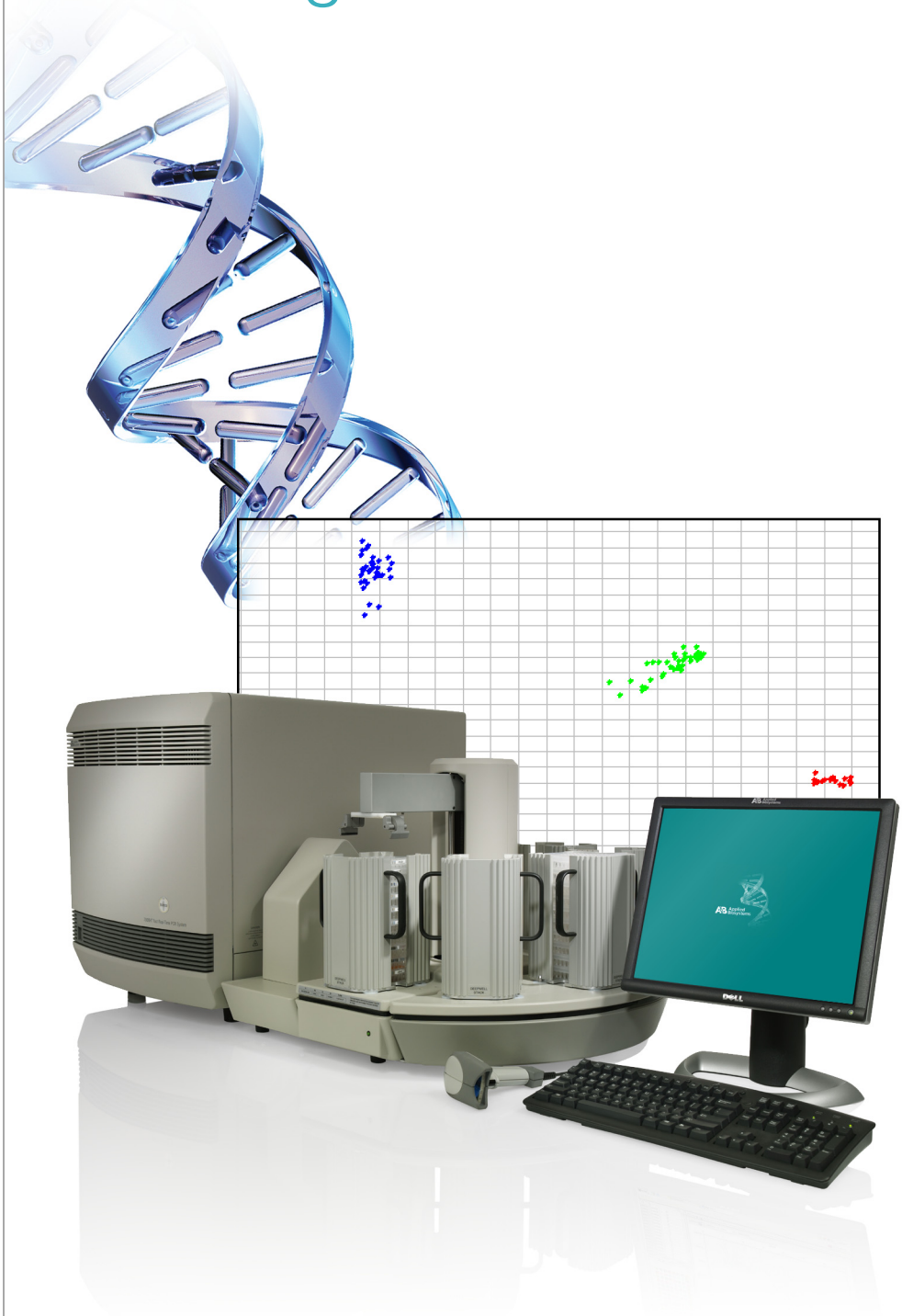
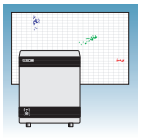
Applied Biosystems

