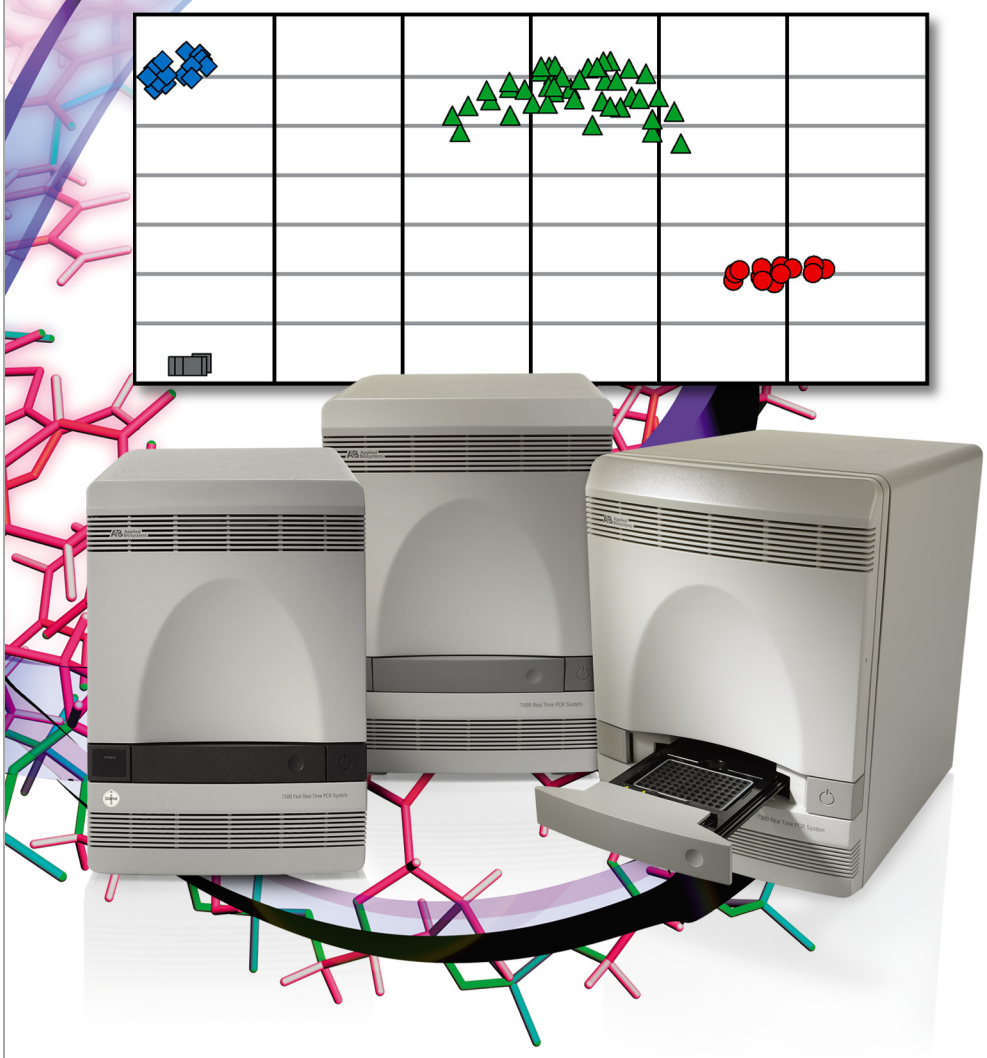
Applied Biosystems

Allelic Discrimination Getting Started Guide



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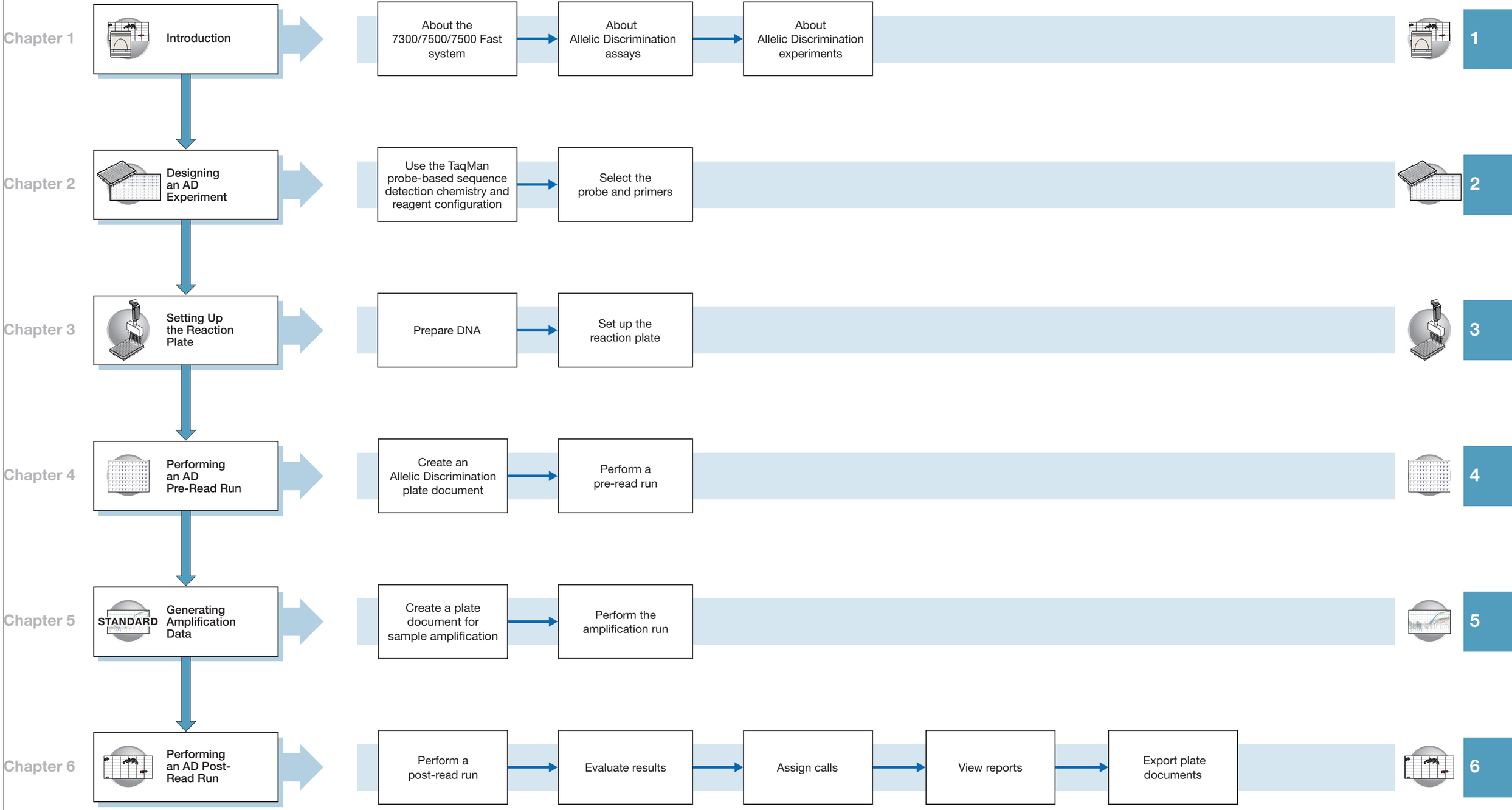
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Preface

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Purpose of This Guide

This manual is written for principal investigators and laboratory staff who conduct allelic discrimination assays using the Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System (7300/7500/7500 Fast system).

Assumptions

This guide assumes that you have:

- Familiarity with Microsoft® Windows® XP operating system.
- Knowledge of general techniques for handling DNA samples and preparing them for PCR.
- A general understanding of hard drives and data storage, file transfers, and copying and pasting.
- Networking experience, if you want to integrate the 7300/7500/7500 Fast system into your existing laboratory data flow system.

Text Conventions

This guide uses the following conventions:

- **Bold** indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow bracket (>) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File > Open > Spot Set**.

User Attention Words

The following user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note – Provides information that may be of interest or help but is not critical to the use of the product.

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

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

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

Safety

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

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

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 24 hours a day.

To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
    - **Open** – To view the document
    - **Print Target** – To print the document
    - **Save Target As** – To download a PDF version of the document to a destination that you choose
4. To have a copy of a document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.

After you enter the required information, click **View/Deliver Selected Documents Now**.

Refer to the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Getting Started Guide* and the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Site Preparation Guide* for important safety information.

How to Obtain More Information

Related Documentation

For more information about using the 7300/7500/7500 Fast system, refer to the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Online Help* or the documents shown below.

Document Title	Online Help P/N	P/N
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Plus/Minus Detection Getting Started Guide	4347821	4378652
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantification Getting Started Guide	4347824	4378655
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4347825	4378656
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Site Preparation Guide	4347823	4378654
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide	4347828	4378657
Real-Time PCR Systems Chemistry Guide	4348358	4378658
Applied Biosystems 7500 FAST Real-Time PCR System, QRC	4362285	4378659
Applied Biosystems Real-Time System Computer Set Up Guide, QRC	4365367	4378660

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techpubs@appliedbiosystems.com

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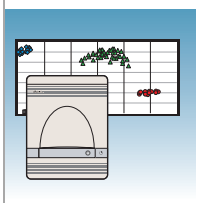
For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

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



Introduction

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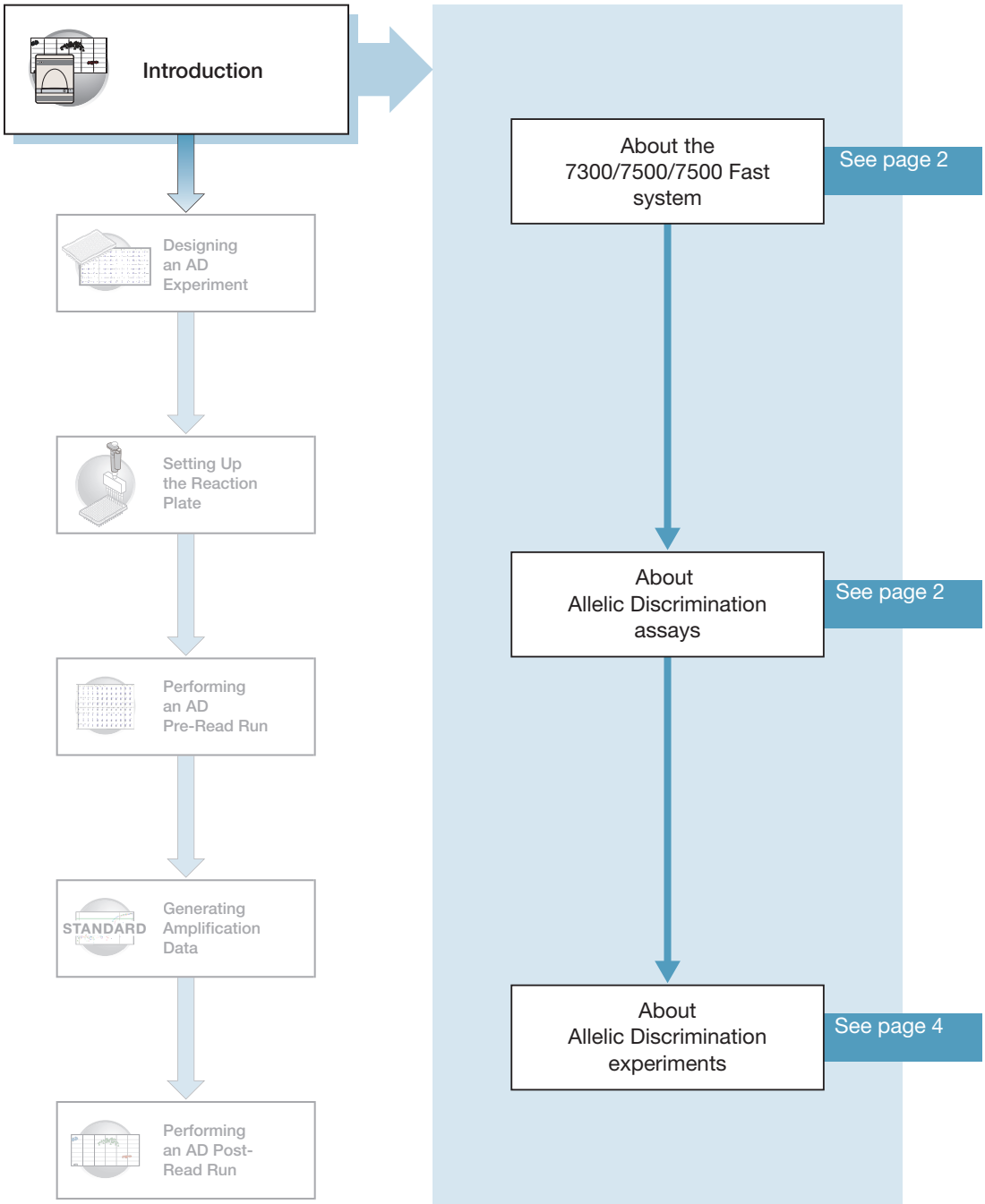

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About the 7300/7500/7500 Fast System

Description

The Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System (7300/7500/7500 Fast system) uses fluorescent-based PCR chemistries to provide:

- Quantitative detection of nucleic acid sequences using real-time analysis.
- Qualitative detection of nucleic acid sequences using end-point and dissociation-curve analysis.

Allelic Discrimination Assay

The 7300/7500/7500 Fast system allows you to perform a number of assay types using plates in the 96-well format. This guide describes the allelic discrimination (AD) assay.

Note: For information about the other assay types, refer to the *Applied Biosystems Real-Time PCR Systems Chemistry Guide* and the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Online Help* (Online Help).

Note: Allelic Discrimination Assays may be run on a 7500 Fast system using standard reagents; Allelic Discrimination Assays are not supported using Fast reagents and protocols.

About Allelic Discrimination (AD) Assays

Definition

An allelic discrimination (AD) assay is a multiplexed (more than one primer/probe pair per reaction), end-point (data is collected at the end of the PCR process) assay that detects variants of a single nucleic acid sequence. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants at the single-nucleic polymorphism (SNP) site in a target template sequence. The actual quantity of target sequence is not determined.

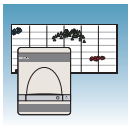
For each sample in an AD assay, a unique pair of fluorescent dye detectors is used, for example, two TaqMan® MGB probes that target an SNP site. One fluorescent dye detector is a perfect match to the wild type (allele 1) and the other fluorescent dye detector is a perfect match to the mutation (allele 2).

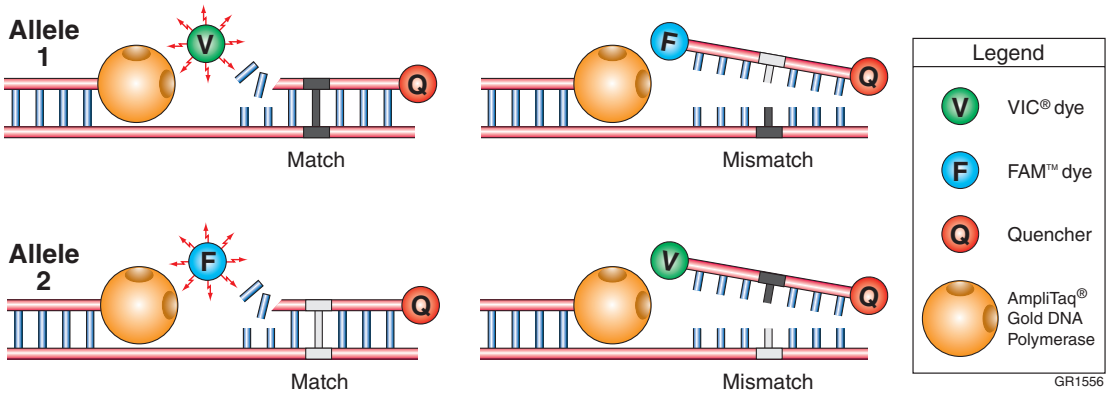
The Allelic Discrimination assay classifies unknown samples as:

- Homozygotes (samples having only allele 1 or allele 2)
- Heterozygotes (samples having both allele 1 and allele 2)

The AD assay measures the change in fluorescence of the dyes associated with the probes. The figure on the next page illustrates results from matches and mismatches between target and probe sequences in TaqMan® Gene Expression Assays (Livak *et al.,* 1995).

Notes





The table below shows the correlation between fluorescence signals and sequences in the sample.

A substantial increase in…	Indicates…
VIC® dye fluorescence only	Homozygosity for allele 1
FAM™ dye fluorescence only	Homozygosity for allele 2
Both fluorescence signals	Heterozygosity allele 1-allele 2

Terms Used in AD Analysis

Term	Definition
No template control (NTC)	A sample that does not contain template. Shows background signal and is used as the negative control. Provides a means of measuring contamination that might give a false positive signal.
Nucleic acid target (target template or target)	Nucleotide sequence that you want to genotype.
Unknown sample (sample of interest)	The sample for which you want to determine the genotype a specific target.
Passive reference	A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations caused by changes in concentration or in volume.
Reporter dye	The dye attached to the 5′ end of a TaqMan® probe. Provides a fluorescence signal that indicates specific amplification.
Normalized reporter (R_n)	The ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye.

