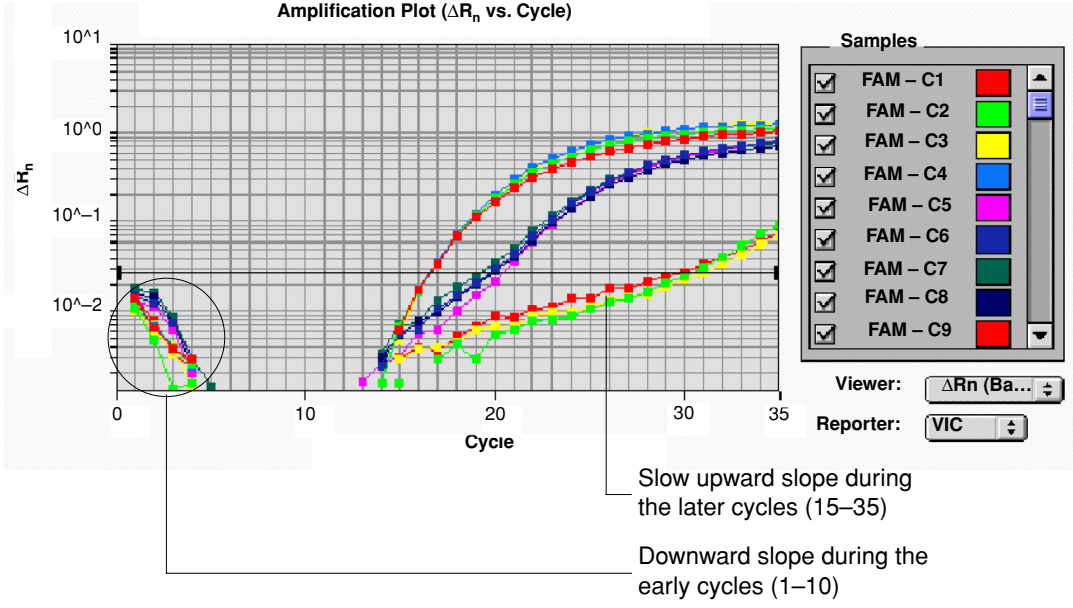


Figure 6-3 Incorrect Plate Format

Troubleshooting Data Analysis *(continued)*

Observation	Possible Cause	Recommended Action
Some growth curves slope down toward the later cycles of large growth curves (Figure 6-4)	The instrument is becoming saturated.	Correcting the problem is optional. The problem cannot be corrected for the current run. To avoid this problem in future runs, follow the procedure “Adjusting the Exposure Time for Card Runs” on page 3-2.

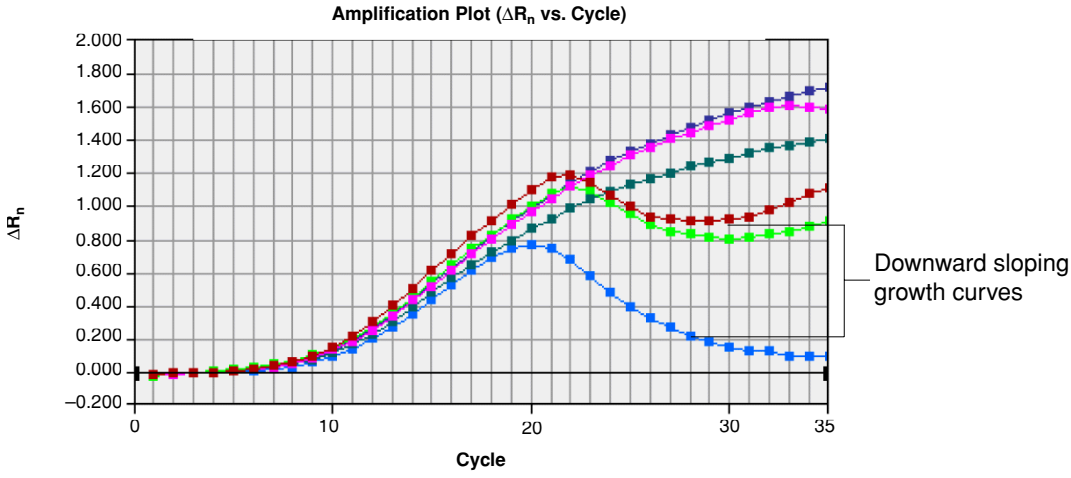


Figure 6-4 Instrument Saturation

Troubleshooting Data Analysis *(continued)*

Observation	Possible Cause	Recommended Action
Poor to no amplification in the wells of the top row (wells A1–A12)	The alignment pins on the 7700 Sample Block may be interfering with heat transfer. The pins of earlier instruments (see Figure 6-5) can prevent the lens plate of the ABI PRISM Card Adaptor from aligning with rows A and B.	Use Figure 6-5 to determine whether the pins on your instrument must be replaced. If your instrument does not contain the correct type of pins, replace them with the new pins supplied in the TaqMan Card Upgrade Package.