Allelic Discrimination Getting Started Guide

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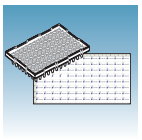
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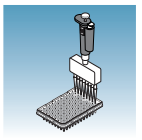
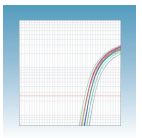
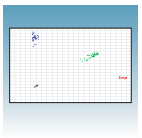

Preparing the Samples and Reaction Plate

Performing an Allelic Discrimination Pre-Read Run


Performing an Amplification Run


Performing and Analyzing an Allelic Discrimination Post-Read Run

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Authorized Thermal Cycler

The Applied Biosystems 7900HT Fast Real-Time PCR System Base Unit, Serial No_____, in combination with its immediately attached sample block modules, comprise an instrument that is an Authorized Thermal Cycler. The purchase price of this Base Unit includes the up-front fee component of a license under the non-U.S. counterparts of U.S. Patents Nos. 4,683,202, 4,683,195 and 4,965,188 owned by F. Hoffmann-La Roche Ltd. (Roche) covering the Polymerase Chain Reaction (PCR) process to practice the PCR process for internal research and development using this instrument. The running royalty component of that license may be purchased from Applied Biosystems or obtained by purchasing Authorized Reagents. This instrument is also an Authorized Thermal Cycler for use with applications licenses available from Applied Biosystems. Its use with Authorized Reagents also provides a limited PCR license in accordance with the label rights accompanying such reagents. Purchase of this product does not itself convey to the purchaser a complete license or right to perform the PCR process. Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

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Preface

How to Use This Guide

Purpose of This Guide

This guide provides procedures for conducting allelic discrimination assays using the Applied Biosystems 7900HT Fast Real-Time PCR System (7900HT Fast System).

Audience

This guide is intended for principal investigators and laboratory staff who conduct allelic discrimination assays using the 7900HT 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 plan to integrate the 7900HT Fast System into your existing laboratory data flow

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**.

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.

CAUTION 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.

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

Safety

Follow specific safety practices when using this instrument. For safety guidelines, refer to the "Safety and EMC Compliance" section in the *Applied Biosystems 7900HT Fast Real-Time PCR System Site Preparation Guide* (PN 4351923).

For any chemical manufactured or distributed by Applied Biosystems, you can obtain the MSDS from Applied Biosystems. This service is available free 24 hours a day. To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**.
2. In the MSDS Search pane:
   a. Type in the chemical name, part number, or other information that would appear in the MSDS of interest.
   b. Select the language of your choice.
   c. Click **Search**.
3. In the Search Results page, find the document of interest.
4. To view, print, or download the document:
   a. Right-click the document title.
   b. Select one of the following options:
      – **Open** – To view the document
      – **Print Target** – To print the document
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5. To have a copy of the document sent by fax or e-mail:
   a. Select the **Fax** or **Email** checkbox beneath the document title.
   b. Click **RETRIEVE DOCUMENTS** at the end of the document list.
   c. Enter the required information.
   d. Click **View/Deliver Selected Documents Now**.

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

How to Obtain More Information

Related Documentation

For more information about using the 7900HT Fast System and performing assays, refer to:

- *Applied Biosystems 7900HT Fast Real-Time PCR System Allelic Discrimination Getting Started Guide* (PN 4364015)
- *Applied Biosystems 7900HT Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide* (PN 4364014)
- *Applied Biosystems 7900HT Fast Real-Time PCR System Plus-Minus Getting Started Guide* (PN 4364017)
- *Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide* (PN 4364016)
- *Sequence Detection Systems Software version 2.3 Online Help (SDS Online Help)*
- *Applied Biosystems 7900HT Fast Real-Time PCR System Maintenance and Troubleshooting Guide* (PN 4365542)
- *Applied Biosystems 7900HT Fast Real-Time PCR System Site Preparation Guide* (PN 4351923)
- *ABI PRISM® 6100 Nucleic Acid PrepStation Users Guide* (PN 4326242)
- *Assays-by-Design Service for SNP Assays Protocol* (PN 4334431)
- *DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol* (PN 4343586)
- *NucPrep® Chemistry Protocol: Isolation of Genomic DNA from Animal and Plant Tissue* (PN 4333959)
- *Primer Express® Software v3.0 Getting Started Guide* (PN 4362460)
- *Real-Time PCR Systems Chemistry Guide* (PN 4348358)
- *TaqMan® Low Density Array Getting Started Guide* (PN 4319399)
- *TaqMan® SNP Genotyping Assays Protocol* (PN 4332856)
- *TaqMan® Universal PCR Master Mix Protocol* (PN 4304449)
- *TransPrep Chemistry Protocol: Purification of gDNA from Filtrates Obtained After the Isolation of RNA from Homogenized Animal or Plant Tissue Samples* (PN 4326965)