Notes

About AD Experiments

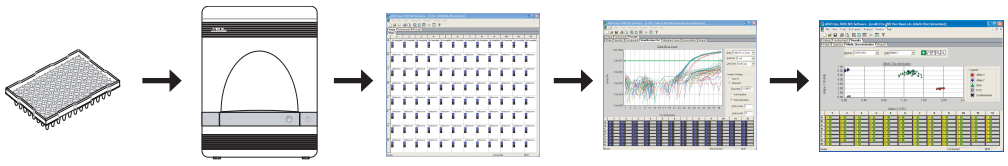
AD Experiment Workflow

This document uses the term "AD experiment" to refer to the entire process of analyzing samples of extracted DNA from data collected at the end of the PCR process.

After you design the experiment and isolate DNA, an AD assay involves performing:

- **A pre-read run** on an AD plate document to determine the baseline fluorescence associated with primers and probes before amplification.
- **An amplification run** using an AQ plate document to generate real-time PCR data, which can be used to analyze and troubleshoot the PCR data for the AD assay, if needed.
- **A post-read run** using the original AD plate document. The post-read run automatically subtracts the baseline fluorescence determined during the pre-read run, then assigns allele calls (automatically or manually) using the amplified data.

The following figure illustrates the complete process.

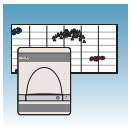


Reaction Plate | 7300/7500/7500 Fast System | Pre-Read Run | Amplification Run | Post-Read Run

Required User-Supplied Materials

Item	Source
Any of the following DNA isolation and purification chemistry systems:	
• ABI PRISM® 6100 Nucleic Acid PrepStation	Applied Biosystems (PN 6100-01)
• BloodPrep™ Chemistry (genomic DNA from fresh or frozen blood)	Applied Biosystems (PN 4346860)
• NucPrep® Chemistry (DNA from animal and plant tissue)	Applied Biosystems (PN 4340274)
TaqMan® Reagents appropriate for your probes and primers:	
• For TaqMan® Gene Expression Assays: TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 200 reactions	Applied Biosystems (PN 4324018)
• For Custom probes/primer design with Primer Express®: TaqMan® Universal PCR Master Mix	Applied Biosystems (PN 4304437)

Notes



Item	Source
• For Custom TaqMan® Gene Expression Assays: – TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 200 reactions – TaqMan® Universal PCR Master Mix	 • Applied Biosystems (PN 4324018) • Applied Biosystems (PN 4304437)
Labeled primers and probes from one of the following sources: • TaqMan® Gene Expression Assays (predesigned primers and probes) • Custom TaqMan® Gene Expression Assays (predesigned primers and probes) • Primer Express® Software (custom-designed primers and probes)	 • Applied Biosystems Web site • Contact your Applied Biosystems Sales Representative • PN 4330710 (1-user license) PN 4330709 (10-user license) PN 4330708 (50-user license)
MicroAmp® Optical 96-Well Reaction Plate Optical Adhesive Cover	Applied Biosystems (PN 4306757) Applied Biosystems (PN 4311971)
Reagent tubes with caps, 10-mL	Applied Biosystems (PN 4305932)
Centrifuge with adapter for 96-well plates	Major laboratory supplier (MLS)
Gloves	MLS
Microcentrifuge	MLS
Microcentrifuge tubes, sterile 1.5-mL	MLS
Nuclease-free water	MLS
Pipette tips, with filter plugs	MLS
Pipettors, positive-displacement	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Vortexer	MLS

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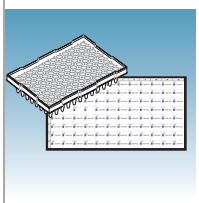
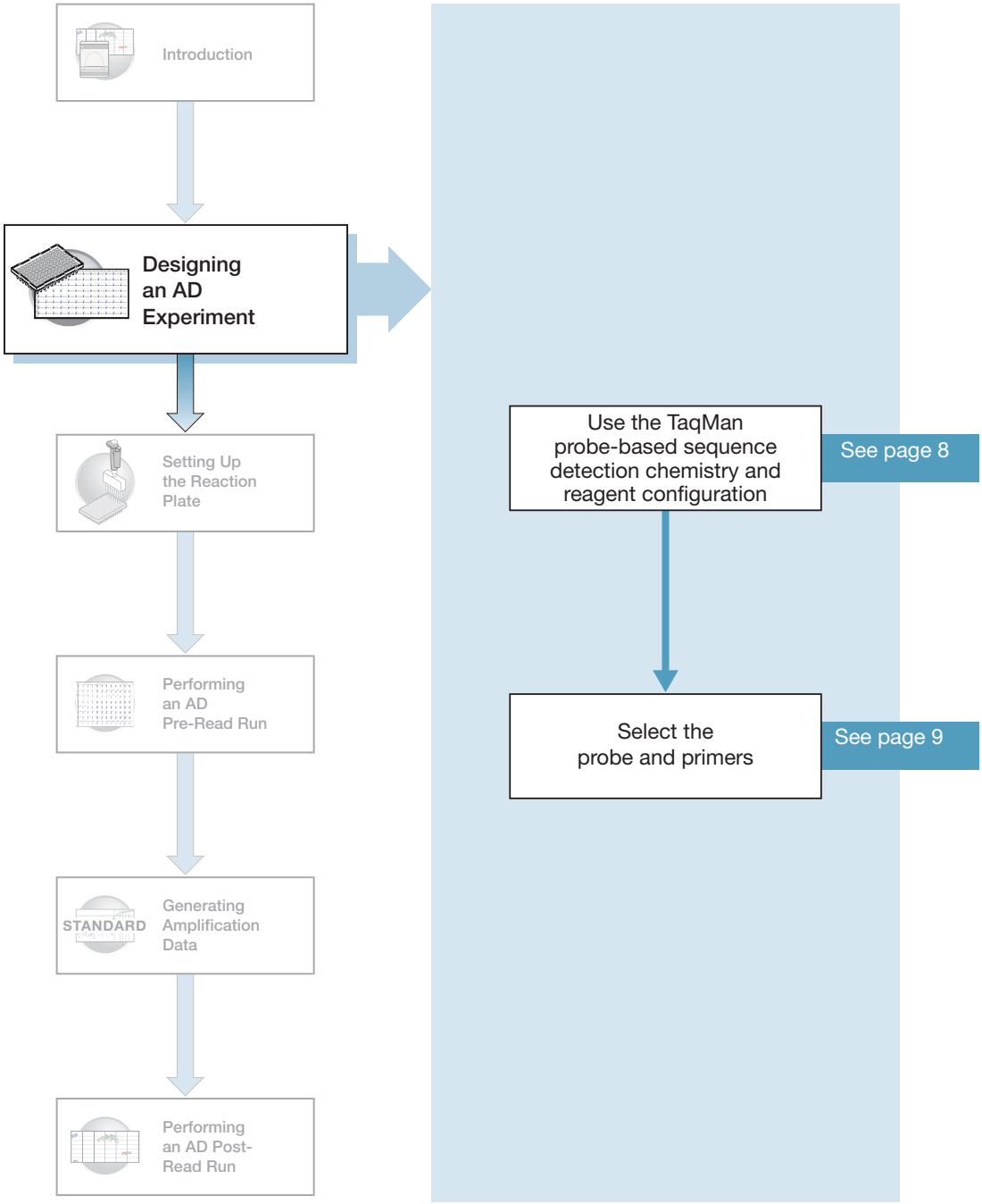


Designing an AD Experiment



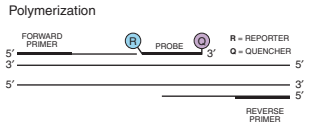
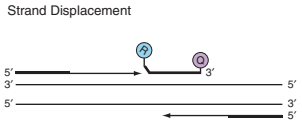
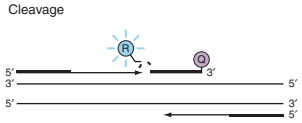
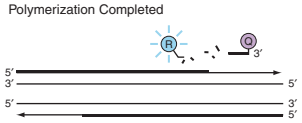
Notes

Using TaqMan® Probe-based Reagent Configuration

About the Chemistry

AD assays use the fluorogenic 5′ nuclease chemistry (also known as TaqMan® probe-based chemistry).

Note: The SYBR® Green I dye chemistry is not supported for AD assays.

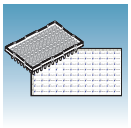
Chemistry	Process
TaqMan® reagents or kits **Description** TaqMan® reagent-based chemistry uses a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles.	 **Step 1**: A reporter (R) and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe.  **Step 1** (continued): When both dyes are attached to the probe, reporter dye emission is quenched.  **Step 2**: During each extension cycle, the AmpliTaq Gold® DNA polymerase cleaves the reporter dye from the probe.  **Step 3**: After being separated from the quencher, the reporter dye emits its characteristic fluorescence.

For more information about the TaqMan probe-based chemistries, refer to the *Real-Time PCR Systems Chemistry Guide*.

Chemistry Kits for Allelic Discrimination

- **For TaqMan® Gene Expression Assays** – TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 200 reactions (PN 4324018)
- **For custom probes/primers designed with Primer Express® Software** – TaqMan® Universal PCR Master Mix (PN 4304437)
- **For Custom TaqMan® Gene Expression Assays** – TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 200 reactions (PN 4324018) or TaqMan® Universal PCR Master Mix (PN 4304437)

Notes



Selecting the Probes and Primers

You must select primer/probe sets for both target sequences (one for allele 1 and the other for allele 2). Applied Biosystems provides three options for selecting probes and primers:

- **TaqMan® Gene Expression Assays** – Provide biologically informative, fully validated, QC tested, TaqMan® probe-based assays for genotyping single nucleotide polymorphisms (SNPs). For information on available primer/probe sets, go to:

  http://www.appliedbiosystems.com, then click the **TaqMan® Gene Expression Assays** link in the right column.
- **Custom TaqMan® Gene Expression Assays** – Designs, synthesizes, formulates, and delivers quality-controlled primer and probe sets. Use this service if the assay you need is not currently available. To place an order, contact your Applied Biosystems representative.
- **Primer Express® Software** – Helps you design primers and probes for your own assays. For more information about using this software, refer to the *Primer Express Software v3.0 Getting Started Guide* (PN 4362460).

  Applied Biosystems provides Assay Design Guidelines, which have been developed specifically for quantification assays (pertinent to the amplification step in AD assays). When used in their entirety, these steps provide a rapid and reliable system for assay design and optimization. For information about the Assay Design Guidelines, refer to the *Real-Time PCR Systems Chemistry Guide*.

Sample Experiment

In the example AD experiment, the genotype of the ApoE gene associated with lipoproteinemia is determined in DNA isolated from blood. Possible genotypes are AA, AG, and GG.

Two primer and probe pairs are used in each reaction to genotype the two possible variants at the SNP site in the target sequence.

Primers and probes for the example experiment are ordered from TaqMan Gene Expression Assays (AB Assay ID C 3084818 10). The probe for allele A is labeled with FAM™ dye; the probe for allele G is labeled with VIC™ dye.

Notes

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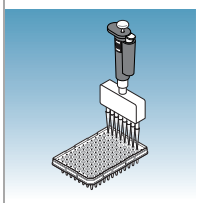
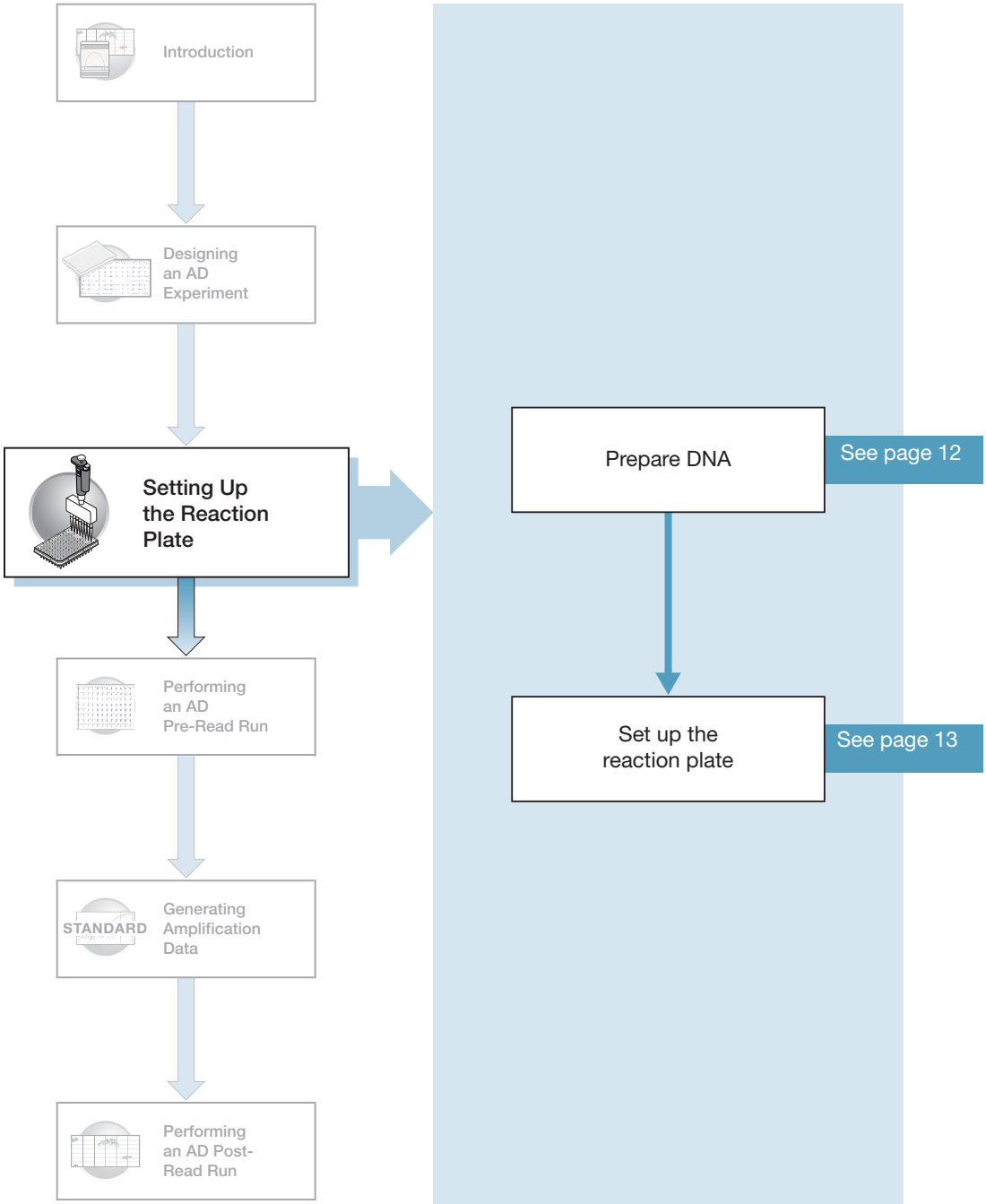


Setting Up the Reaction Plate



Notes

Preparing DNA

Systems and Chemistries for DNA Isolation

Applied Biosystems supplies several instrument systems and chemistries for DNA isolation from a variety of starting materials, such as blood, tissue, cell cultures, and plant material.

System	Part Number
BloodPrep™ Chemistry (genomic DNA from fresh or frozen blood)	4346860
NucPrep® Chemistry (DNA from animal and plant tissue)	4340274
ABI PRISM® 6100 Nucleic Acid PrepStation	6100-01

For more information, refer to:

- *DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol* (PN 4343586)
- *NucPrep® Chemistry: Isolation of Genomic DNA from Animal and Plant Tissue: Protocol* (PN 4333959)

Quality of DNA

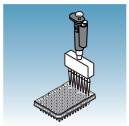
Ensure that the DNA you use for the AD experiments:

- Is extracted from the raw material you are testing with an optimized protocol
- Does not contain PCR inhibitors
- Has an $A_{260/280}$ ratio greater than 1.7
- Is intact as visualized by gel electrophoresis
- Has not been heated above 60 °C; heat can cause degradation

Sample Experiment

Genomic DNA for the example AD experiment is isolated from blood using a BloodPrep™ Chemistry Kit. The recommended template for TaqMan® Gene Expression Assays is purified genomic DNA (1 to 20 ng). The final concentration of genomic DNA for all samples in the example experiment is 10 ng/µL.

Notes



Setting Up the Reaction Plate

This section describes how to set up the 96-well reaction plate for an AD assay with samples and reaction mix.

A reaction plate contains the following for an AD assay:

- No Template Controls (NTCs)
- Known genomic DNA controls (optional, not included in example experiment)
- Unknown genomic DNA samples

Preparing the Reaction Mix for Custom TaqMan Gene Expression Assays

If you use the Primer Express Software to design probes and primers for your SNP genotyping assay, follow instructions in the *TaqMan Universal PCR Master Mix Protocol* (PN 4304449) and the *Real-Time PCR Systems Chemistry Guide* to optimize primer and probe concentrations. If you obtain your assay from the Custom TaqMan® Gene Expression Assays service, follow instructions in the *Assays-by-Design Service For SNP Assays Protocol* (PN 4334431).

Preparing the Reaction Mix for TaqMan Gene Expression Assays

The AD reaction mix contains:

- SNP Genotyping Assay Mix
- TaqMan® Universal PCR Master Mix (No AmpErase® UNG)
- Nuclease-free water

IMPORTANT! Do not use TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG.