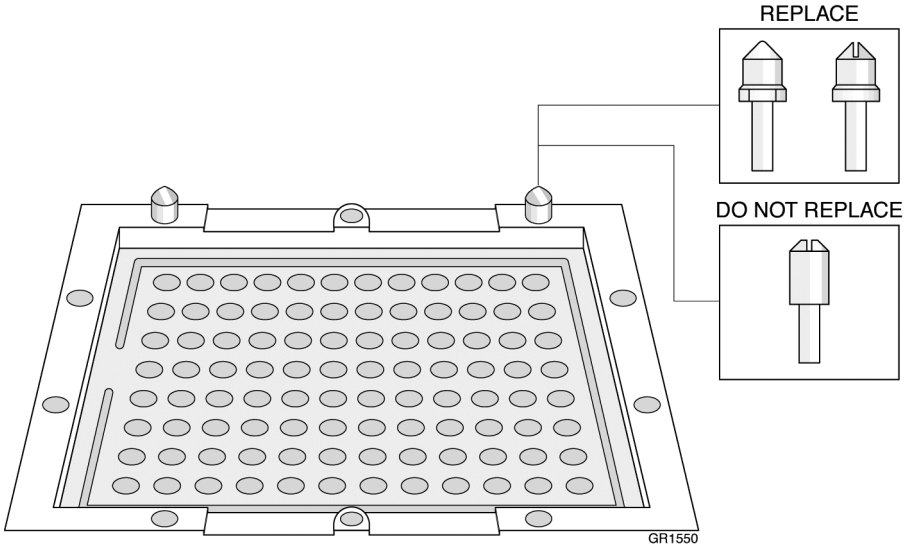


Figure 6-5 Pin Replacement

Correcting for Plate Document Type

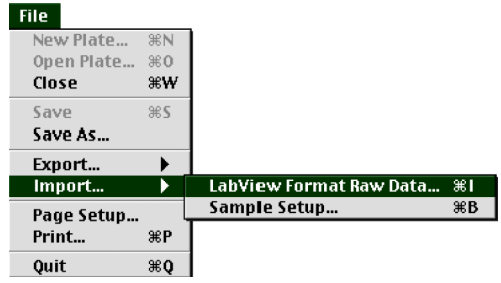
To correct a mislabeled document:

Step	Action
1	**Save** and **Close** the current plate document.
2	From the **File** menu, select **New ⌘–N**.

To correct a mislabeled document: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>3</td><td>Configure the new file as follows:

<table>
<tr><th>From menu…</th><th>Select…</th></tr>
<tr><td>Plate Type</td><td>Single Reporter</td></tr>
<tr><td>Plate Format</td><td>The Card</td></tr>
<tr><td>Run</td><td>Real Time</td></tr>
</table>
</td></tr>
<tr><td>4</td><td>From the Import submenu off of the File menu, select Labview Format Raw Data.



</td></tr>
<tr><td>5</td><td>Using the browser, navigate to the SDS Runs folder of the SDS software folder on the hard disk of your 7700 instrument.</td></tr>
<tr><td>6</td><td>Select the Labview file from the problem run.

The SDS software labels Labview files by the time and date of the run. Select the file with the correct creation date and time.</td></tr>
<tr><td>7</td><td>Click OK.

The SDS software imports the data from the Labview file.</td></tr>
<tr><td>8</td><td>Reanalyze the file as described in Chapter 4, “Data Analysis.”</td></tr>
</table>

Interpreting Results

Troubleshooting Interpreting Results:

Observation	Possible Cause	Recommended Action
Unable to import card file into the ABI PRISM® 7700 Relative Quantification Software	Files may not be the proper format	Verify that the file you are attempting to import is a tab-delimited results file exported from a card run and not the SDS run file. If necessary, export the files as explained in “Exporting the Analyzed Run as a Results File” on page 4-14.  Re  SDS run file
	Files may not be configured with correct sample types (TARG and ENDO)	While viewing the card run within the SDS software, verify the following: ♦ The TARG sample type must be applied to all wells in the FAM dye layer (see page 3-19 for the FAM dye layer setup). ♦ The ENDO sample type must be applied to all wells in the VIC dye layer (see page 3-19 for the VIC dye layer setup). If the ENDO and TARG sample types are incorrectly applied or are absent from the Sample Type palette: a. Add the sample types to the palette (see “Adding the TARG and ENDO Sample Types to the Dye Palette” on page 6-11). b. Apply the correct sample types to the SDS file. c. Re-analyze and export the results.
	Threshold values within the FAM and VIC dye layers may not be identical for all of the results files	While viewing the card run within the SDS software, verify the following: ♦ The VIC threshold value must match the VIC thresholds for the other cards in the analysis. ♦ The FAM threshold value must match the FAM thresholds for the other cards in the analysis. If the threshold values are not consistent with the other cards in the analysis, set them accordingly (see “Setting the Threshold Values” on page 4-4).

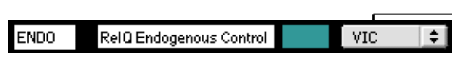
Observation	Possible Cause	Recommended Action
Average endogenous control C_Ts are ≥23	♦ Not enough sample in the card ♦ Not enough cDNA from total RNA.	a. Verify the type and quantity of the cDNA sample loaded into the TaqMan Human Cytokine Card. (See pages 3-4 to 3-5 for the correct template information.) b. Increase the concentration of the cDNA sample loaded into the card.