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At the Support page, you can:

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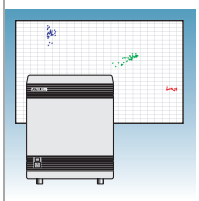
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates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



Introduction

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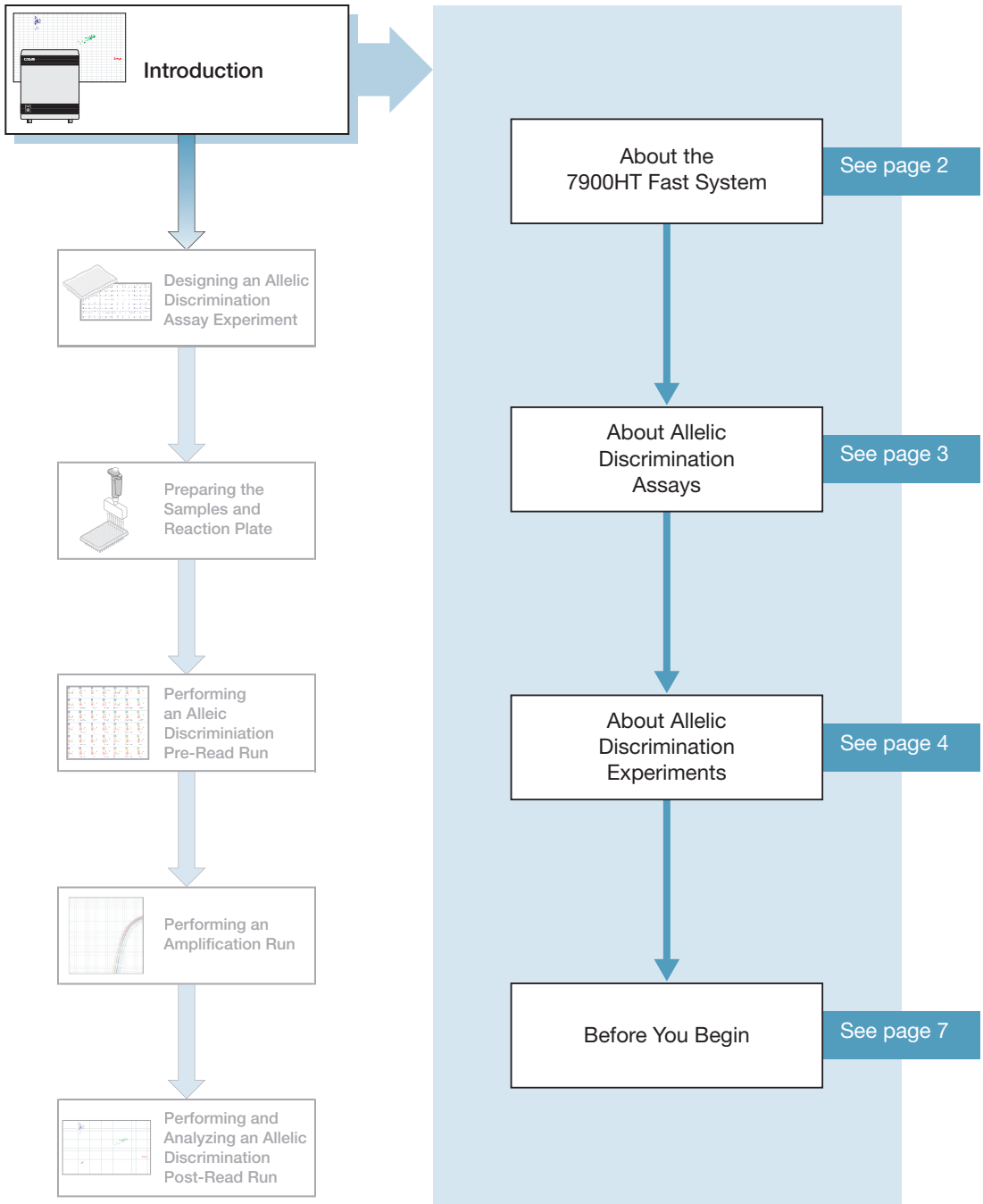