The recommended reaction size is 25 µL for a 96-well setup.

The instructions below are excerpted from the *TaqMan® Gene Expression Assays Protocol* (PN 4332856), for wet DNA samples.

Note: If you are using dried-down DNA samples, refer to the *TaqMan® Gene Expression Assays Protocol* for instructions on preparing the reaction mix.

Preparing the Reaction Mix

1. Calculate the number of reactions to be performed for each assay.

   Note: Include at least two NTCs and optional known genomic DNA controls on each reaction plate for optimal performance of TaqMan® Gene Expression Assays.

Notes

2. Calculate the volume of components needed for all wells on the reaction plate:

Component	Volume (µL/reaction)
2× TaqMan Universal PCR Master Mix, No AmpErase UNG	12.50
20× SNP Genotyping Assay Mix	1.25
Total	13.75

Note: Add extra reactions to provide excess volume for the loss that occurs during reagent transfers.

3. Swirl the bottle of 2× TaqMan Universal PCR Master Mix, No AmpErase UNG, gently to resuspend.

CAUTION **CHEMICAL HAZARD. TaqMan Universal PCR Master Mix (2×) No AmpErase UNG** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

4. Vortex and centrifuge the 20× SNP Genotyping Assay Mix briefly.

WARNING **CHEMICAL HAZARD. SNP Genotyping Assay Mix** contains **formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

5. Pipette the volumes required for all wells on the reaction plate (plus additional reactions to compensate for reagent transfer loss) of 2× TaqMan Universal PCR Master Mix (No AmpErase UNG), and 20× SNP Genotyping Assay Mix into a microcentrifuge tube. Cap the tube.

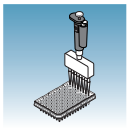
Sample Experiment

Volumes prepared for the example experiment:

Component	Volume for 106 Reactions‡
2× TaqMan Universal PCR Master Mix, No AmpErase UNG	1.33 mL
20× SNP Genotyping Assay Mix	132.5 µL
Total	1.46 mL

‡ Extra volume is included to account for pipetting losses.

Notes

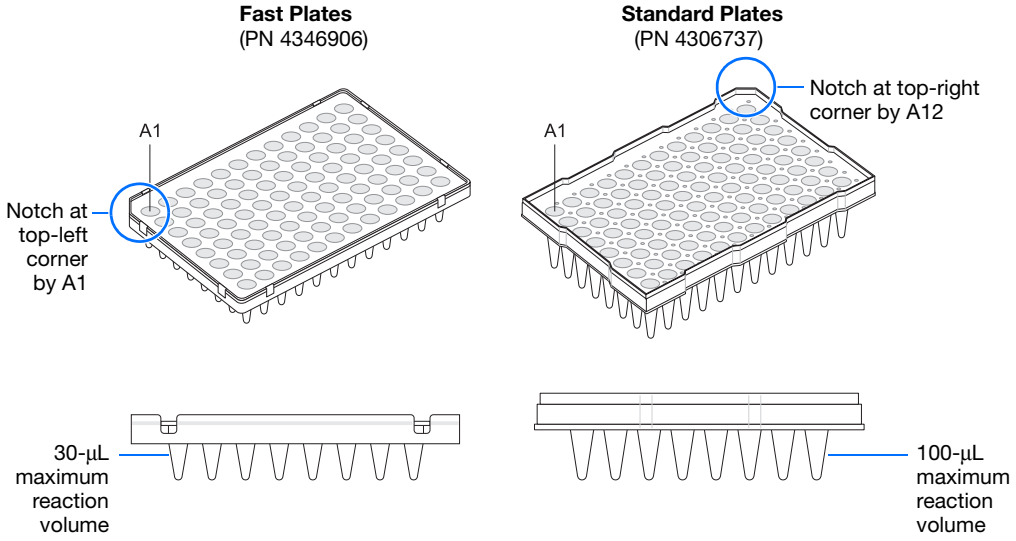


Preparing the Plate

Standard vs. Fast Plates

IMPORTANT! Ensure you use the standard Optical 96-Well Plate on the 7500 Real-Time PCR system. Optical 96-Well Fast Plates will *not* fit into the standard block correctly and will result in loss of data.

IMPORTANT! Ensure you use the Optical 96-Well Fast Plate on the 7500 Fast Real-Time PCR system. Standard plates will not function properly and may be crushed when using the 96-Well Fast Block.

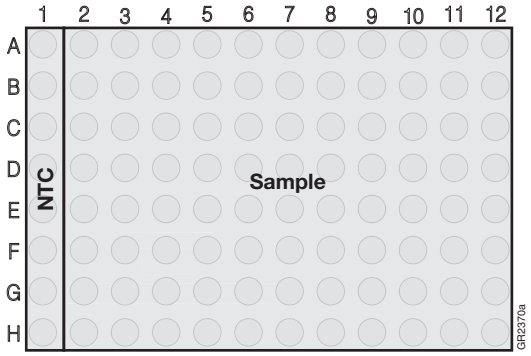


Notes

To prepare the plate:

1. Invert the reaction mix tube prepared in the previous section.
2. Centrifuge the tube briefly to spin down the contents and to eliminate air bubbles.
3. Pipette 13.75 µL of reaction mix into each well in a 96-well reaction plate.
4. Dilute 1 to 20 ng of each purified genomic DNA sample into nuclease-free water for a total sample volume of 11.25 µL.
5. Pipette 11.25 µL of the following solutions into the indicated wells:

Wells	Add
A1 through H1 (No Template Control)	Nuclease-free water or TE (Tris-EDTA) buffer
Remaining wells (Sample)	Diluted sample DNA



IMPORTANT! Use a calibrated, positive-displacement pipettor to minimize contamination and error. Change tips between samples to prevent cross-contamination.

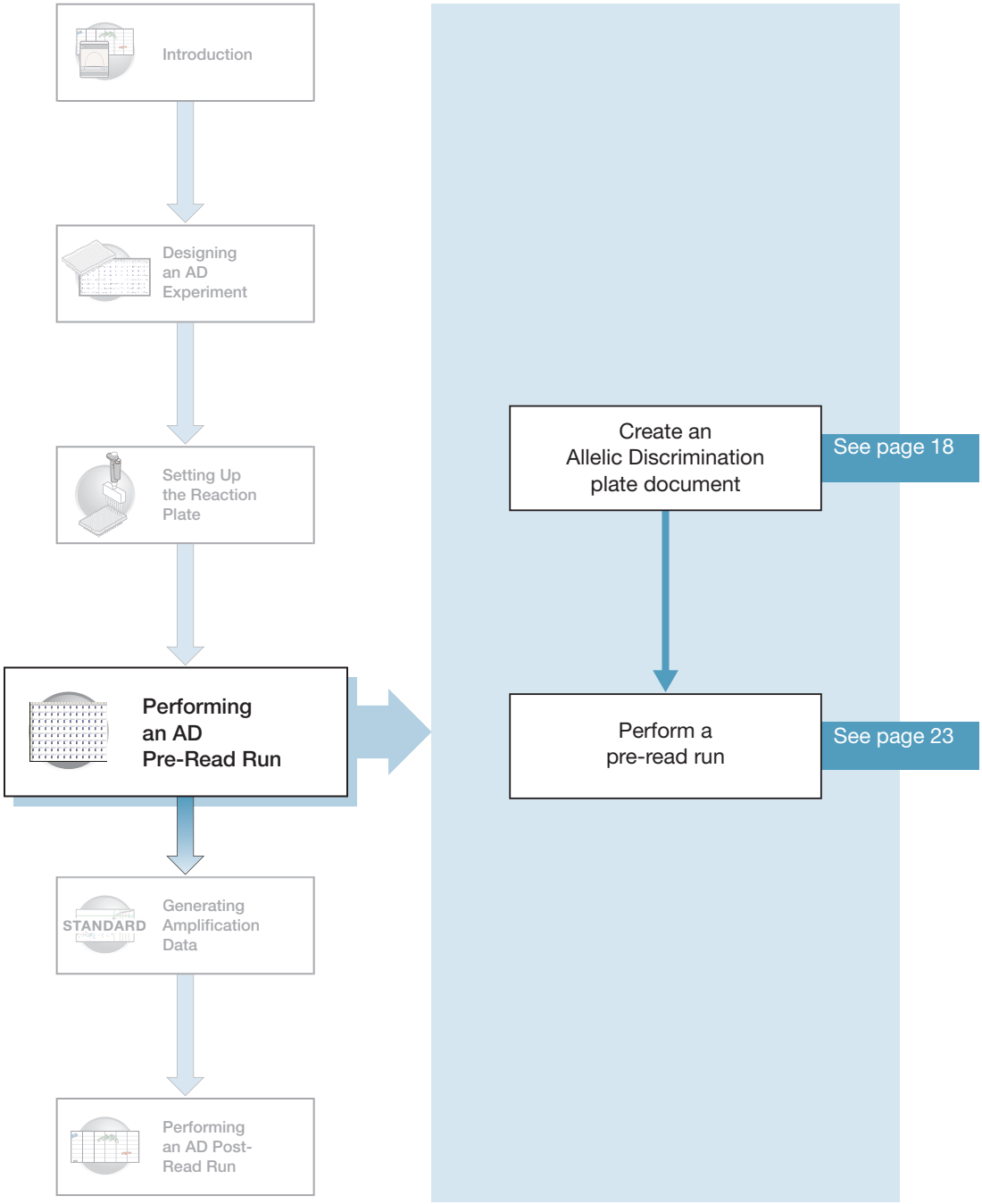
6. Cover the reaction plate with an optical adhesive cover or optical caps.
7. Keep the reaction plate on ice until loading in the 7300/7500/7500 Fast system.

Sample Experiment

The recommended template for TaqMan Gene Expression Assays is purified genomic DNA (1 to 20 ng). The final concentration of genomic DNA for all samples in the example experiment is 10 ng/µL.

Notes

Performing an AD Pre-Read Run



Notes

The Pre-Read Run

A pre-read run records the background fluorescence of each well of the AD plate document before PCR. During the post-read run, the pre-read fluorescence is subtracted from the post-read fluorescence to account for pre-amplification background fluorescence, ensuring accurate results.

Before You Begin

Check that background and pure-dye runs have been performed regularly to ensure optimal performance of the 7300/7500/7500 Fast system. For more information about calibrating the 7300/7500/7500 Fast system, refer to the Online Help.

Creating an Allelic Discrimination (AD) Plate Document

An AD plate document stores data collected from an AD run for a single reaction plate. An AD plate document also stores other information about the run, including sample names, markers, and detectors.

Detectors, Markers, and Tasks

AD plate documents use:

- **Detector** – In SDS Software 1.3.1, a virtual representation of a TaqMan® probe and primer set and associated fluorescent dye that detects a single target nucleic acid sequence.
- **Markers** – A set of two detectors that discriminate between different alleles of a common locus. Allele 1 is detected by one detector (for example, FAM™), and allele 2 is detected by the second detector (for example, VIC®).
- **Task** – A setting that you apply to the markers in a well of a plate document and that determines the way the SDS Software 1.3.1 uses the data collected from the well during analysis.

  AD plate document markers use two types of tasks:

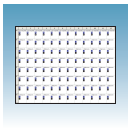
  – **Unknown** – Applied to markers of wells that contain PCR reagents for the amplification of target sequences. The SDS Software 1.3.1 indicates unknown targets with a U.

  – **No Template Control** – Applied to markers of wells that contain no target template. The SDS Software 1.3.1 indicates no template controls by an **NTC**.

Creating a New AD Plate Document

You can enter sample information into a new plate document, import sample information from existing plate documents, or use a template document to set up new plate documents. This section describes setting up new plate documents. Refer to the Online Help for information about importing sample information or using template documents.

Notes



To create a new AQ plate document:

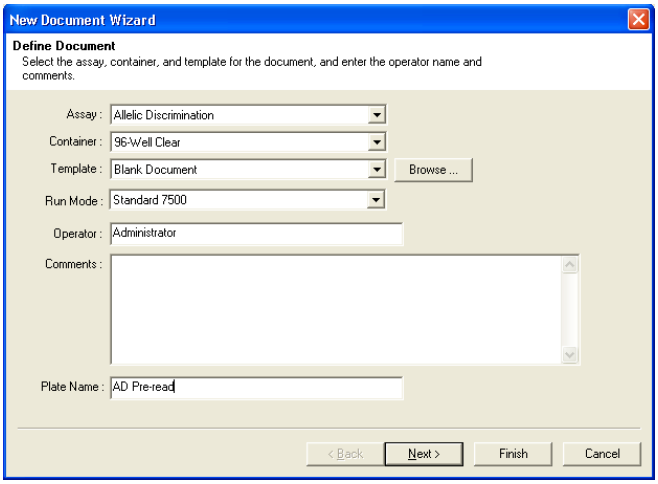
1. Select **Start > Programs > 7300/7500/7500 Fast System > 7300/7500/7500 Fast System Software** ( )to start the 7300/7500/7500 Fast system SDS Software 1.3.1.

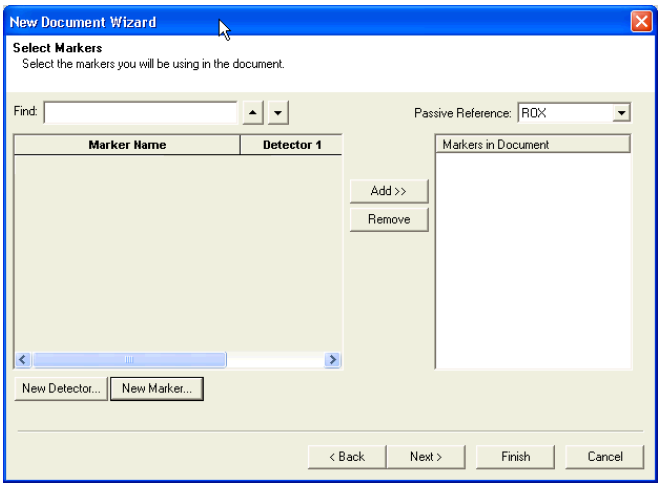
2. Select **File** > **New**.

3. In the New Document Wizard:
   a. Click the Assay drop-down list, then select **Allelic Discrimination**.
   b. Accept the default settings for the Container and Template fields (**96-Well Clear** and **Blank Document**).
   c. In the Plate Name field, type **AD Pre-Read.**

4. Click **Next>** to access the Select Markers page.

   If the Markers list in the Select Markers page contains a marker suitable for your application, skip to step 6.





Notes

5. If the Markers list does not contain a marker suitable for your application, create detectors and marker:

   a. Click **New Detector**.

   b. In the New Detector dialog box, type **Allele A** for Name.

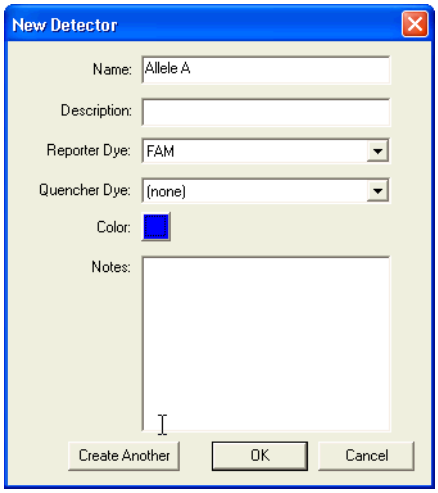
   c. Leave the Reporter Dye set to **FAM**.

   d. Click the color button, select blue, then click **OK**.

   e. Click **Create Another**.

   f. For Name, type **Allele G**.

   g. Select **VIC** for the Reporter Dye.



   Note: Select different Reporter dyes for the detectors. A marker (which you create next) cannot contain detectors with the same Reporter dye.

   h. Click the color button, select green, then click **OK**.

   Note: The names you assign to the detectors are displayed on the axes of the Allelic Discrimination plot in results and listed in the Call column in reports. It is good practice to assign the actual allele names to the detectors.

   For more information on creating detectors, see Appendix A, Creating Detectors.

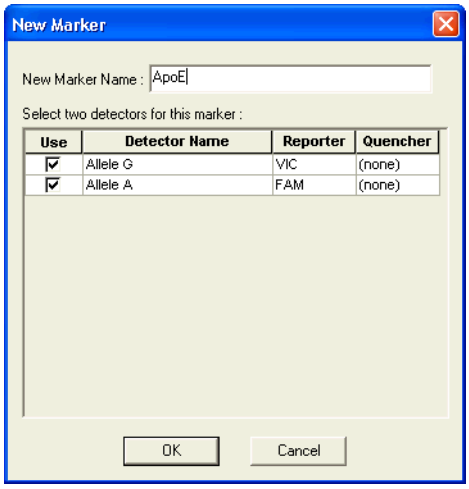
   i. Click **New Marker**.

   j. In the New Marker dialog box, type **ApoE** for Name.

   k. Select the **Allele A** and **Allele G** detectors you created above.

   l. Click **OK**.

   For more information on creating markers, see the Online Help.