Adding the TARG and ENDO Sample Types to the Dye Palette

To add new dyes to the dye palette:

Step	Action
1	From the **Setup** menu, select **Sample Type Palette**. The **Sample Type Palette** dialog box appears.
2	Click **Sample Type Setup**. The **Sample Type Setup** dialog box appears.
3	Add the **TARG – RelQ Target** sample type to the palette: a. Click **Add**. A new row appears at the bottom of the dye list. b. Click the **Acronym** text field and type **TARG**.  c. Click the **Name** text field and type **RelQ Target**.  d. Click the **Color** field. When the Color pallet dialog box appears, Select a color for the new dye and click **OK**.  The color field for the new dye fills with the new color. e. From the **Reporter** pull-down menu, select **FAM** 

To add new dyes to the dye palette: *(continued)*

Step	Action
4	Add the **ENDO – RelQ Endogenous Control** sample type to the palette: a. Click **Add**. A new row appears at the bottom of the dye list. b. Click the **Acronym** text field and type **ENDO**. c. Click the **Name** text field and type **RelQ Endogenous Control**. d. Click the **Color** field. The Color pallet Dialog Box appears. e. Select a color for the new dye and click **OK**. The color field for the new dye fills with the new color. f. From the **Reporter** pull-down menu, select **VIC** 
5	Click **OK**. The new sample types will now be available from the **Sample Type** pop-up menu.

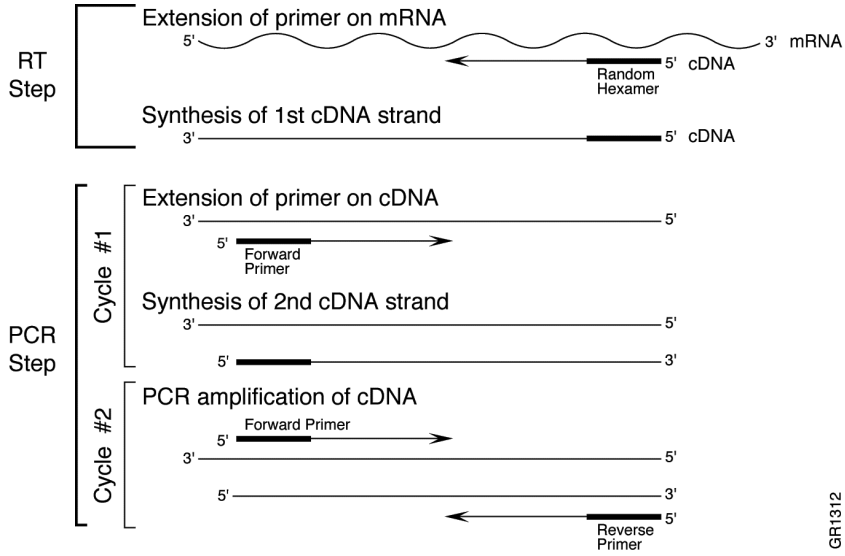
Theory of Operation A

Overview This appendix provides an overview of the theoretical basis for the TaqMan® Human Cytokine Card chemistry and ABI PRISM® 7700 Sequence Detection System data collection.

TaqMan Human Cytokine Card Chemistry

RT-PCR

The TaqMan Human Cytokine Card evaluates gene expression in a two-step reverse transcription-polymerase chain reaction (RT-PCR). The figure below illustrates the assay steps.

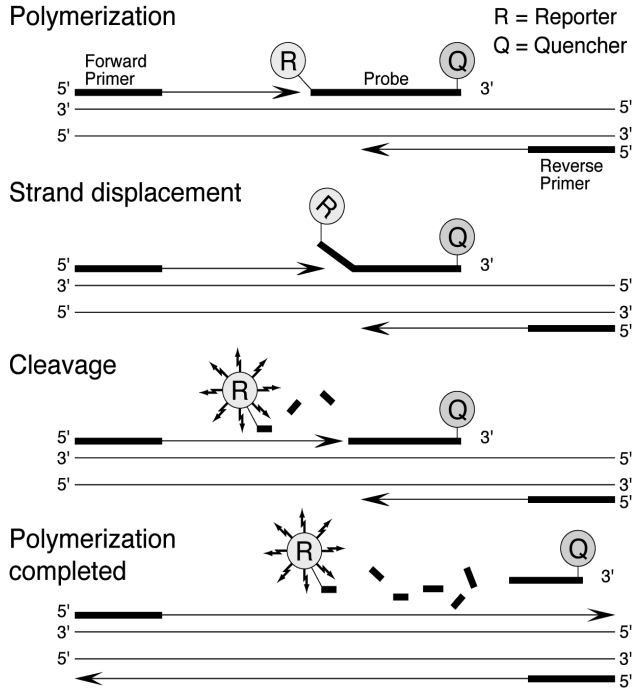


In the RT step, cDNA is reverse transcribed from total RNA samples using random hexamers from the TaqMan Reverse Transcription Reagents. In the PCR step, products are synthesized from cDNA samples using the TaqMan Universal PCR Master Mix and target specific primers/probes.

Basics of the 5´ Nuclease Assay

The PCR reaction exploits the 5´ nuclease activity of AmpliTaq Gold DNA Polymerase to cleave a TaqMan® probe during PCR. The TaqMan probe incorporates a reporter dye (VIC or FAM) at the 5´ end of the probe and a quencher (TAMRA, or MGB) at the 3´ end of the probe.

During the reaction, cleavage of the probe separates the reporter dye and the quencher, which results in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye as shown below.



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´→3´ nucleolytic activity of the AmpliTaq Gold DNA Polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3´ end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product.

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, any nonspecific amplification is not detected.

About AmpliTaq Gold DNA Polymerase

AmpliTaq Gold is a thermal stable DNA polymerase. The enzyme has a 5´→3´ nuclease activity, but lacks a 3´→5´ exonuclease activity (Innis *et al.*, 1988; Holland *et al.*,1991). With AmpliTaq Gold enzyme, Hot Start PCR and Time Release PCR can be introduced into existing amplification systems with little or no modification of cycling parameters or reaction conditions. These techniques improve amplification of most templates by lowering background and increasing amplification of specific products.