Notes

About the 7900HT Fast System

System Description

The Applied Biosystems 7900HT Fast Real-Time PCR System (7900HT 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

For more information on the 7900HT Fast System, refer to the *Sequence Detection Systems Software version 2.3 Online Help (SDS Online Help)* and the *Applied Biosystems 7900HT Fast Real-Time PCR System Maintenance and Troubleshooting Guide* (PN 4365542).

Note: To access the *SDS Online Help*, select **Help > SDS Online Help** from the SDS software menu bar.

Supported Assays and Consumables

You can perform several assay types on the 7900HT Fast System using reactions plates in the 96-well, 384-well, or TaqMan® Low Density Array format. This guide describes the allelic discrimination assay.

	Reaction Plate and System Block Options			
Assay Type	Fast 96-well	Standard 96-well	Standard 384-well	TaqMan® Low Density Array (TLDA)
Standard curve (AQ)	Yes	Yes	Yes	No
Comparative C_T (RQ)	Yes	Yes	Yes	Yes
Allelic discrimination	No	Yes	Yes	No
Plus/minus	No	Yes	Yes	No

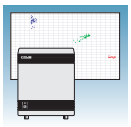
Allelic Discrimination Assay Configuration

Allelic discrimination assays can be run on the 7900HT Fast System using standard 96-well and standard 384-well reaction plates with standard reagents and standard protocols.

IMPORTANT! Allelic discrimination assays are not supported using Fast reaction plates, Fast reagents, Fast protocols, or the TaqMan® Low Density Array.

IMPORTANT! Be sure you use standard reaction plates on the 7900HT Fast System with a standard block. Fast 96-well reaction plates do not fit into the standard 96-well block and standard 96-well reaction plates do not fit into the Fast 96-well block.

Notes



About Allelic Discrimination Assays

Definition

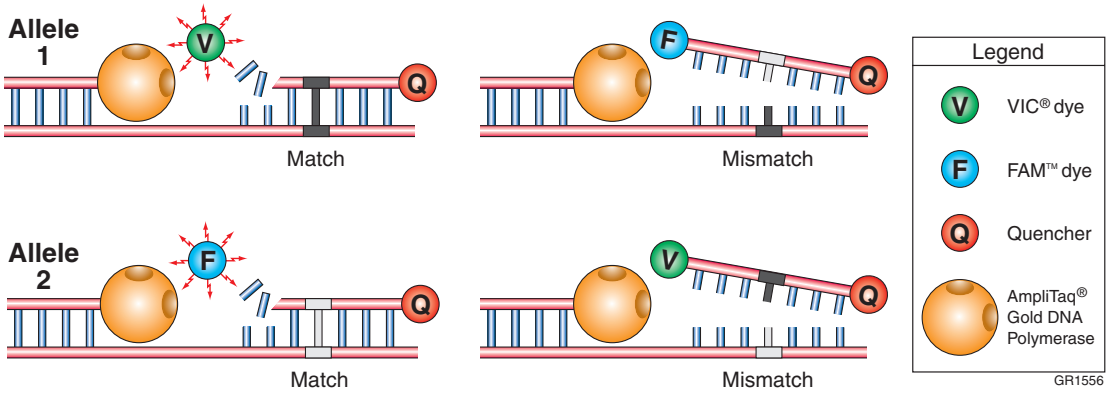
An allelic discrimination assay is a multiplexed (more than one primer/probe pair per reaction) end-point (data is collected at the end of the PCR process) assay that detects variants of a single nucleic acid sequence. The presence of two primer/probe pairs in each reaction allows genotyping of the two 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 allelic discrimination assay, a unique pair of fluorescent dye detectors is used, for example, two TaqMan® MGB (minor groove binder) probes that target an SNP site (Afonina *et al.,* 1997; Kutyavin *et al.*, 1997). 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 allelic discrimination assay measures the change in fluorescence of the dyes associated with the probes. The figure below illustrates results from matches and mismatches between target and probe sequences in TaqMan® SNP Genotyping Assays (Livak *et al.,* 1995).