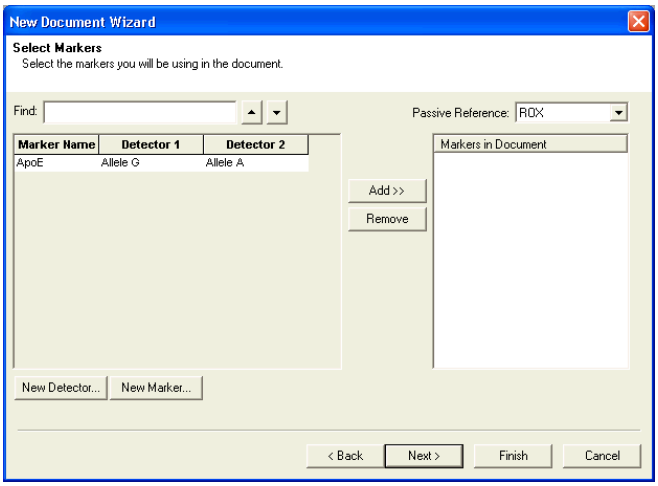


Notes

6. In the Select Markers window, select either the **ApoE** marker you created above or a suitable marker, then click **Add>>**.

   Note: To remove a marker, select it, then click **Remove**.

7. Click **Next>**.



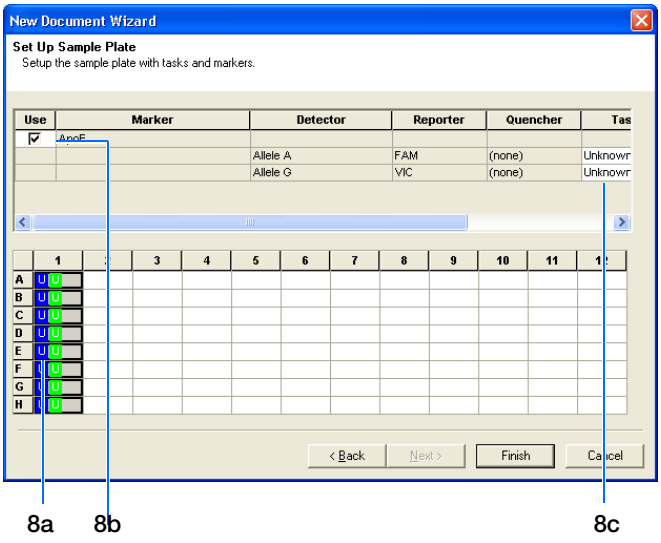
Sample Experiment

In the example AD experiment, detectors are named Allele A and Allele G and the marker is named ApoE. You can use appropriate names that represent the detectors and markers for your experiment.

8. In the Setup Sample Plate page, select the marker for wells:

   a. Click-drag to select wells **A1** through **H1**.
   b. Select the **Use** box for the marker.
   c. Click the Task field for one of the detectors, then select **NTC** for task.
   d. Select the remaining wells.
   e. Select the **Use** box for the marker. Leave the Task set to **Unknown**.

9. Click **Finish**.



Notes

10. Enter the sample names.

a. Click  or select **View > Well Inspector**.

b. Click-drag to select wells **A1** through **H1**.

c. Type **NTC** for the Sample Name.

d. Select remaining wells, then type **Unknown** for the Sample Name.

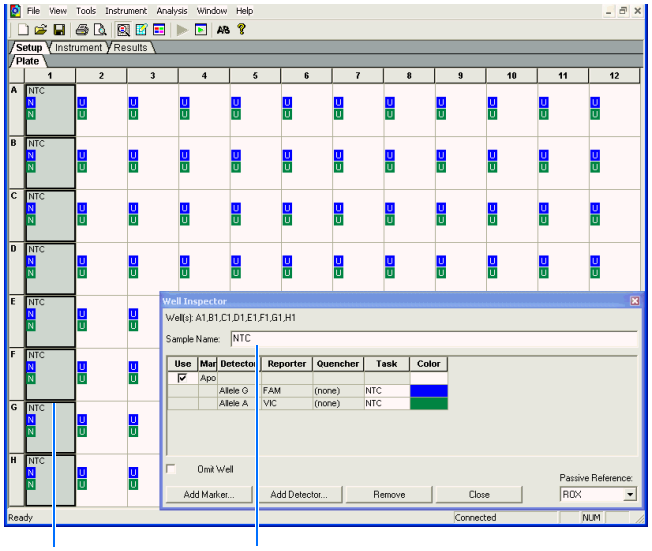
e. Leave the Passive Reference dye set to ROX™ dye.

Note: If your experiment does not use all the wells in a plate document, do not omit the wells from use at this point. You can omit unused wells after the run is completed. For more information about omitting wells, refer to the Online Help.

Note: You can change the sample setup information (sample name, detector, task) after a run is complete.

f. Click  to close the Well Inspector.

11. Verify the information on each well in the Setup tab.



Notes

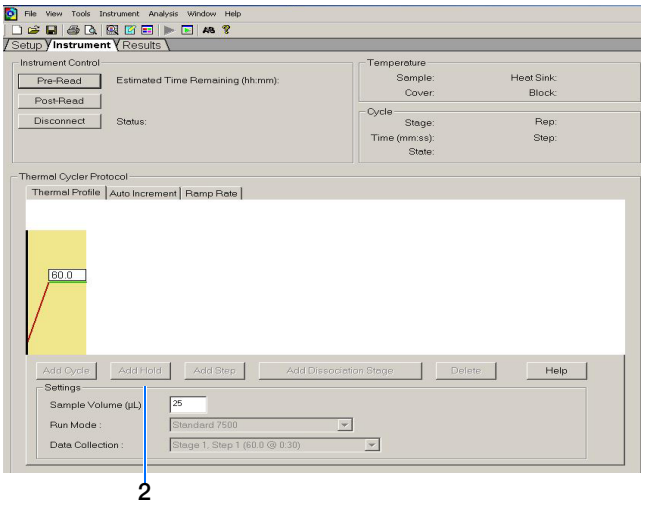
Performing the Pre-Read Run

1. Select the **Instrument** tab.

2. If your assay uses TaqMan® Gene Expression Assays probes and primers or Custom TaqMan® Gene Expression Assays probes and primers, change the Sample Volume to **25** µL.

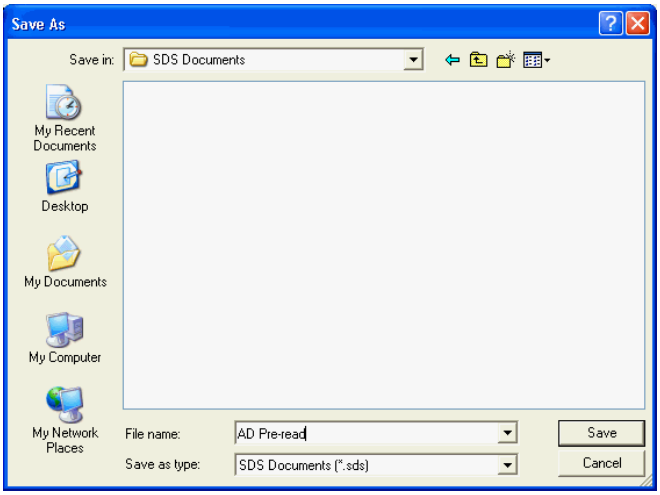
   Note: The recommended sample volume for the 7500 Fast system is 20 µL.

   If your assay uses probes and primers designed with Primer Express® software, adjust the Sample Volume to the sample volume you added to reaction plate.



3. Select **File** > **Save**, then click **Save** to retain the name you assigned when you created the plate document.

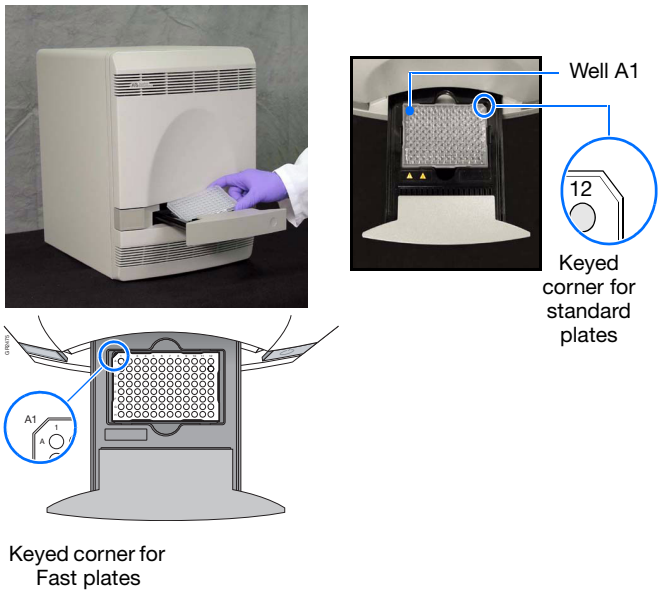
   (Optional) If you want to use this plate document again, select **File** > **Save As**, type the **File Name**, then select (*.sdt) **for Save As** type to save the file as a template.



Notes

4. Load the reaction plate into the instrument.

   Note: The A1 position is in the top-right side of the instrument tray for the 7300/7500 system. The A1 position is on the top-left side of the instrument tray for the 7500 Fast system.

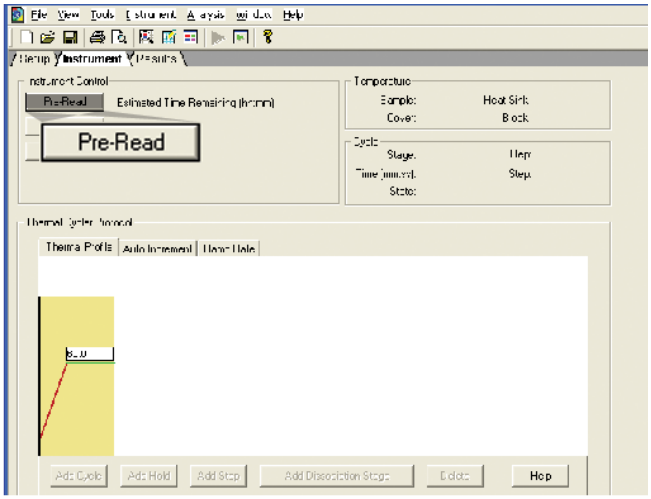


5. Click **Pre-Read**.

   During the pre-read run, the instrument collects one fluorescent scan per well.

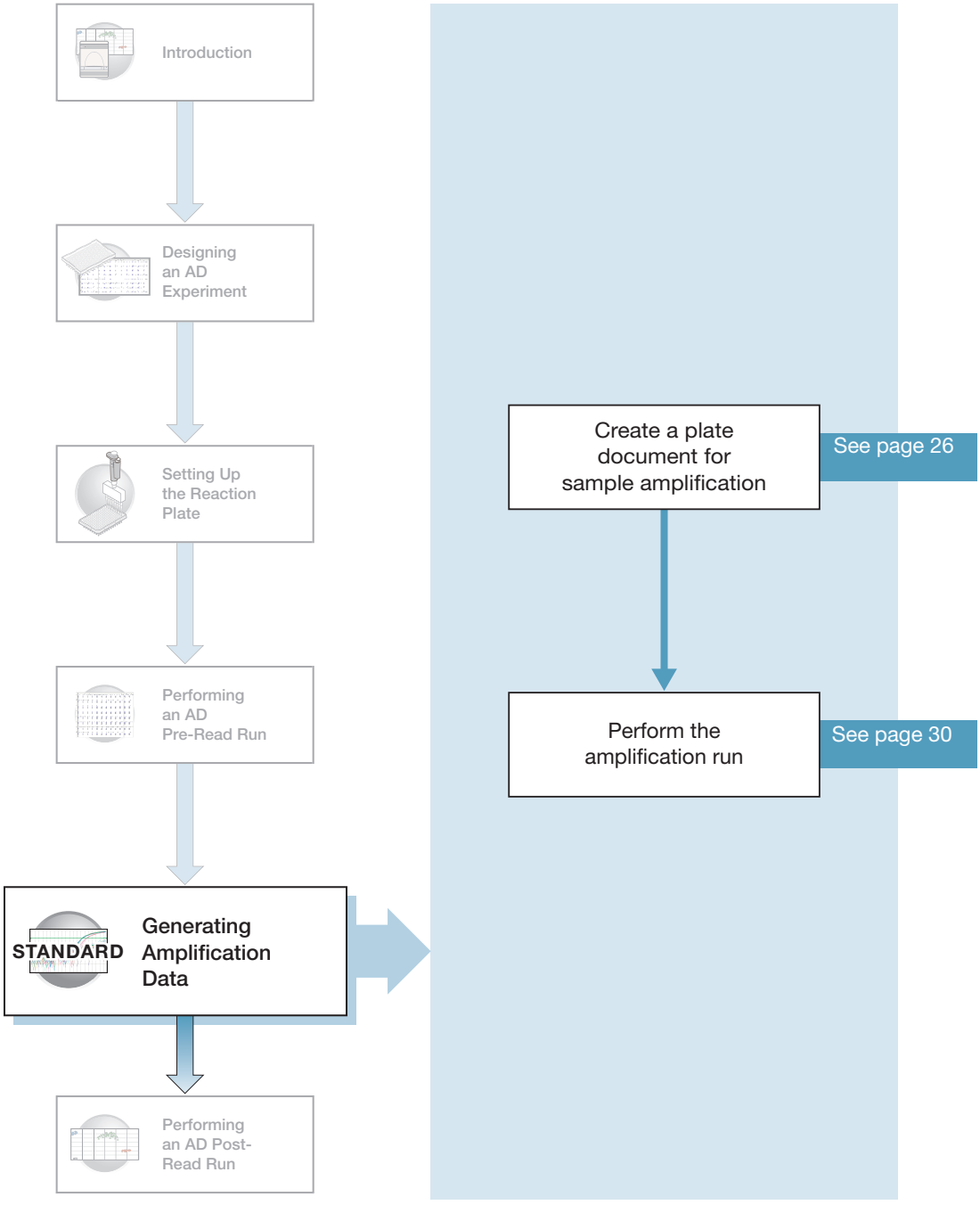
   As the instrument performs the run, it displays status information in the Instrument tab. After the run is finished, the status values and the buttons are grayed out and a message indicates whether or not the run is successful.

6. Select **File>Close**.



Notes

Generating Amplification Data



Notes

Creating an Absolute Quantification (AQ) Plate Document

Benefits of Real-Time Amplification

Because the AD assay is an end-point assay, you can amplify the target sequences offline using any thermal cycler. However, using the 7300/7500/7500 Fast system to amplify the target sequences provides Real-Time PCR data. When you perform allele-calling (described in Chapter 6 on page 37), you can study the amplification plots if you observe questionable calls or do not observe data for a well.

Using AQ Plate Documents for Amplification

You create and use AQ Plate documents to store real-time data for AD assays. Because the AQ plate document is used only to amplify target sequences (not to quantify the PCR data), you do not need a standard curve for the AQ plate.

Notes

Creating an AQ Plate Document

1. Select **File** > **New**. The New Document Wizard opens.

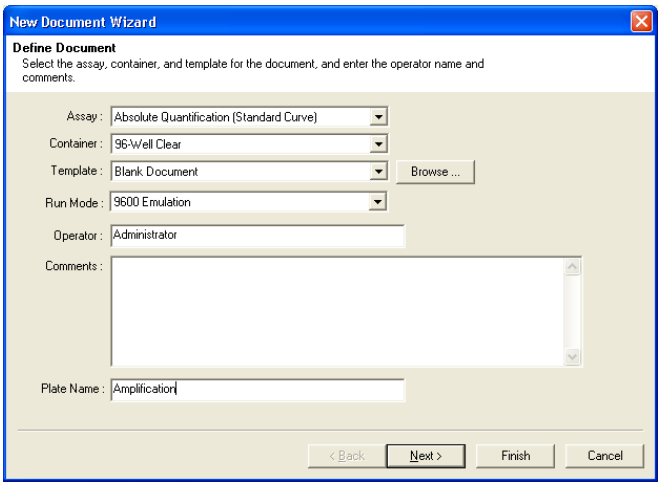
2. In the New Document Wizard:

   a. Click the Assay drop-down list, then select **Absolute Quantification (Standard Curve)**.

   Note: A standard curve is not needed for a non-quantification amplification run.

   b. Accept the default settings for the Container and Template fields (**96-Well Clear** and **Blank Document**).

   c. In the Plate Name field, type **Amplification**.



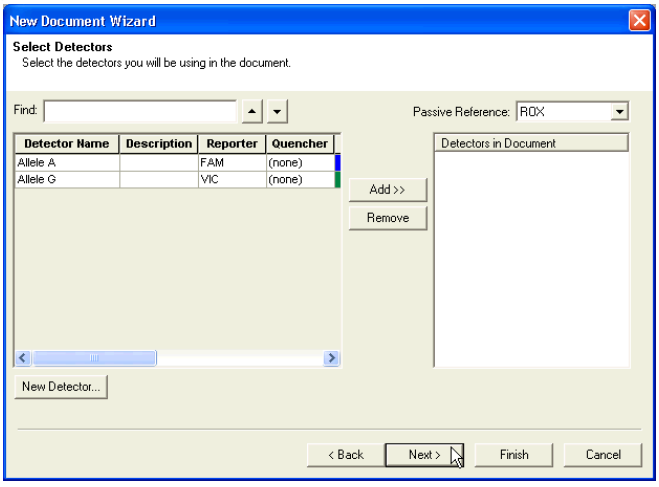
3. Click **Next>**.