Multicomponent Analysis

Fluorescent Detection

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

Function	Dye
Reporters	FAM, TET, VIC
Quenchers	TAMRA, MGB
Passive reference	ROX

Passive Reference

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

Normalization

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

Variable	Description
R_n^+	The R_n value of a reaction containing all components including the template.
R_n^-	The R_n value of an unreacted sample. This value may be obtained from the early cycles of a Real Time run, those cycles prior to a detectable increase in fluorescence. This value may also be obtained from a reaction not containing template.
ΔR_n	The difference between the R_n^+ value and the R_n^- value. It reliably indicates the magnitude of the signal generated by the given set of PCR conditions.

The following equation expresses the relationship of these terms:

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

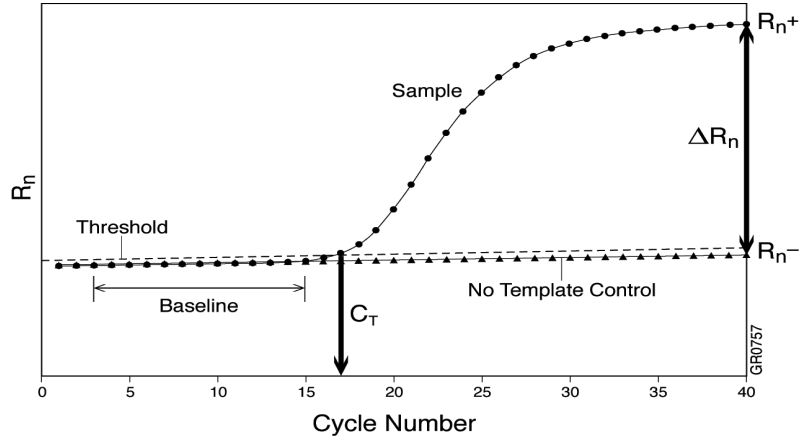
where:

$$R_n^+ = \frac{\text{emission intensity of reporter}}{\text{emission intensity of passive reference}}$$ PCR with template

$$R_n^- = \frac{\text{emission intensity of reporter}}{\text{emission intensity of passive reference}}$$ PCR without template or early cycles of a Real Time reaction

Real Time Detection

The threshold cycle or C_T value is the cycle at which a statistically significant increase in ΔR_n is first detected. Threshold is defined as the average standard deviation of R_n for the early cycles, multiplied by an adjustable factor. On the graph below, the threshold cycle occurs when the Sequence Detection Systems software begins to detect the increase in signal associated with an exponential growth of PCR product.



Demonstrating Performance with Control RNA

B

Overview TaqMan Human Control Total RNA is available from Applied Biosystems for demonstrating the performance of the TaqMan® Human Cytokine Card. This appendix includes the performance guarantee statement and protocols for analyzing the Control RNA data.

System Performance Guarantee

Statement

If TaqMan Human Control Total RNA is run in the card using the conditions in the following protocol, the average C_T value for the 18S endogenous control will be fewer than 12 cycles and the ΔC_T values for five cytokine targets will be as follows:

Target	ΔC_T
IL-10	Below 20
Lymphotoxin-B	Below 20
TGF-B	Below 17
TNF-a	Below 20
TNF-B	Below 17

Protocol for Analyzing TaqMan Human Control Total RNA

Verifying C_T Values Using the SDS Software

Card performance can be verified immediately after running a control card by examining the threshold cycle (C_T) values of each reporter dye layer within the Sequence Detection System (SDS) software.

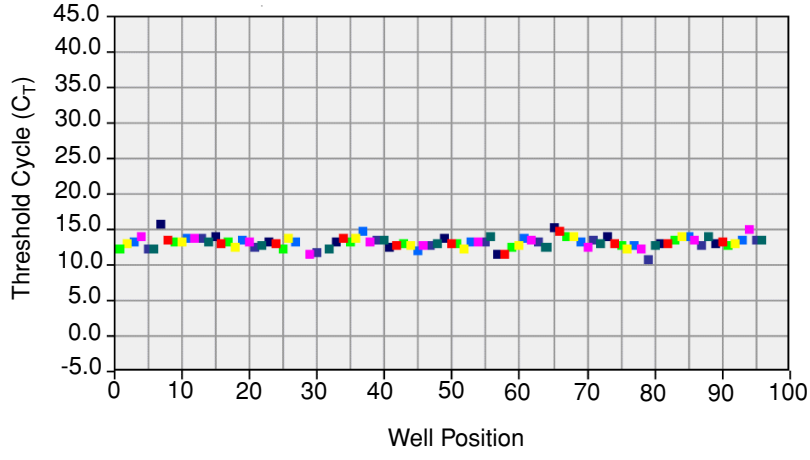
To verify the C_T values:

Step	Action
1	Perform reverse transcription as described in Chapter 2 using 2 µg of TaqMan Human Control Total RNA in a 100 µL reaction.
2	Make a sample-specific PCR reaction mix using 150 µL TaqMan Universal PCR Master Mix, 30 µL of 20X 18S Primer and Probe Mix, 118 µL water, and 2 µL cDNA as described in Chapter 3, "PCR." Then, fill and run a TaqMan Human Cytokine Card with the reaction mix.