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

Notes

Terms Used in Allelic Discrimination Analysis

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 (Kwok and Higuchi, 1989).
Nucleic acid target (also called “target template” or “target”)	Nucleotide sequence that you want to genotype.
Unknown sample (U; also called “sample of interest”)	The sample for which you want to determine the genotype of 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.

About Allelic Discrimination Experiments

Allelic Discrimination Experiment Workflow

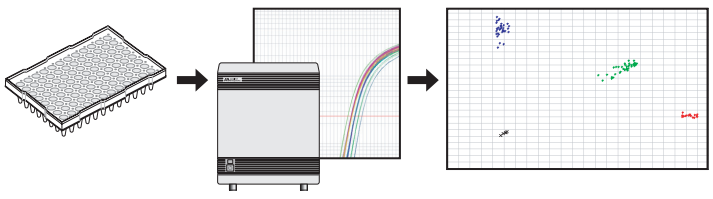
After you design an allelic discrimination experiment and prepare the DNA samples, you need to perform:

- **An amplification run** using a Standard Curve (AQ) plate document to generate real-time PCR data. The real-time PCR data can be used to analyze and troubleshoot the PCR data for the allelic discrimination assay, if needed.

  Note: The amplification run can be performed either on the 7900HT Fast System or offline using any thermal cycler.

- **An allelic discrimination run** using an Allelic Discrimination (AD) plate document. The SDS software analyzes the data, then you assign allele calls (automatically or manually).

The following figure illustrates the complete process:



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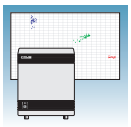

Sample Experiment

A sample experiment representing a typical allelic discrimination experiment is provided in Appendix A on page 73. You can use the summarized procedures of the sample experiment in Appendix A to familiarize yourself with the entire allelic discrimination assay workflow.

References to the sample experiment are provided in Chapter 2 through Chapter 6, where applicable.

Required User-Supplied Materials

Chemistry/Reagents

Item	Source
Any of the following DNA isolation and purification chemistry systems:	
• ABI PRISM® 6100 Nucleic Acid PrepStation	Applied Biosystems (PN 6100-01)
• ABI PRISM® TransPrep System (purification of gDNA after isolation of RNA from animal and plant tissue)	Applied Biosystems web site
• 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® SNP Genotyping Assays, Custom TaqMan® SNP Genotyping Assays, and custom probes/primer design with Primer Express® Software, use either:	
• TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 200 reactions	Applied Biosystems (PN 4324018)
• TaqMan® Universal PCR Master Mix	Applied Biosystems (PN 4304437)
For TaqMan® Pre-Developed Assay Reagents for Allelic Discrimination (TaqMan® PDARs for Allelic Discrimination) use:	
• TaqMan® Universal PCR Master Mix	Applied Biosystems (PN 4304437)
Labeled primers and probes from one of the following sources:	
• TaqMan® SNP Genotyping Assays (predesigned primers and probes)	Applied Biosystems web site
• TaqMan® PDARs for Allelic Discrimination	Applied Biosystems web site
• Custom TaqMan® SNP Genotyping Assays (predesigned primers and probes)	Applied Biosystems web site
• Primer Express® Software (custom-designed primers and probes)	Contact your Applied Biosystems sales representative.

Notes

Reaction Plates and Covers

IMPORTANT! Do not use MicroAmp® caps (domed) or Optical Tubes with the 7900HT Fast System. You can use Optical Caps (PN 4323032) *only* on the standard 96-well plates with the 7900HT Fast System.