4. In the Select Detectors page, select the same detectors you added to the marker in the AD plate document (Allele A and Allele G).

   a. Ctrl-click to select multiple detectors.

   b. Click **Add>>**. The detectors are added to the plate document.

   c. Click **Next>**.



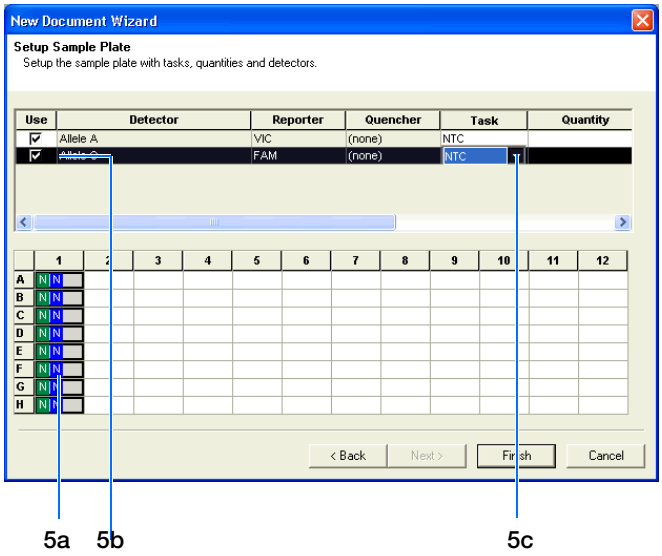
Notes

5. In the Setup Sample Plate page, set detector tasks:

   a. On the plate, click-drag to select wells **A1** through **H1**.

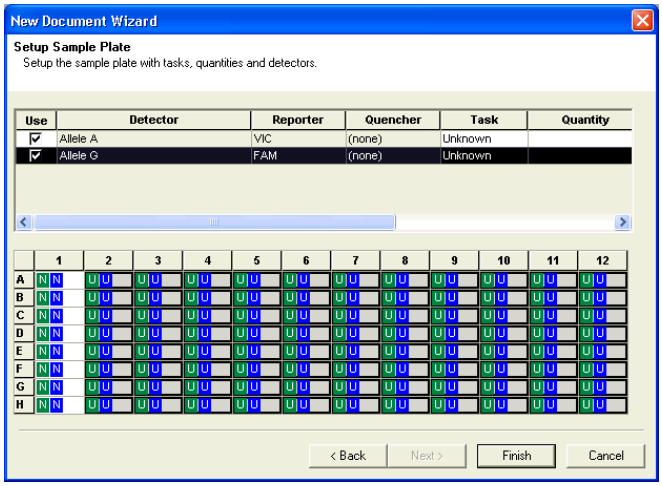
   b. Select the **Use** box for the Allele A and Allele G detectors.

   c. Click the Task field for each of the detectors, then select **NTC** for task.



   d. Select the remaining wells.

   e. Select the **Use** box for both detectors. Leave the Task set to Unknown.

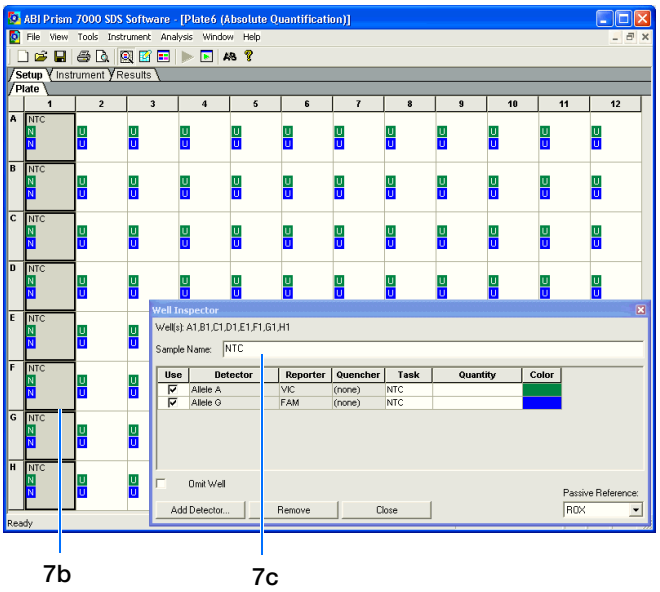


6. Click **Finish**.

   The 7300/7500/7500 Fast SDS software creates the plate document.

Notes

7. Enter the sample names.
   a. Click  or select **View > Well Inspector**.
   b. Click-drag to select wells **A1** through **H1**.
   c. Type **NTC** for the Sample Name.
   d. Select remaining wells, then type **Unknown** for the Sample Name.
   e. Leave the Passive Reference dye set to ROX™ dye.
   f. Click  to close the Well Inspector.
8. Verify the information on each well in the Setup tab.



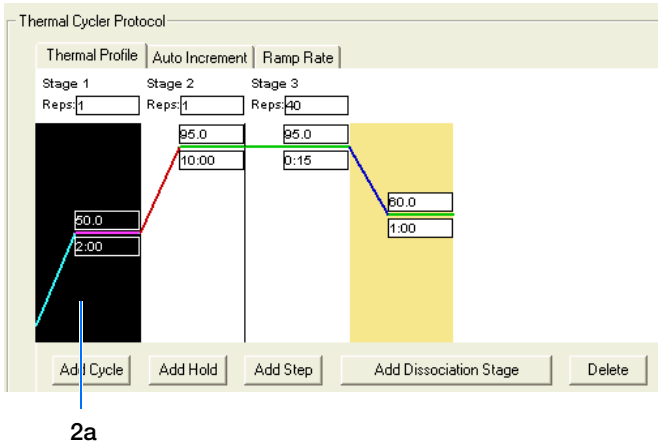
Notes

Performing the Amplification Run

1. Select the **Instrument** tab.

2. If your assay uses TaqMan® Gene Expression Assays or Custom TaqMan® Gene Expression Assays probes and primers and the TaqMan® Universal PCR Master Mix, No AmpErase® UNG Kit, modify the data in the instrument tab in the following manner:

   Note: The recommended Universal PCR Master Mix, No UNG (PN 4324018) does not contain Amperase® UNG; therefore the default first stage is not needed. However, if you use Custom TaqMan® Gene Expression Assays probes and the TaqMan® Universal PCR Master Mix Kit (PN 4304437) (which contains AmpErase UNG), the first stage is needed. Do not delete.

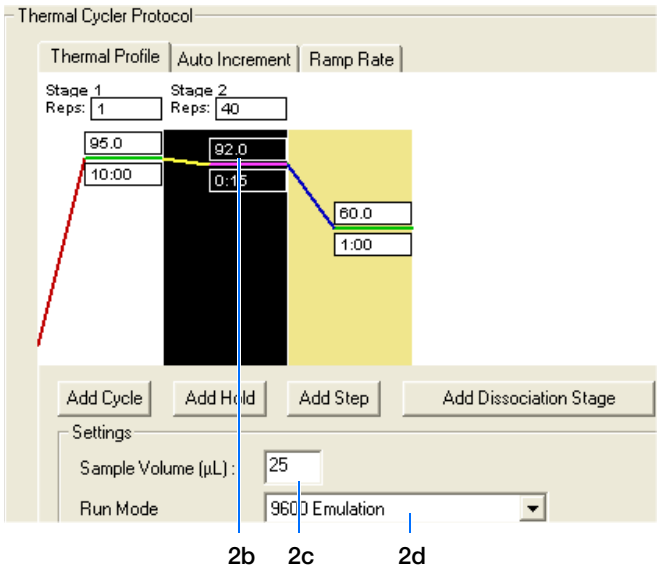


   a. Delete the default first stage by **Shift+clicking** near the bottom of the stage box to select it, then clicking **Delete**.

   b. Change the temperature for the second step to **92** by clicking the second box in the second stage, then typing **92**.

   c. Set the Sample Volume to **25** µL.

      Note: The recommended sample volume for the 7500 Fast system is 20 µL.



   d. Verify that 9600 Emulation is selected as the run mode.

      Note: In the 7300 instrument, the 9600 Emulation feature is not available.

   e. Accept the remaining default times and temperatures for the PCR step and go to step 3 on page 32.

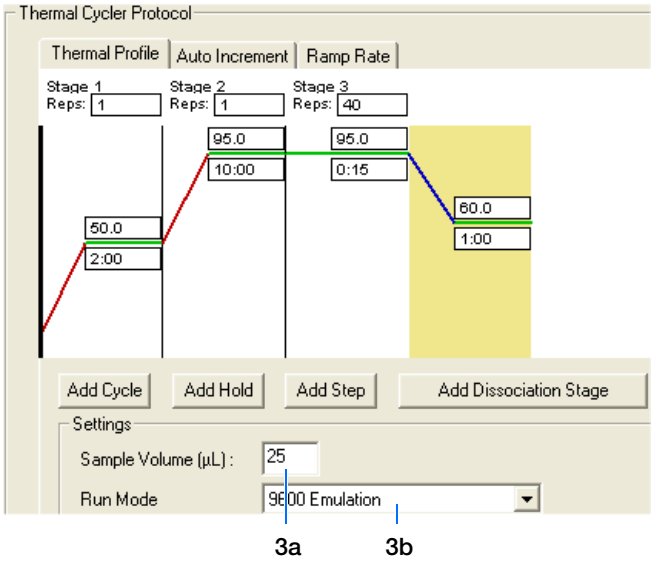
Notes

Times and Temperatures		
Initial Steps	PCR (Each of 40 cycles)	
AmpliTaq Gold® DNA Polymerase Activation	Melt	Anneal/Extend
HOLD	CYCLE	
10 min @ 95 °C	15 sec @ 92 °C	1 min @ 60 °C

If your assay uses probes and primers designed with Primer Express® software and uses the TaqMan Universal PCR Master Mix Kit:

a. Adjust the Sample Volume to the sample volume you added to reaction plate.

b. Verify that 9600 Emulation is selected as the run mode.



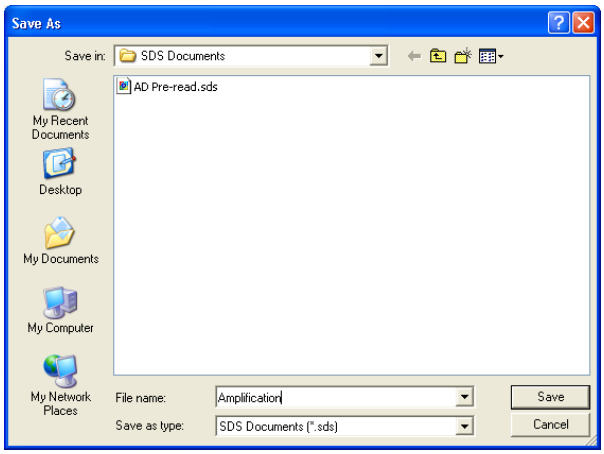
Note: In the 7300 instrument, the 9600 Emulation feature is not available.

c. Accept the remaining default times and temperatures for the PCR step and go to step 3.

Times and Temperatures			
Initial Steps		PCR (Each of 40 cycles)	
AmpErase® UNG Activation	AmpliTaq Gold® DNA Polymerase Activation	Melt	Anneal/ Extend
HOLD	HOLD	CYCLE	
2 min @ 50 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

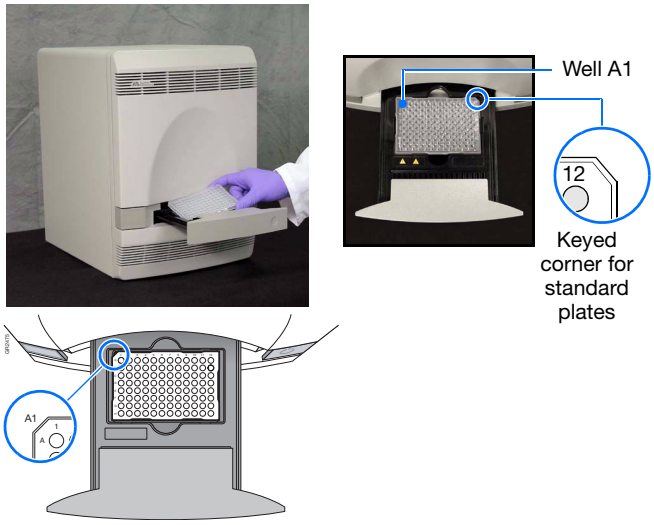
Notes

3. Select **File** > **Save**, then click **Save** to retain the name you assigned when you created the plate document.



4. Load the reaction plate into the instrument.

Note: The A12 position is notched in the top-right side of the instrument tray for the 7300/7500 system. The A1 position is notched on the top-left side of the instrument tray for the 7500 Fast system.



5. Click **Start**.

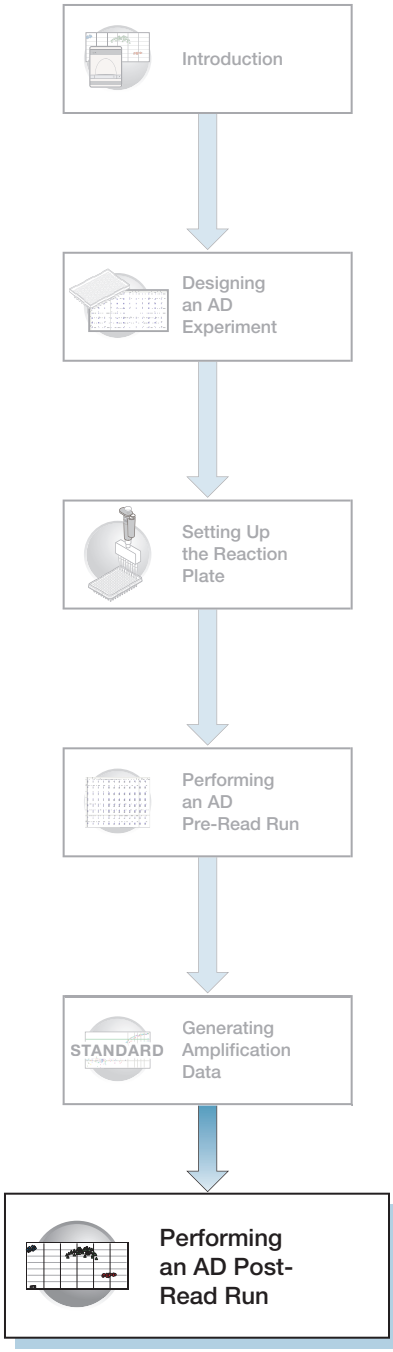
   As the instrument performs the PCR run, it displays real-time status information in the Instrument tab and records the fluorescence resulting from cleavage of TaqMan® probes in the presence of the target sequences.

   After the run, the status values and buttons are grayed-out, the Analysis button is enabled ( ), and a message indicates whether or not the run is successful.

   All data generated during the run are saved to the AQ plate document that you specified in step 3 and can be analyzed later for troubleshooting purposes.

Notes

Performing an AD Post-Read Run



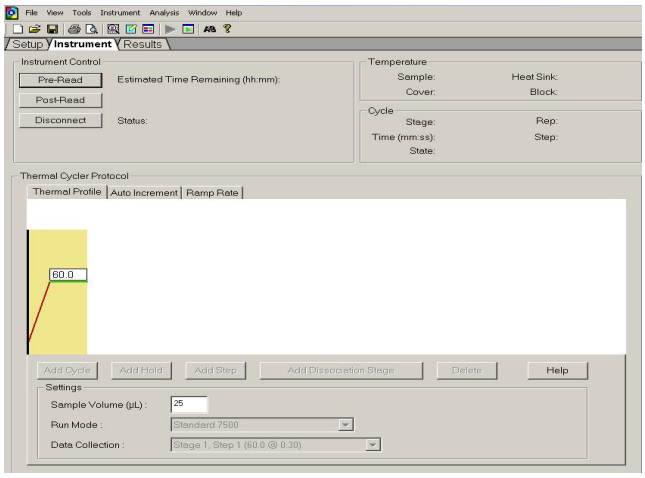
Perform a post-read run — See page 34
Evaluate results — See page 36
Assign calls — See page 37
View reports — See page 40
Export plate documents — See page 41

Notes

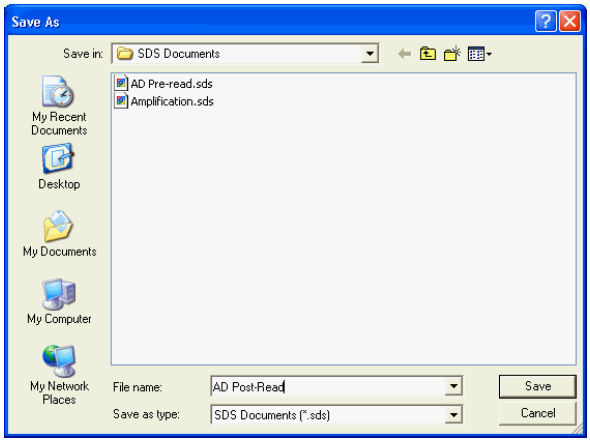
Performing the Post-Read Run

1. Open the pre-read plate document.

2. Select the **Instrument** tab.



3. Select **File** > **Save As**, type **AD Post-Read** for the name for the AD plate document, then click **Save**.



Notes

4. Load the reaction plate into the instrument.

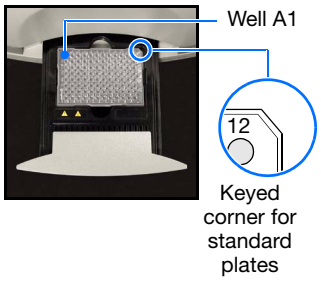
   Note: The A12 position is notched in the top-right side of the instrument tray for the 7300/7500 system. The A1 position is notched on the top-left side of the instrument tray for the 7500 Fast system.