To verify the C_T values: *(continued)*

Step	Action
3	After the run is complete, activate the Spectral Compensation feature: a. Select **Advanced Options** from the **Diagnostics** submenu off of the **Instrument** menu. The **Advanced Options** dialog box appears. b. From the **Miscellaneous** group box, click the **Use Spectral Compensation for Real Time** check box. c. Click **OK**.
4	Select **Analyze (⌘-L)** from the **Analysis** menu. The SDS software analyzes the raw data and displays an amplification plot (log ΔR_n vs. Cycle).
5	From the **Reporter** menu, select **VIC**. The SDS software displays the amplification plot (log ΔR_n vs. Cycle) for the VIC dye layer.
6	From the **Viewer** menu, select **C_T vs. Well**. The SDS software displays the Threshold Value (C_T) vs. Well Position view for the VIC dye. Because of the high abundance of rRNA, performance integrity across a entire card can be best assessed by examining the C_T from the 18S rRNA endogenous control. The card is performing properly if the average VIC C_T is <12 cycles.

Amplification – TaqMan Human Total RNA (VIC)



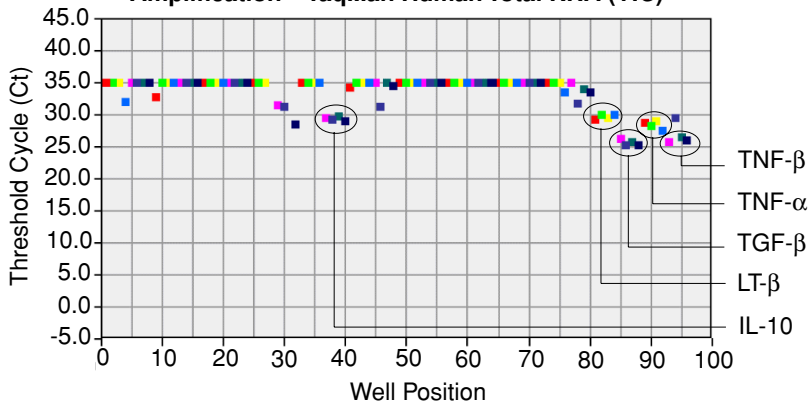
To verify the C_T values: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>7</td><td>From the Reporter menu, select FAM.

The card is performing properly if the following FAM C_Ts are <30 cycles:

<table>
<tr><th>Cytokine Target</th><th>Wells</th></tr>
<tr><td>IL-10</td><td>37–40</td></tr>
<tr><td>LT-β</td><td>81–84</td></tr>
<tr><td>TGF-β</td><td>85–88</td></tr>
<tr><td>TNF-α</td><td>89–92</td></tr>
<tr><td>TNF-β</td><td>93–96</td></tr>
</table>

Note The TaqMan Human Total RNA sample expresses some cytokines other than those listed above to a limited extent.

Amplification – TaqMan Human Total RNA (VIC)


</td></tr>
<tr><td>8</td><td>Export the analyzed data as explained in “Exporting the Analyzed Run as a Results File” on page 4-14.</td></tr>
</table>

Verifying the Expression Profile of Control RNA

Note For the purpose of demonstration, the following procedure illustrates how to generate a profile of median ΔC_T values using Microsoft's Excel spreadsheet utility. Other software packages can be used to create the profile, however the keystrokes and command pathways may differ.

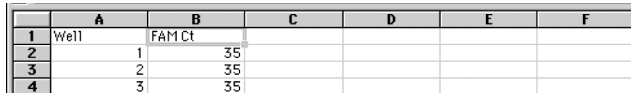
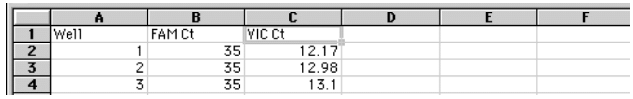
After exporting the results of the control card run, the performance of the card can be verified again by generating a profile of the median ΔC_T values using a spreadsheet utility. Because the control RNA sample lacks a calibrator sample for comparison, relative quantities cannot be derived from the results of the control card run. Instead, the data from the control run can be used to generate a profile of the normalized, median C_T values for the cytokine assays.

Transferring Data from the Results File

To transfer data from the results file to the spreadsheet:

Step	Action
1	Launch Microsoft Excel.
2	Open the results file from the control card run: a. From the **File** menu, select **Open**. The **Open** dialog box appears. b. Use the browser to navigate to and select the results file from the control card run. c. Click **Open**. The software displays the data within the SDS results file.
3	From the **File** menu, select **New**. A new spreadsheet appears.
4	From the **Window** menu, select the SDS results file. The cytokine card results spreadsheet reappears.
5	Select cells **A1–A97**.
6	From the **Edit** menu, select **Copy**.
7	From the **Window** menu, select the new spreadsheet. The new spreadsheet file reappears.
8	Click cell **A1**.
9	From the **Edit** menu, select **Paste**. Excel pastes the data into the new spreadsheet.

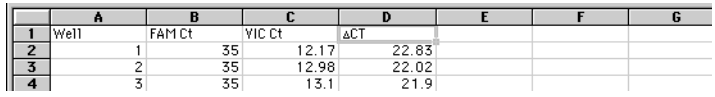
To transfer data from the results file to the spreadsheet: *(continued)*

Step	Action
10	Repeat steps 3-7 to copy the FAM C_T values in cells **F1–F97** from the results file and paste them into cell **B1–B97** of the new file. 
11	Click cell **B1** and type **Ct FAM**.
12	Repeat steps 3-7 to copy the VIC C_T values in cells **F111–F207** from the results file and paste them into cell C1 of the new Excel file. 
13	Click cell **C1** and type `Ct VIC`.