Item	Source
Standard 96-well reaction plates	
• MicroAmp® 96-Well Optical Reaction Plate with Barcode (code 128), 20 plates	Applied Biosystems (PN 4306737)
• MicroAmp® 96-Well Optical Reaction Plate with Barcode (code 128), 25-Pack, 500 plates Includes 25 of PN 4306737, MicroAmp® 96-Well Optical Reaction Plates with Barcode	Applied Biosystems (PN 4326659)
• MicroAmp® 96-Well Optical Reaction Plate with Barcode (code 128) and ABI PRISM® Optical Adhesive Covers, 100 plates/100 covers Includes 100 ABI PRISM® Optical Adhesive Covers (PN 4311971) and 5 of PN 4306737, MicroAmp® 96-Well Optical Reaction Plates with Barcode	Applied Biosystems (PN 4314320)
• MicroAmp® Splash Free Support Base for 96-Well Reaction Plates, 10 bases	Applied Biosystems (PN 4312063)
Standard 384-well reaction plates	
• 384-Well Clear Optical Reaction Plate with Barcode (code 128), 50 plates	Applied Biosystems (PN 4309849)
• 384-Well Clear Optical Reaction Plate with Barcode (code 128), 10-Pack, 500 plates Includes 10 of PN 4309849, 384-Well Clear Optical Reaction Plates with Barcode	Applied Biosystems (PN 4326270)
Optical adhesive covers	
• ABI PRISM® Optical Adhesive Cover Starter Kit, 20 covers Includes 20 ABI PRISM® Optical Adhesive Covers, an Applicator, and an ABI PRISM® Optical Cover Compression Pad.	Applied Biosystems (PN 4313663)
• ABI PRISM® Optical Adhesive Covers, 100 covers	Applied Biosystems (PN 4311971)
• ABI PRISM® Optical Adhesive Covers, 25 covers	Applied Biosystems (PN 4360954)
• Optical Caps, 8 Caps/Strip, 2400 Caps/300 Strips	Applied Biosystems (PN 4323032)

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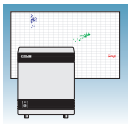

Other Consumables and Equipment

Item	Source
Centrifuge with adapter for standard 96-well or standard 384-well reaction 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

Before You Begin

Background and Pure Dye Calibrations

Check that background and pure dye calibrations have been performed regularly to ensure optimal performance of the 7900HT Fast System. For more information about background and pure dye calibrations, refer to the *Sequence Detection Systems version 2.3 Software Online Help (SDS Online Help)* and the *Applied Biosystems 7900HT Fast Real-Time PCR System Maintenance and Troubleshooting Guide* (PN 4365542).

Accessing the SDS Online Help

Some steps in this chapter refer you to the *SDS Online Help* for more information. To access the *SDS Online Help*, select **Help > SDS Online Help** from the SDS software menu bar.

Automation Options

The 7900HT Fast System can run prepared reaction plates individually or in groups using the Automation Accessory with the Zymark® Twister Microplate Handler. If you are not using the Automation Accessory, you must run reaction plates individually.

For clarity, this chapter only illustrates running an individual reaction plate. For information on running multiple reaction plates, see Appendix B on page 83. For information on automated operation of the 7900HT Fast System using the Automation Accessory, see the *SDS Online Help*.