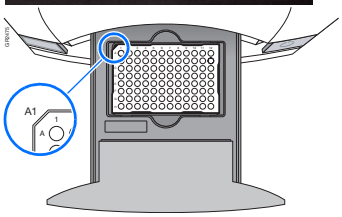
5. Click **Post-Read**.

   After the run is finished, the status values and the buttons are grayed-out and a message indicates whether or not the run is successful.

6. Click the green analysis button ( ) to start analysis.

   All data generated during the run are saved to the AD plate document that you specified in step 3.





Keyed corner for Fast plates

Notes

Evaluating Results

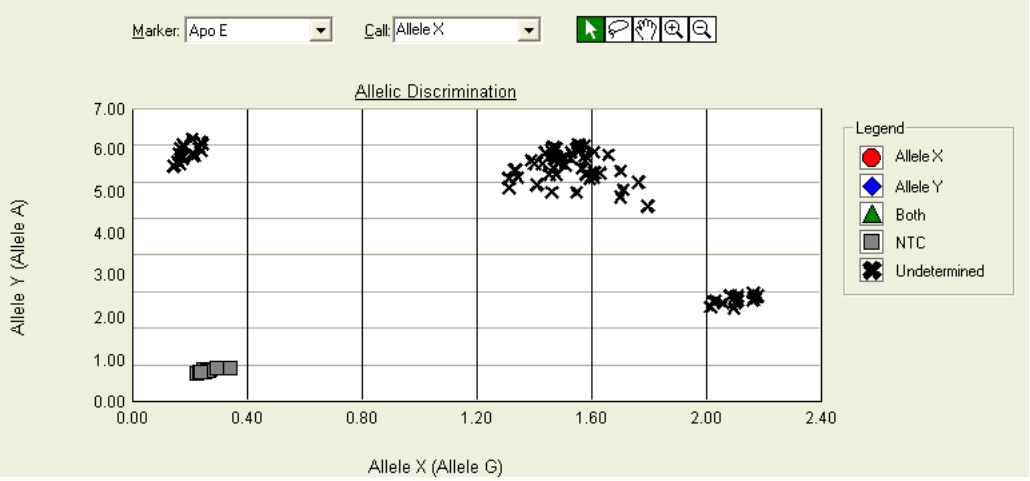
After an AD post-read run, the 7300/7500/7500 Fast SDS software analyzes raw data. During the analysis, the SDS software converts the raw data, expressed in terms of fluorescence signal versus filters, to pure dye components using the extracted pure dye standards.

After identifying the dye components, the SDS software determines the contribution of each dye in the raw data using the multicomponent algorithm.

Cluster Variations

The SDS software plots the results of the allelic discrimination run on a scatter plot of Allele X versus Allele Y. Each well of the 96-well reaction plate is represented with an ✕ (Undetermined) on the plot. The clustering of points can vary along the horizontal axis (Allele X), vertical axis (Allele Y), or diagonal (Allele X/Allele Y). This variation is due to differences in the extent of reporter dye fluorescent intensity after PCR amplification.

The example below shows variation in clustering due to the genotype of the target allele.

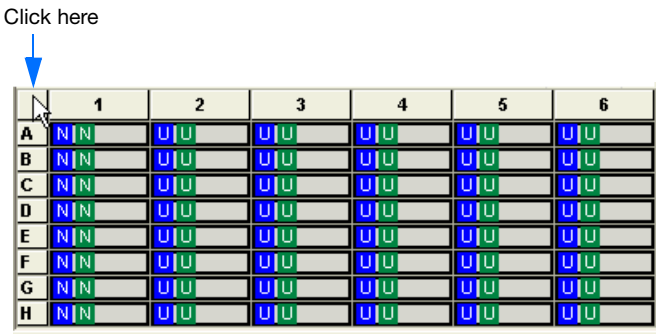


Notes

Assigning Calls

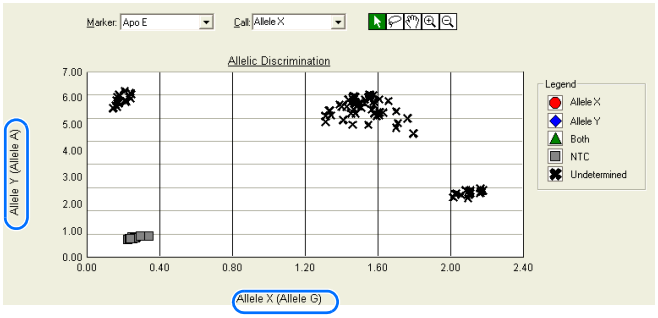
Assigning Calls Automatically

1. In the AD plate document that contains the post-read data, select the **Results** tab.
2. Select the **Allelic Discrimination** tab.
3. To view all results for the plate, select all 96 wells in the plate document by clicking the upper-left corner of the plate.



Before alleles are identified, each selected well is represented as an ✕ (Undetermined) on the Allelic Discrimination plot.

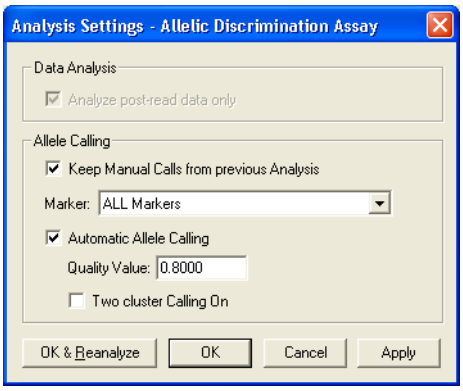
The names you assigned to the detectors on page 20 are displayed on the axes of the plot.



Note: You can customize the symbols and colors associated with alleles by double-clicking the axis of the plot, then modifying Graph Settings

4. Select **Analysis > Analysis Settings**.
5. Select **Automatic Allele Calling**. If desired, increase the Quality Value for more stringent allele calling.
6. Click **OK & Reanalyze**.

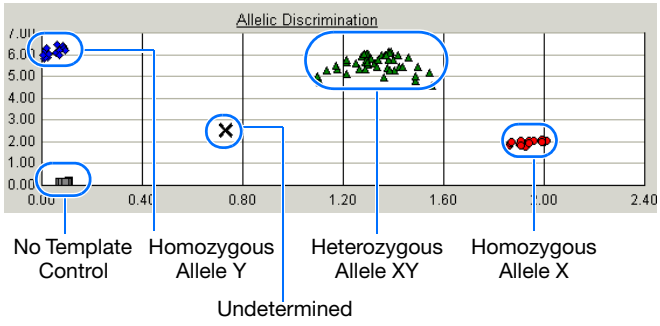
   Alleles are identified on the plot.



Notes

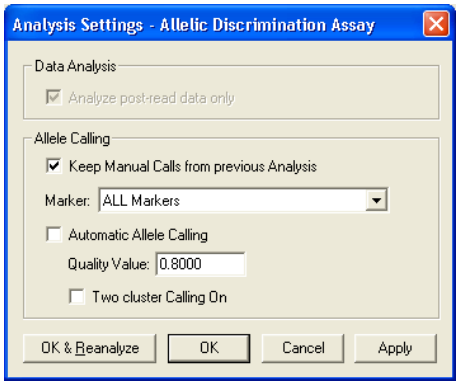
Samples are grouped:

Samples Containing...	Are Grouped In...
Allele X	Lower right corner of the plot
Allele Y	Upper left corner of the plot
Both (Allele X and Allele Y – heterozygote)	Approximately midway between the Allele X and Allele Y groups
No Template Control (NTC)	Bottom left corner of the plot
Undetermined	Anywhere on plot

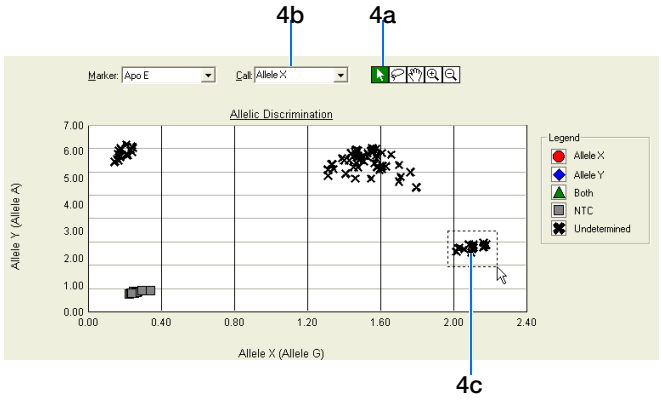


Assigning Calls Manually

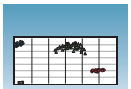
1. Select all 96 wells in the plate document by clicking the upper-left corner of the plate.
2. Select **Analysis > Analysis Settings**.
3. Deselect **Automatic Allele Calling**.



4. To assign calls:
   a. Click the selection tool, then click-drag a box around the allele data points in the lower-right of the plot.
   b. In the Call drop-down list, select **Allele X**.
   c. Click-drag a box around the allele data points in the upper-left of the plot.
   d. In the Call drop-down list, select **Allele Y**.
   e. Click-drag a box around the allele data points in the center of the plot.
   f. In the Call drop-down list, select **Both**.



Notes



g. Click-drag a box around any allele data points that are not included in any of the grouped data points (not shown in the example).

h. In the Call drop-down list, select **Undetermined**.

Determining the Genotype

To determine the genotype for each sample, you can select a well, or view reports (see page 40).

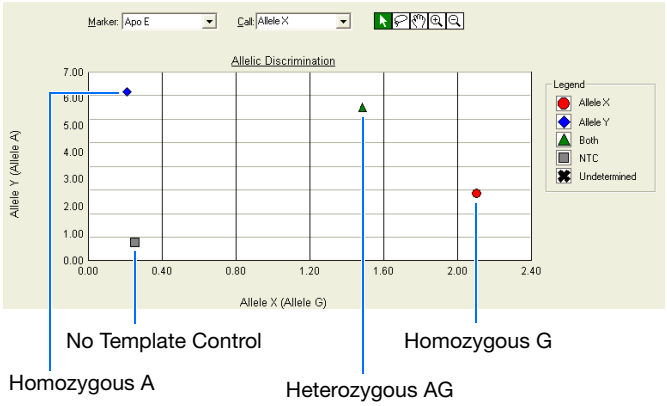
The figure below shows a plot with four wells selected. From this plot, you can derive the genotype of the sample in each well:

– Allele X – Homozygous Allele G (as indicated by the detector name associated with the Allele X axis on the plot)

– Allele Y – Homozygous Allele A (as indicated by the detector name associated with the Allele Y axis on the plot)

– Both – Heterozygous Alleles A and G

– NTC – No template control



For More Information

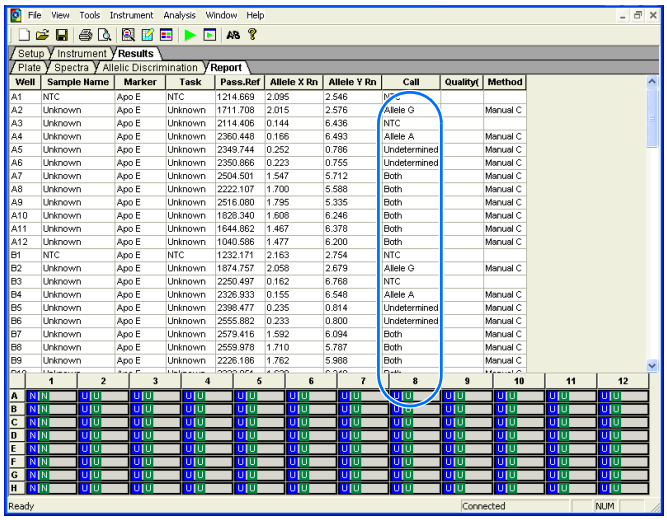
For more information on the tools in the Allelic Discrimination plot, see the Online Help.

6

Notes

Viewing Reports

In the AD plate document that contains the post-read data, select the **Results** tab.



The Report tab displays the results in table form.

Note that the name of the detectors you specified for the markers are listed in the Call column.

You can export the report by exporting results (see page 41).

Notes

Exporting Plate Documents

You can export numeric data from AD plates into text files, which can then be imported into spreadsheet applications such as Microsoft® Excel.

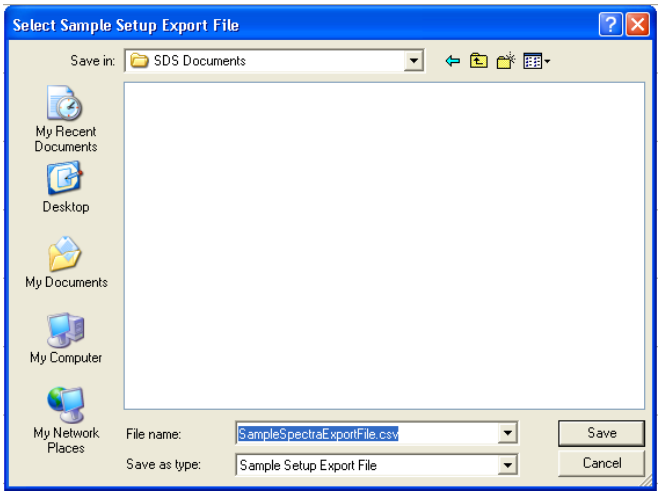
1. Select **File** > **Export**, then select the data type to export:
   - **Sample Setup** (*.txt)
   - **Calibration Data** (*.csv)
   - **Spectra** (*.csv)
   - **Component** (*.csv)
   - **Rn** (*.csv)
   - **Results** (*.csv) (exports reports)

   Typically, you export sample setup data for newly created and newly run plates; other data types are exported for existing plates.

2. Enter a file name for the export file.

   Note: The name of the dialog box depends on the type of data you want to export.

3. Click **Save**.



For more information on exporting, see the Online Help.

Notes

Notes

Creating Detectors

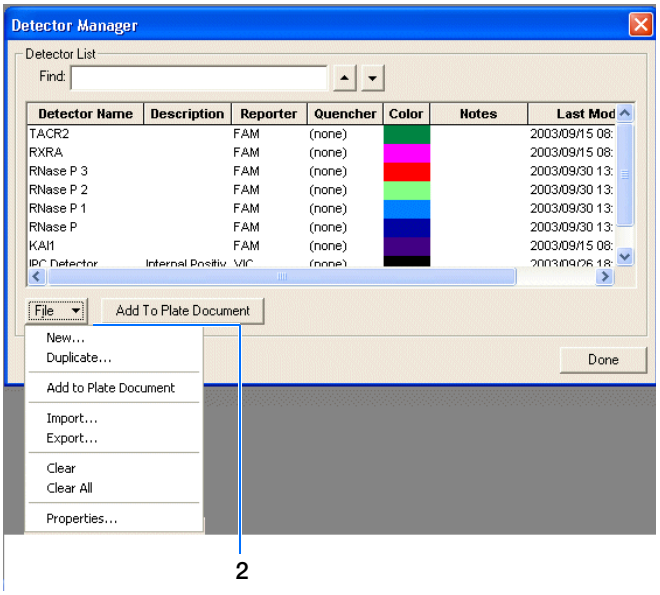
Before you can use a plate document to run a plate, you need to create and apply detectors for all samples on the plate. A detector is a virtual representation of a gene- or allele-specific nucleic acid probe reagent used for analyses performed on instruments.

To create a detector:

1. Select **Tools > Detector Manager**.

   Note: A plate document (any type) must be open before you can access the Tools menu.

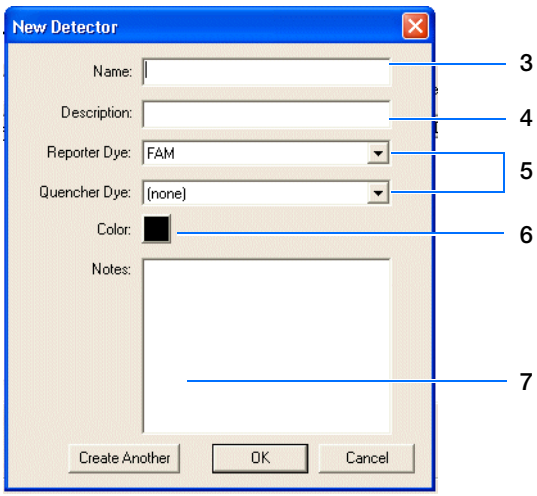
2. In the Detector Manager, select **File > New**.



3. In the New Detector dialog box, enter a name for the detector.

   IMPORTANT! The name of the detector must be unique and should reflect the target locus of the assay (such as GAPDH or RNase P). Do not use the same name for multiple detectors.

4. Optionally, click the **Description** field, then enter a brief description of the detector.



Notes

5. In the Reporter Dye and Quencher Dye drop-down lists, select the appropriate dyes for the detector.

Note: The dyes that appear on the Reporter and Quencher Dye lists are those that have been previously entered using the Dye Manager. If the dye that you want to use does not appear in a list, use the Dye Manager to add the dye and then return to this step in this procedure. Refer to the Online Help for more information.

Note: Select TAMRA™ as the quencher for TaqMan® probes and None for TaqMan® MGB probes.