Calculating ΔC_T

ΔC_T is calculated with the equation: $\Delta C_T = C_{T\,FAM} - C_{T\,VIC}$. To calculate ΔC_Ts, the formula must be entered into column D.

To enter the equation into the spreadsheet:

Step	Action
1	Click cell **D2**.
2	Type the equation **=B2-C2** and press **Return**.
3	Click cell **D2**.
4	From the **Edit** menu, select **Copy**.
5	Select cells **D3–D97** by clicking and dragging the mouse cursor down the spreadsheet document.
6	From the **Edit** menu, select **Paste**. Excel pastes copies of the equation into cells **D2-D97** of the new spreadsheet. The program automatically calculates ΔC_T values. 
7	Click cell **D1** and type **ΔC_T**.
8	From the **File** menu, select **Save As**. Save the spreadsheet document.

Creating an Average ΔC_T Table

Now that ΔC_T values have been calculated from the cytokine card data, the results must be organized under the cytokines they detect.

To create a Median ΔC_T table:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Click cell F1 and type Target.</td></tr>
<tr><td>2</td><td>Click and type the following entries into the designated cells:
<table>
<tr><th>Click Cell</th><th>Type</th><th>Click Cell</th><th>Type</th></tr>
<tr><td>F2</td><td>IL-1alpha</td><td>F14</td><td>IL-13</td></tr>
<tr><td>F3</td><td>IL-1beta</td><td>F15</td><td>IL-15</td></tr>
<tr><td>F4</td><td>IL-2</td><td>F16</td><td>IL-17</td></tr>
<tr><td>F5</td><td>IL-3</td><td>F17</td><td>IL-18</td></tr>
<tr><td>F6</td><td>IL-4</td><td>F18</td><td>G–CSF</td></tr>
<tr><td>F7</td><td>IL-5</td><td>F19</td><td>GM–CSF</td></tr>
<tr><td>F8</td><td>IL-6</td><td>F20</td><td>M–CSF</td></tr>
<tr><td>F9</td><td>IL-7</td><td>F21</td><td>IFN–gamma</td></tr>
<tr><td>F10</td><td>IL-8</td><td>F22</td><td>LT–beta</td></tr>
<tr><td>F11</td><td>IL-10</td><td>F23</td><td>TGF–beta</td></tr>
<tr><td>F12</td><td>IL-12p35</td><td>F24</td><td>TNF–alpha</td></tr>
<tr><td>F13</td><td>IL-12p40</td><td>F25</td><td>TNF–beta</td></tr>
</table>
</td></tr>
</table>

To create a Median ΔC_T table: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>3</td><td>Click and type the following equations into the designated cells:

<table>
<tr><th>Cell</th><th>Type</th><th>Cell</th><th>Type</th></tr>
<tr><td>G2</td><td>=MEDIAN(D2:D5)</td><td>G14</td><td>=MEDIAN(D50:D53)</td></tr>
<tr><td>G3</td><td>=MEDIAN(D6:D9)</td><td>G15</td><td>=MEDIAN(D54:D57)</td></tr>
<tr><td>G4</td><td>=MEDIAN(D10:D13)</td><td>G16</td><td>=MEDIAN(D58:D61)</td></tr>
<tr><td>G5</td><td>=MEDIAN(D14:D17)</td><td>G17</td><td>=MEDIAN(D62:D65)</td></tr>
<tr><td>G6</td><td>=MEDIAN(D18:D21)</td><td>G18</td><td>=MEDIAN(D66:D69)</td></tr>
<tr><td>G7</td><td>=MEDIAN(D22:D25)</td><td>G19</td><td>=MEDIAN(D70:D73)</td></tr>
<tr><td>G8</td><td>=MEDIAN(D26:D29)</td><td>G20</td><td>=MEDIAN(D74:D77)</td></tr>
<tr><td>G9</td><td>=MEDIAN(D30:D33)</td><td>G21</td><td>=MEDIAN(D78:D81)</td></tr>
<tr><td>G10</td><td>=MEDIAN(D34:D37)</td><td>G22</td><td>=MEDIAN(D82:D85)</td></tr>
<tr><td>G11</td><td>=MEDIAN(D38:D41)</td><td>G23</td><td>=MEDIAN(D86:D89)</td></tr>
<tr><td>G12</td><td>=MEDIAN(D42:D45)</td><td>G24</td><td>=MEDIAN(D90:D93)</td></tr>
<tr><td>G13</td><td>=MEDIAN(D46:D49)</td><td>G25</td><td>=MEDIAN(D94:D97)</td></tr>
</table></td></tr>
<tr><td>4</td><td>Click cell G1 and type MedianΔCt.</td></tr>
<tr><td>5</td><td>Compare the ΔC_Ts of the targets in the spreadsheet to the values in the table below.