6. Click the **Color** box, select a color to represent the detector using the Color dialog box, then click OK.

7. Optionally, click the **Notes** field, then enter any additional comments for the detector.

8. Click **OK** to save the detector and return to the Detector Manager.

9. Repeat steps 2 through 8 for the remaining detectors.

10. In the Detector Manager, click **Done** when you finish adding detectors.

Note: TaqMan® Gene Expression Assays are shipped with an assay information file (AIF). This text-based file contains information about the assays that you ordered, including the Applied Biosystems Assay ID number, well-location of each assay, and primer concentration. The file also indicates the reporter dyes and quenchers (if applicable) that are used for each assay. When creating detectors, you use the reporter dye and quencher information (and optionally, the gene name or symbol for the sample name). You can view the contents of AIFs in a spreadsheet program, such as Microsoft® Excel.

Sample Experiment

In the example AD experiment, two detectors are created for the marker used in the assay. One detector is named Allele A, assigned a blue color, and labeled with FAM™ dye. The other detector is named Allele G, assigned a green color, and labeled with VIC® dye. No quencher dye is necessary.

Notes

Viewing Amplification Data

If you observe questionable allele calls, you can analyze, then view the amplification data (generated using the AQ plate in Chapter 5).

Configuring Analysis Settings

Before you analyze, specify parameters to enable auto-baseline and auto-threshold calculations.

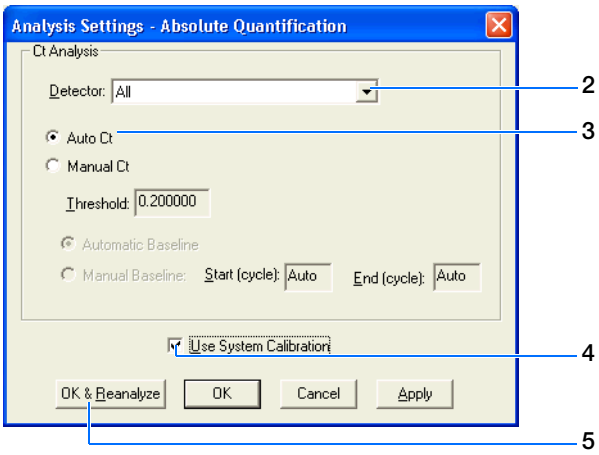
To configure analysis settings:

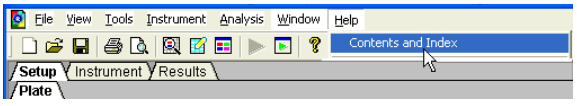
1. Select **Analysis** > **Analysis Settings.**
2. In the Analysis Settings dialog box, select **All** from the Detectors drop-down list.
3. Select **Auto Ct** to set the SDS software to automatically generate baseline and threshold values for all detectors in the study.

   IMPORTANT! After analysis, you must verify that the baseline and threshold were called correctly for each detector, as explained in the Online Help.

   Alternatively, you can select Manual Ct and specify the threshold and baseline manually.
4. Select **Use System Calibrator** if you want to use the calibration files (Background and Pure Dye) that are stored on the computer rather than the calibration information that is stored in the plate document.

   For more information about system calibration files, refer to the Online Help.





Notes

5. Click **OK & Reanalyze**.

6. Examine the amplification plot. For more information on adjusting the baseline and threshold, refer to the Online Help.

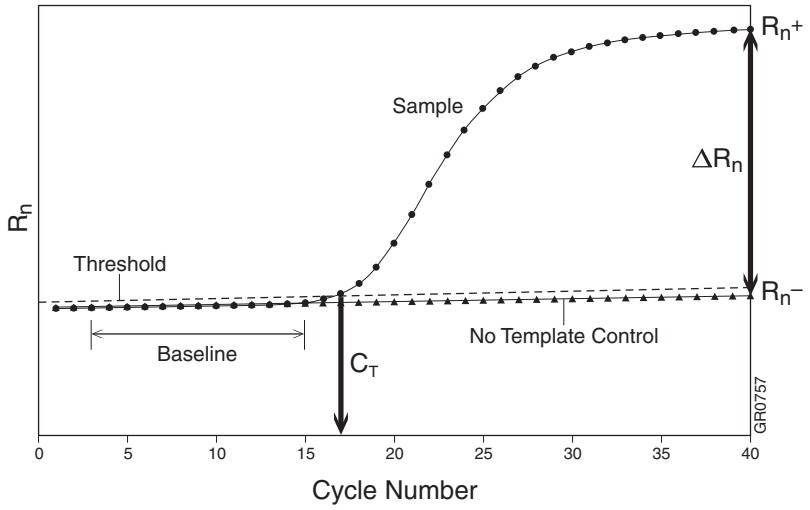
Analyzing the Amplification Data (AQ Plate)

Terms Used in Qualitative Analysis

The following terms are commonly used in quantification analysis.

Term	Definition
Baseline	A line fit to fluorescence intensity values during the initial cycles of PCR, in which there is little change in the fluorescence signal.
Threshold cycle (C_T)	The fractional cycle number at which the fluorescence intensity exceeds the threshold intensity.
Passive reference	A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations caused by changes in concentration or of volume.
Reporter dye	The dye attached to the 5′ end of a TaqMan® probe. The dye provides a signal that indicates of specific amplification.
Normalized reporter (R_n)	The ratio of the fluorescence intensity of the reporter dye signal to the fluorescence intensity of the passive reference dye signal.
Delta R_n (ΔR_n)	The magnitude of the signal generated by a set of PCR conditions. ($\Delta R_n = R_n$ –baseline)

The figure below shows a representative amplification plot that includes some of the terms defined above.



Notes

Viewing the Amplification Data

About the Results Tab

In the Results tab, you can view the results of the run and change the parameters to run the plate document again or reanalyze the data.

The Results tab has seven secondary tabs. Details about each tab are provided in the Online Help.

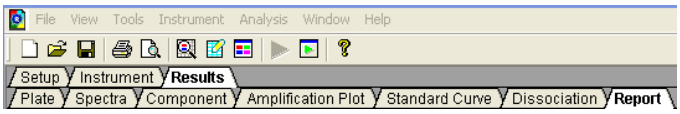
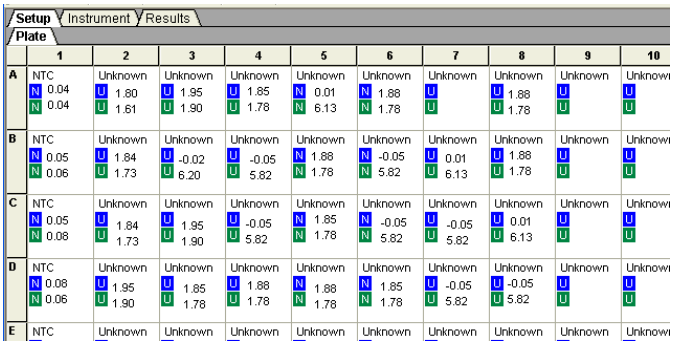


Plate Tab

Displays the results data of each well, including:

- The sample name and detector task and color for each well.
- A calculated value – quantity (default; displays Not Determined for runs without standard curves), ΔRn, or Ct. Select **Analysis > Display** to select the value to display.



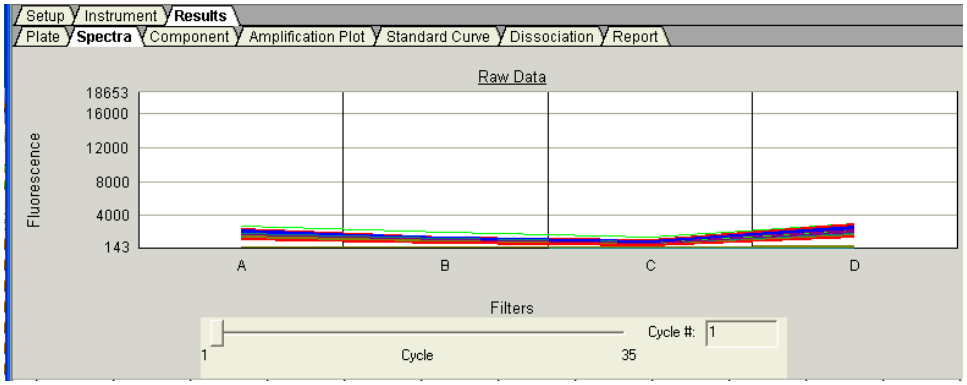
Notes

Spectra Tab

Displays the fluorescence spectra of selected wells.

- The Cycles slider allows you to see the spectra for each cycle by dragging it with the pointer.
- The Cycle # text box shows the current position of the slider.

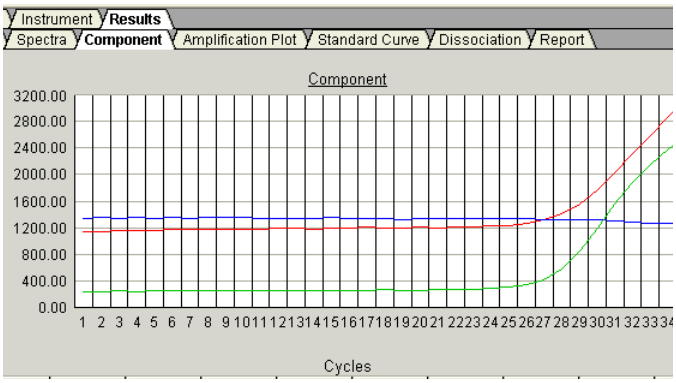
Double-clicking the y-axis opens the Graph Settings dialog box where you can reset the Y- and X-axes or allow autoscaling.



Component Tab

This tab displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run. Only the first selected well is shown at one time.

Double-clicking the y-axis displays the Graph Settings dialog box.

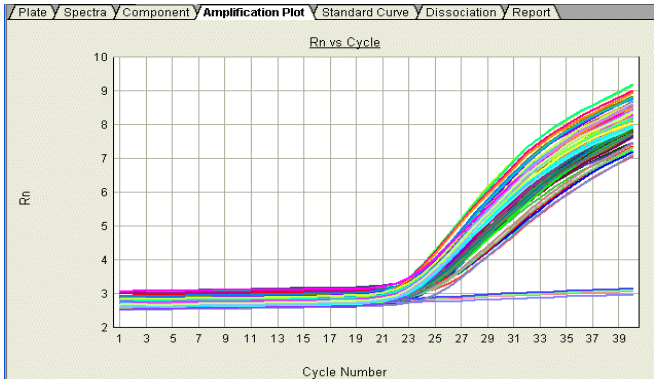


Amplification Plot Tab

The three Amplification Plots allow you to view both real-time and post-run amplification of specific samples. The Amplification plots display all samples in the selected wells.

Notes

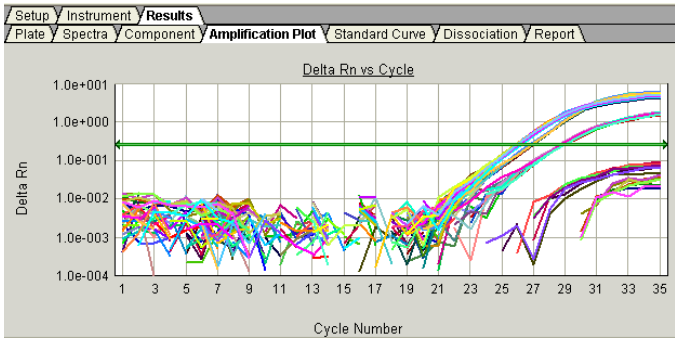
Rn vs. Cycle (Linear)



The Rn vs. Cycle plot displays normalized reporter (R_n) dye fluorescence as a function of cycle. You can use this plot to identify and examine irregular amplification.

For more information about R_n, refer to the *Real-Time PCR Systems Chemistry Guide*.

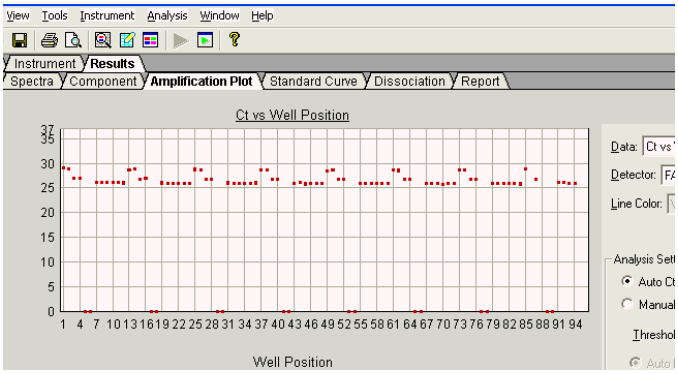
ΔRn vs.Cycle (Log)



The ΔRn vs.Cycle plot displays Rn dye fluorescence as a function of cycle number. You can use this plot to identify and examine irregular amplification and to manually set the threshold and baseline parameters for the run.

Notes

Ct vs. Well Position



The Ct vs. Well Position plot displays threshold cycle (C_T) as a function of well position. You can use this plot to locate outliers in detector data sets.

Report Tab

This tab displays data for selected wells in table format. The data columns associated with the report are determined by the assay type. For AQ assays, the following data columns are available: Well, Sample Name, Detector, Task, Ct, StdDev Ct, Qty, Mean Qty, and StdDev Qty.

The Report Settings dialog box formats the display of the report and how the report is printed. Refer to the Online Help for more information about this dialog box.

Setup | Instrument | **Results**

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dis

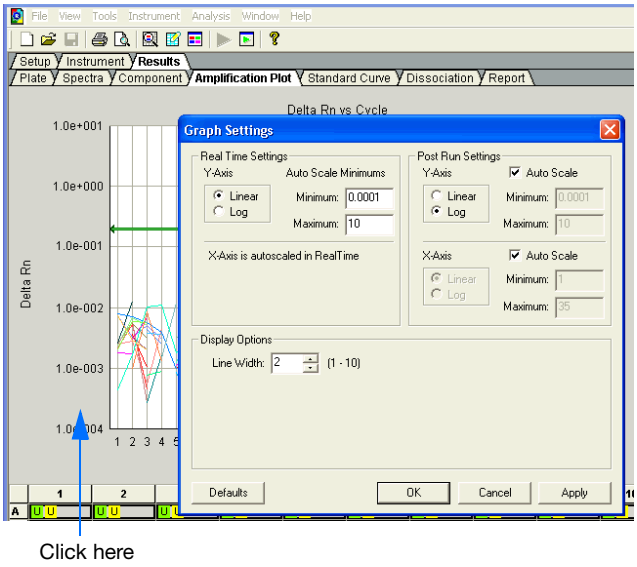
Well	Sample Name	Detector	Task	Ct	St
A1	Wildtype	VIC	Unknown	27.61	0.0
		FAM	Unknown	29.00	0.1
A2	Wildtype	VIC	Unknown	27.70	0.0
		FAM	Unknown	28.91	0.1
A3	Mutation	VIC	Unknown	Undet.	
		FAM	Unknown	26.89	0.0
A4	Mutation	VIC	Unknown	Undet.	
		FAM	Unknown	26.92	0.0
A5	NTC	VIC	NTC	Undet.	
		FAM	NTC	Undet.	
A6	NTC	VIC	NTC	Undet.	
		FAM	NTC	Undet.	
A7	Heterozygotes	VIC	Unknown	27.67	0.2
		FAM	Unknown	26.09	0.0
A8	Heterozygotes	VIC	Unknown	27.46	0.2
		FAM	Unknown	26.08	0.0

Notes

Adjusting Graph Settings

Clicking on the Spectra, Component, Amplification Plot, Standard Curve, and Dissociation plots displays the Graph Settings dialog box, which allows you to adjust the plot settings.

The adjustable settings depend on which plot you are viewing. Refer to the Online Help for more information about specific settings.



Notes

Notes

Example AD Experiment

Overview

To better illustrate how to design, perform, and analyze AD experiments, this section guides you through an example experiment. The example experiment represents a typical AD experiment setup that you can use as a quick-start procedure to familiarize yourself with the AD workflow. Detailed steps in the AD workflow are described in the subsequent chapters of this guide. Also in the subsequent chapters are Example Experiment boxes that provide details for some of the related steps in the example experiment.

Description

The objective of the example AD experiment is to investigate a genetic variant of Apolipoprotein E (ApoE), a gene associated with lipoproteinemia. Possible genotypes are AA, AG, and GG.

The experiment uses multiplex PCR. Primers and probes are ordered from TaqMan® Gene Expression Assays (AB Assay ID C 3084818 10).

Reactions are set up for PCR using the TaqMan® Universal PCR Master Mix and appropriate primers and probes.

The example AD experiment data and results are generated using a 7500 system by performing:

- **A pre-read run** on an AD plate document to determine the baseline fluorescence associated with primers and probes before amplification.
- **An amplification run** using an AQ plate document to generate Real-Time PCR data, which can be used to analyze and troubleshoot the PCR data for the AD assay, if needed.
- **A post-read run** using the original AD plate document, which automatically subtracts the baseline fluorescence determined during the pre-read run, then assigns allele calls (automatically or manually) using the amplified data.

Notes

Example AD Experiment Procedure

Design the experiment and prepare DNA:

1. Design the experiment as explained in Chapter 2.
   a. Order the TaqMan® Universal PCR Master Mix.
   b. Select and order the probes and primers.
2. Extract the DNA from samples (see "Preparing DNA" on page 12).

   The sample DNA for this experiment was extracted using the BloodPrep™ Chemistry Kit (PN 4346860) to obtain a final concentration of 10 ng/µL of DNA for each sample.
3. Prepare the reaction mix. The final reaction volume in each well is 25 µL.

Note: The recommended reaction volume the 7500 Fast system is 20 µL.

Note: This section describes preparing reaction mix for a TaqMan® SNP Genotyping Assay using the TaqMan® Universal PCR Master Mix, No AmpErase® UNG Kit (PN 4324018). If your assay is custom-designed and uses the TaqMan® Universal PCR Master Mix Kit, refer to "Preparing the Reaction Mix for Custom TaqMan Gene Expression Assays" on page 13.

CAUTION CHEMICAL HAZARD. **TaqMan Universal PCR Master Mix (2×) No AmpErase UNG** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Component	Volume (µL/reaction)	Volume for 106 Reactions‡
2× TaqMan Universal PCR Master Mix, No AmpErase UNG	12.50	1.33 mL
20× SNP Genotyping Assay Mix	1.25	132.5 µL
Total	13.75	1.46 mL

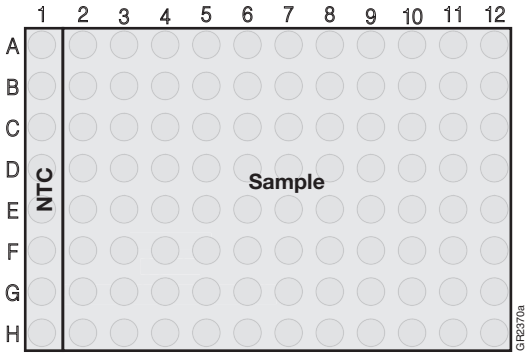
‡ Extra volume is included to account for pipetting losses.

Notes

a. Pipette 13.75 µL of reaction mix into each well of a 96-well reaction plate.

b. Pipette 11.25 µL of the following solutions into the indicated wells:

Wells	Add
A1 through H1 (No Template Control)	Nuclease-free water or TE (Tris-EDTA) buffer
Remaining wells (Sample)	Sample DNA



Notes

Perform the pre-read run:

1. Select **Start > Programs > 7300/7500/7500 Fast System > 7300/7500/7500 Fast System Software** ( )to start the 7300/7500/7500 Fast system SDS software.

2. Create an AD plate document.

   Follow the instructions in "Creating an Allelic Discrimination (AD) Plate Document" on page 18. Briefly:

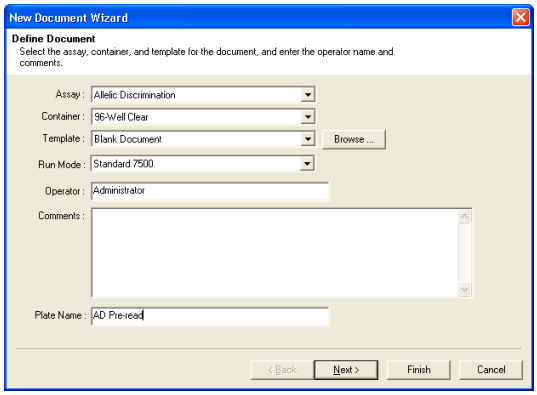
   a. Select **File > New**.

   b. Select **Allelic Discrimination** in the Assay drop-down list.

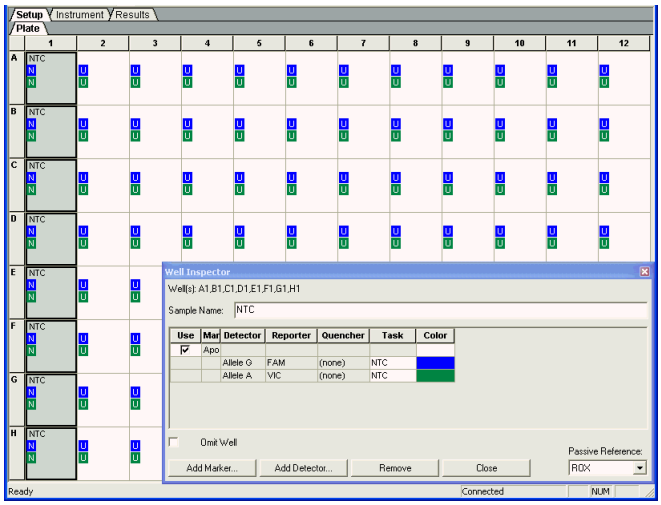
   c. In the Plate Name field, type **AD Pre-Read**, then click **Next**.

   d. Add a marker to the plate document, then click **Next**.

   e. Specify the markers and tasks for each well, then click **Finish**.



3. Enter the sample names and specify tasks in the Well Inspector (**View > Well Inspector**).



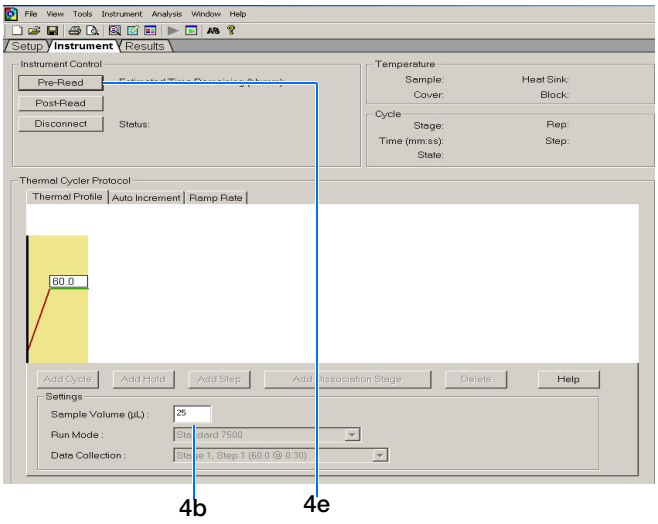
IMPORTANT! If your experiment does not use all the wells in a plate document, do not omit the wells from use at this point. You can omit unused wells after the run is completed. For more information about omitting wells, refer to the Online Help.

Notes

4. Perform the AD pre-read run.
   a. Select the **Instrument** tab.
   b. Change the Sample Volume to **25** µL.

      Note: The recommended sample volume is 20 µL for the 7500 Fast system.

   c. Select **File > Save**, then click **Save** to retain the name you assigned when you created the plate document.
   d. Load the reaction plate into the instrument.
   e. Click **Pre-Read**.



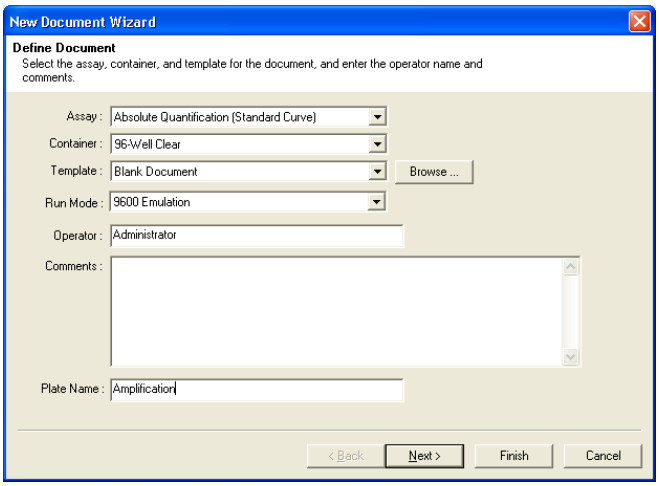
Amplify the DNA:

1. Create an AQ plate document for amplifying samples.

   Follow the instructions in "Creating an Absolute Quantification (AQ) Plate Document" on page 26. Briefly:
   a. Select **File > New**.
   b. Select **Absolute Quantification (Standard Curve)** in the Assay drop-down list.

      Note: A standard curve is not needed for a non-quantification amplification run.

   c. In the Plate Name field, type **Amplification**, then click **Next.**
   d. Add detectors to the plate document, then click **Next**.
   e. Specify the detectors and tasks for each well, then click **Finish**.



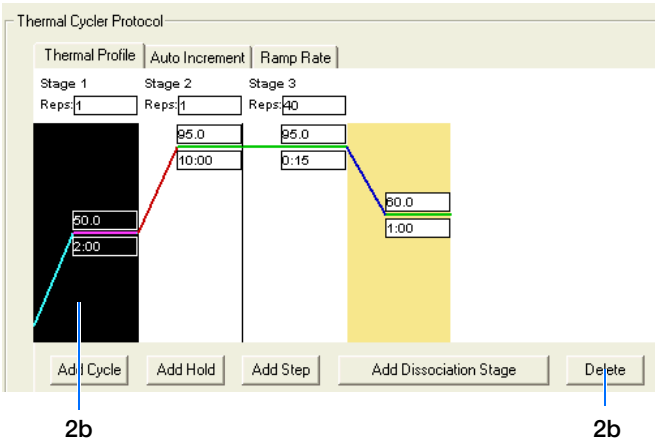
Notes

2. Perform the amplification run.

Note: This section describes amplifying a TaqMan Gene Expression Assay using the TaqMan Universal PCR Master Mix, No AmpErase UNG Kit for the 7500 system. If your assay is custom-designed and uses the TaqMan Universal PCR Master Mix Kit, refer to "Performing the Amplification Run" on page 30.

a. Select the **Instrument** tab.

b. Delete the default first stage by Shift+clicking near the bottom of the stage box to select it, then clicking **Delete**.



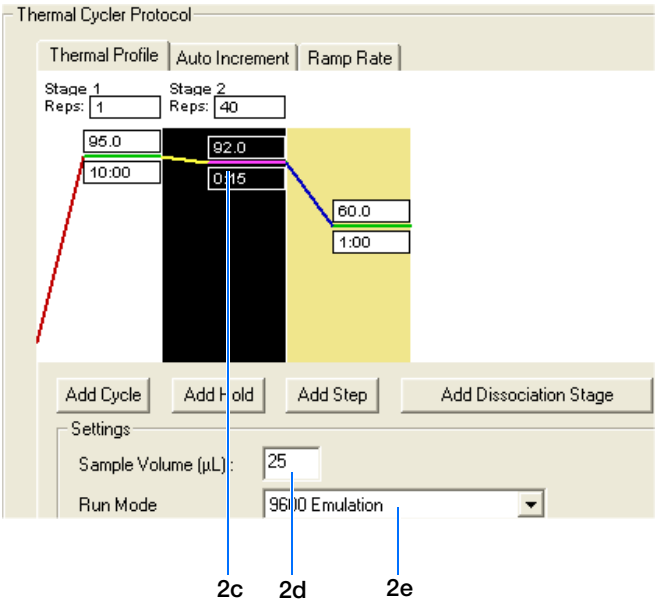
c. Change the temperature for the second step to **92** by clicking the second box in the second stage, then typing **92**.

d. Change the Sample Volume to **25** µL.

Note: The recommended sample volume for the 7500 Fast system is 20 µL.

e. Verify that 9600 Emulation is selected as the run mode.

Note: In the 7300 instrument, the 9600 Emulation feature is not available.



Notes

f. Accept the remaining default times and temperatures for the PCR step.

g. Select **File > Save**, then click **Save** to retain the name you assigned when you created the plate document.

h. Click **Start**.

As the instrument performs the PCR run, it displays real-time status information in the Instrument tab.

Times and Temperatures		
Initial Steps	PCR (Each of 40 cycles)	
AmpliTaq Gold® DNA Polymerase Activation	Melt	Anneal/Extend
HOLD	CYCLE	
10 min. @ 95 °C	15 sec @ 92 °C	1 min. @ 60 °C

Perform the post-read run:

1. Perform a post-read run.

   a. Open the pre-read plate document.

   b. Select the **Instrument** tab.

   c. Verify the Sample Volume is set to **25** µL.

   Note: The recommended sample volume for the 7500 Fast system is 20 µL.

   d. Select **File > Save As**, type **AD Post-Read** for the plate document name, then click **Save**.

   e. Click **Post-Read**.

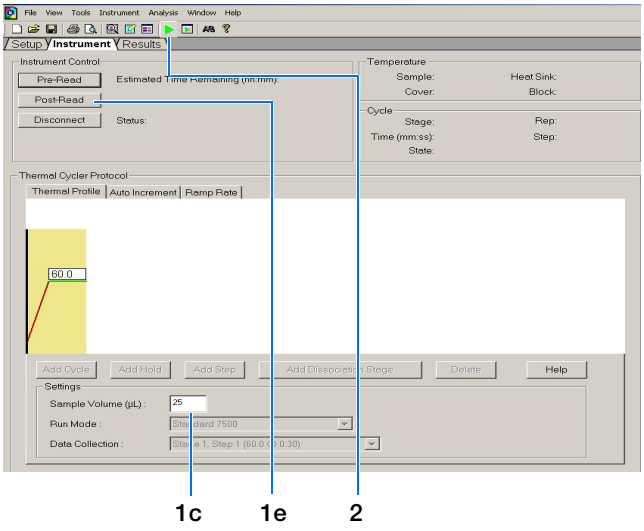
2. Click the green analysis button ( ) to start analysis.

3. Assign calls as described on page 37. Briefly:

   a. Select the **Results** tab.

   b. Select the **Allelic Discrimination** tab.

   c. Click the upper-left corner of the plate to select all wells.



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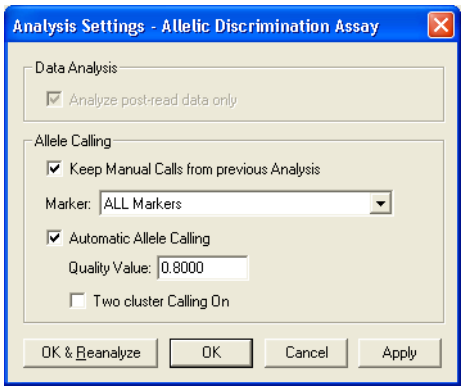
Notes

4. Select **Analysis > Analysis Settings**.

5. Select **Automatic Allele Calling**. If desired, increase the Quality Value for more stringent allele calling.

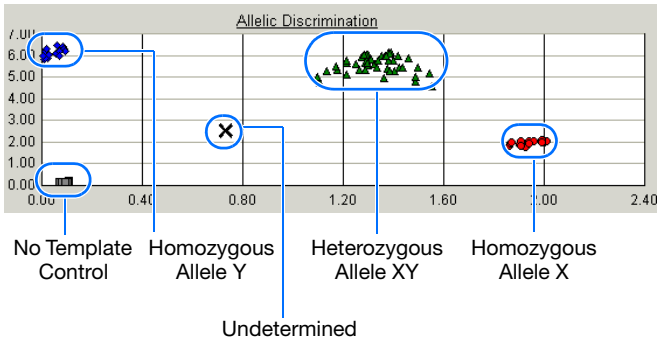
6. Click **OK & Reanalyze**.

   Alleles are identified on the plot.



Samples are grouped:

Samples Containing...	Are Grouped In...
Allele X	Lower right corner of the plot
Allele Y	Upper left corner of the plot
Both (Allele X and Allele Y – heterozygote)	Approximately midway between the Allele X and Allele Y groups
No Template Control (NTC)	Bottom left corner of the plot
Undetermined	Anywhere on plot



To determine the genotype for each sample, you can select a well, or view reports (see page 40).

The figure to the right shows the Allelic Discrimination plot with four wells selected. From this plot, you can derive the genotype of the sample in each well:

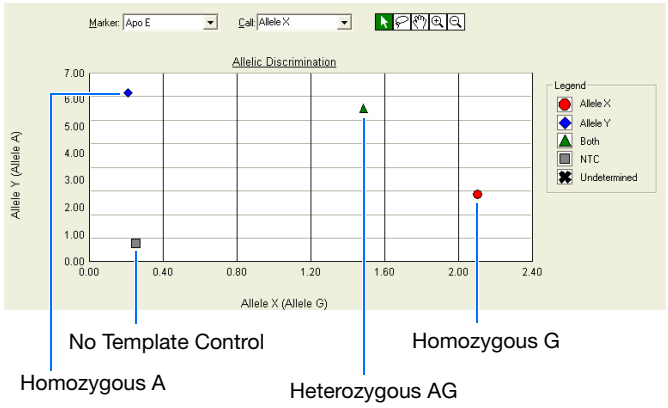
Allele X – Homozygous Allele G (as indicated by the detector name associated with the Allele X axis on the plot).

– Allele Y – Homozygous Allele A (as indicated by the detector name associated with the Allele Y axis on the plot).

– Both – Heterozygous Alleles A and G

– NTC – No template control

For more information, see Chapter 6 on page 36.



Notes

References

Afonina, I., Zivarts, M., Kutyavin, I.*, et al.,* 1997. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Res.* 25:2657–2660.

Kutyavin, I.V., Lukhtanov, E.A., Gamper, H.B., and Meyer, R.B. 1997. Oligonucleotides with conjugated dihydropyrroloindole tripeptides: base composition and backbone effects on hybridization. *Nucleic Acids Res.* 25:3718–3723.

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

Lakowicz, J.R. 1983. Energy Transfer. In *Principles of Fluorescence Spectroscopy*, New York: Plenum Press 303–339.

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

Livak, K.J., Marmaro, J., and Todd, J.A. 1995. Towards fully automated genome-wide polymorphism screening [letter]. *Nat. Genet.* 9:341–342.

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.

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