The card is performing properly if the designated targets achieve the following ΔC_T values:

<table>
<tr><th>Target</th><th>ΔC_T</th></tr>
<tr><td>IL-10</td><td>Below 20</td></tr>
<tr><td>Lymphotoxin-β</td><td>Below 20</td></tr>
<tr><td>TGF-β</td><td>Below 17</td></tr>
<tr><td>TNF-α</td><td>Below 20</td></tr>
<tr><td>TNF-β</td><td>Below 17</td></tr>
</table></td></tr>
</table>

Contacting Technical Support

C

Technical Support

Contacting Technical Support

You can contact Applied Biosystems for technical support:

- By e-mail
- By telephone or fax
- Through the Applied Biosystems web site

You can order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents 24 hours a day. In addition, you can download documents in PDF format from the Applied Biosystems web site. (Please see the section “To Obtain Technical Documents” following the telephone information below)

To Reach Technical Support by E-Mail

To contact Applied Biosystems Technical Support by e-mail for help in the following product areas:

Product/Product Area	E-mail address
Genetic Analysis (DNA Sequencing)	galab@appliedbiosystems.com
Sequence Detection Systems and PCR	pcrlab@appliedbiosystems.com
Protein Sequencing, Peptide, and DNA Synthesis	corelab@appliedbiosystems.com

Product/Product Area	E-mail address
Biochromatography PerSeptive DNA, PNA and Peptide Synthesis systems FMAT™ 8100 HTS System CytoFluor® 4000 Fluorescence Plate Reader Mariner™ Mass Spectrometers Voyager™ Mass Spectrometers MassGenotyping Solution 1™ (MGS1) System	tsupport@appliedbiosystems.com
LC/MS (Applied Biosystems/MDS Sciex)	support@sciex.com
Chemiluminescence (Tropix)	tropix@appliedbiosystems.com

To Reach Technical Support by Phone or Fax (N. America)

To contact Applied Biosystems Technical Support in North America, use the telephone or fax numbers in the table below.

Note To schedule a service call for other support needs, or in case of an emergency, dial **1.800.831.6844**, then press **1**.

Product/Product Area	Telephone	Fax
ABI PRISM® 3700 DNA Analyzer	**1.800.831.6844**, then press **8**[a]	1.650.638.5981
DNA Synthesis	**1.800.831.6844**, press **2**, then press **1**[a]	1.650.638.5981
Fluorescent DNA Sequencing	**1.800.831.6844**, press **2**, then press **2**[a]	1.650.638.5981
Fluorescent Fragment Analysis (including GeneScan® applications)	**1.800.831.6844**, press **2**, then press **3**[a]	1.650.638.5981
Integrated Thermal Cyclers (ABI PRISM® 877 and Catalyst 800 instruments)	**1.800.831.6844**, press **2**, then press **4**[a]	1.650.638.5981
ABI PRISM® 3100 Genetic Analyzer	**1.800.831.6844**, press **2**, then press **6**[a]	1.650.638.5981

Product/Product Area	Telephone	Fax
Peptide Synthesis (433 and 43x Systems)	**1.800.831.6844**, press **3**, then press **1**[a]	1.650.638.5981
Protein Sequencing (Procise® Protein Sequencing Systems)	**1.800.831.6844**, press **3**, then press **2**[a]	1.650.638.5981
PCR and Sequence Detection	**1.800.762.4001**, then press: **1** for PCR[a] **2** for TaqMan® applications and Sequence Detection Systems including ABI Prism, 7700, 7900, and 5700[a] **6** for the 6700 Automated Sample Prep System[a] or **1.800.831.6844**, then press **5**[a]	1.240.453.4613
Voyager™ MALDI-TOF Biospectrometry Workstations Mariner™ ESI-TOF Mass Spectrometry Workstations MassGenotyping Solution 1™ (MGS1) System	**1.800.899.5858**, press **1**, then press **3**[b]	1.508.383.7855
Biochromatography (BioCAD®, SPRINT™, VISION™, and INTEGRAL® Workstations and POROS® Perfusion Chromatography Products)	**1.800.899.5858**, press **1**, then press **4**[b]	1.508.383.7855
Expedite™ Nucleic Acid Synthesis Systems	**1.800.899.5858**, press **1**, then press **5**[b]	1.508.383.7855
Peptide Synthesis (Pioneer™ and 9050 Plus Peptide Synthesizers)	**1.800.899.5858**, press **1**, then press **5**[b]	1.508.383.7855

Product/Product Area	Telephone	Fax
PNA Custom and Synthesis	**1.800.899.5858**, press **1**, then press **5**[b]	1.508.383.7855
FMAT™ 8100 HTS System CytoFluor® 4000 Fluorescence Plate Reader	**1.800.899.5858**, press **1**, then press **6**[b]	1.508.383.7855
Chemiluminescence (Tropix)	**1.800.542.2369** (U.S. only), or **1.781.271.0045**[c]	1.781.275.8581
LC/MS (Applied Biosystems/MDS Sciex)	**1.800.952.4716**	1.508.383.7899

a. 5:30 AM to 5:00 PM Pacific time.

b. 8:00 AM to 6:00 PM Eastern time.

c. 9:00 AM to 5:00 PM Eastern time.

To Reach Technical Support by Phone or Fax (Outside N. America)

To contact Applied Biosystems Technical Support or Field Service outside North America, use the telephone or fax numbers below.

Region	Telephone	Fax
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Australia (Scoresby, Victoria)	**61 3 9730 8600**	**61 3 9730 8799**
China (Beijing)	**86 10 64106608** or **86 800 8100497**	**86 10 64106617**
Hong Kong	**852 2756 6928**	**852 2756 6968**
India (New Delhi)	**91 11 653 3743/3744**	**91 11 653 3138**
Korea (Seoul)	**82 2 593 6470/6471**	**82 2 593 6472**
Malaysia (Petaling Jaya)	**60 3 79588268**	**60 3 79549043**
Singapore	**65 896 2168**	**65 896 2147**
Taiwan (Taipei Hsien)	**886 2 2358 2838**	**886 2 2358 2839**
Thailand (Bangkok)	**66 2 719 6405**	**66 2 319 9788**
Europe		

Region	Telephone	Fax
Austria (Wien)	**43 (0)1 867 35 75 0**	**43 (0)1 867 35 75 11**
Belgium	**32 (0)2 532 4484**	**32 (0)2 582 1886**
Denmark (Naerum)	**45 45 58 60 00**	**45 45 58 60 01**
Finland (Espoo)	**358 (0)9 251 24 250**	**358 (0)9 251 24 243**
France (Paris)	**33 (0)1 69 59 85 85**	**33 (0)1 69 59 85 00**
Germany (Weiterstadt)	**49 (0)6150 101 0**	**49 (0)6150 101 101**
Italy (Milano)	**39 (0)39 83891**	**39 (0)39 838 9492**
Norway (Oslo)	**47 23 12 06 05**	**47 23 12 05 75**
Portugal (Lisboa)	**351.(0)22.605.33.14**	**351.(0)22.605.33.15**
Spain (Tres Cantos)	**34.(0)91.806.1210**	**34.(0)91.806.12.06**
Sweden (Stockholm)	**46 (0)8 619 4400**	**46 (0)8 619 4401**
Switzerland (Rotkreuz)	**41 (0)41 799 7777**	**41 (0)41 790 0676**
The Netherlands (Nieuwerkerk a/d IJssel)	**31 (0)180 392400**	**31 (0)180 392409** or **31 (0)180 392499**
United Kingdom (Warrington, Cheshire)	**44 (0)1925 825650**	**44 (0)1925 282502**

European Managed Territories (EMT)		
Africa, English speaking (Johannesburg, South Africa)	**27 11 478 0411**	**27 11 478 0349**
Africa, French speaking (Paris, France)	**33 1 69 59 85 11**	**33 1 69 59 85 00**
India (New Delhi)	**91 11 653 3743** **91 11 653 3744**	**91 11 653 3138**
Poland, Lithuania, Latvia, and Estonia (Warszawa)	**48 22 866 40 10**	**48 22 866 40 20**
For all other EMT countries not listed (Central and southeast Europe, CIS, Middle East, and West Asia)	**44 1925 282481**	**44 1925 282509**
Japan		

Region	Telephone	Fax
Japan (Hacchobori, Chuo-Ku, Tokyo)	**81 3 5566 6230**	**81 3 5566 6507**
Latin America		
Caribbean countries, Mexico, and Central America	**52 55 35 3610**	**52 55 66 2308**
Brazil	**0 800 704 9004** or **55 11 5070 9654**	**55 11 5070 9694/95**
Argentina	**800 666 0096**	**55 11 5070 9694/95**
Chile	**1230 020 9102**	**55 11 5070 9694/95**
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To Reach Technical Support Via the Applied Biosystems Web Site

To contact Technical Support 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, then click **Frequently Asked Questions**.
3	Click **Contact Support** in the contents list at the left of the screen.
4	Click your geographic region for the product area of interest.
5	In the Personal Assistance form, enter the requested information and your question, then click **Ask Us RIGHT NOW**.
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You can obtain technical documents, such as Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents for free, 24 hours a day. You can obtain documents:

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

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2	Click **SERVICES & SUPPORT** at the top of the page, then click **Documents on Demand**.
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4	In the results screen, do any of the following: ♦ Click the pdf icon to view a PDF version of the document. ♦ Right-click the pdf icon, then select **Save Target As** to download a copy of the PDF file. ♦ Select the **Fax** check box, then click **Deliver Selected Documents Now** to have the document faxed to you. ♦ Select the **Email** check box, then click **Deliver Selected Documents Now** to have the document (PDF format) e-mailed to you. **Note** There is a limit of five documents per fax request, but no limit on the number of documents per e-mail request.

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Step	Action
1	Go to **http://www.appliedbiosystems.com**
2	Click **SERVICES & SUPPORT** at the top of the page, then click **Training**.

References

D

Förster, V.T. 1948. Zwischenmolekulare Energiewanderung und Fluoreszenz. *Annals of Physics (Leipzig)* 2:55–75.

Holland, P.M., Abramson, R.D., Watson, R., and Gelfand, D.H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5´Æ3´ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA 88:7276–7280.

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

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

Lakowicz, J.R. 1983. *Principles of Fluorescence Spectroscopy.* New York: Plenum Press. xiv, 496 pp.

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

Mullis, K.B., and Faloona, F.A. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155:335–350.

Saiki, R.K., Scharf, S., Faloona, F., et al. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–1354.

Limited Warranty Statement

E

Applied Biosystems warrants to the customer that, for a period ending on the earlier of one year from the completion of installation or fifteen (15) months from the date of shipment to the customer (the “Warranty Period”), the TaqMan® Human Cytokine Card Upgrade Package purchased by the customer (the “Instrument”) will be free from defects in material and workmanship, and will perform in accordance with the installation specifications set forth in the ABI PRISM® 7700 and TaqMan Card Upgrade Installation Manual (the “Specifications”).

During the Warranty Period, if the Instrument's hardware becomes damaged or contaminated or it the Instrument otherwise fails to meet the Specifications, Applied Biosystems will repair or replace the Instrument so that it meets the Specifications, at Applied Biosystems expense. However, if the ABI PRISM® Card Filling Station or Card Adaptor become damaged or contaminated or if the performance of the Instrument otherwise deteriorates due to consumables and/or reagents other than those supplied or expressly recommended by Applied Biosystems, Applied Biosystems will return the Instrument to Specification at the customer's request and at the customer's expense. After this service is performed, coverage of the parts repaired or replaced will be restored thereafter for the remainder of the original Warranty Period.

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