Notes

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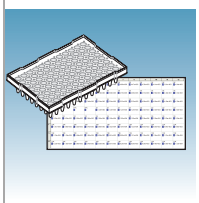
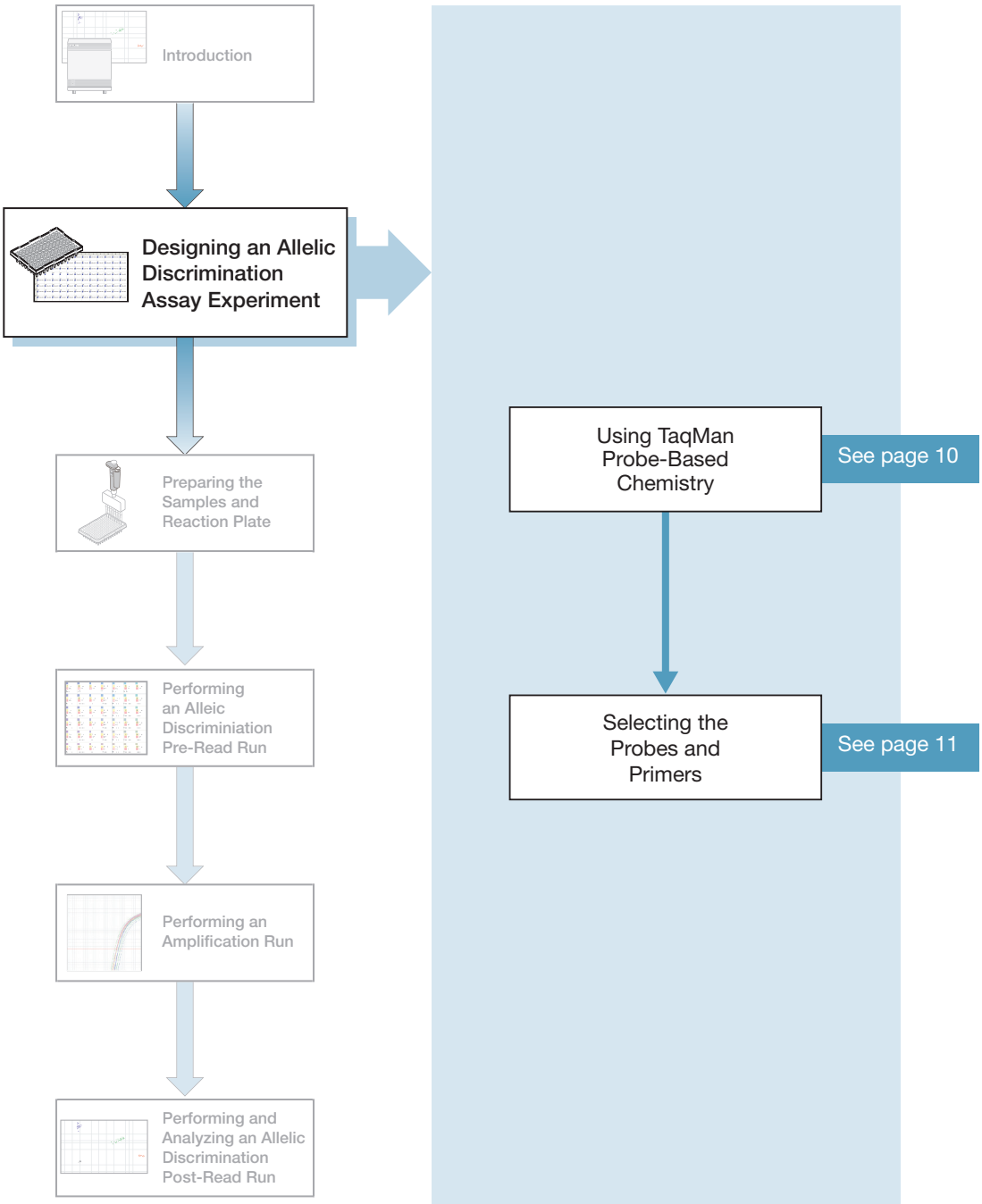


Designing an Allelic Discrimination Assay Experiment



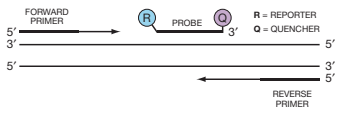
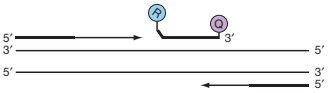
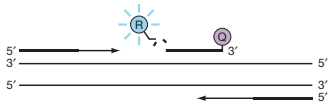
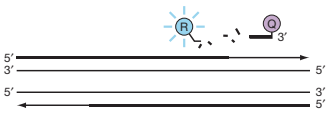
Notes

Using TaqMan Probe-Based Chemistry

About the Chemistry

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

IMPORTANT! The SYBR® Green I dye chemistry and Fast chemistry are not supported for allelic discrimination assays.

Description	Process
TaqMan probe-based chemistry uses a fluorogenic probe to detect specific PCR product as it accumulates during PCR cycles (Lee *et al.,* 1993).	**Step 1: Polymerization** A reporter (R) and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe.  **Step 2: Strand Displacement** When both dyes are attached to the probe, reporter dye emission is quenched.  **Step 3: Cleavage** During each extension cycle, the Applied Biosystems hot-start DNA polymerase system cleaves the reporter dye from the probe.  **Step 4: Polymerization Completed** After being separated from the quencher, the reporter dye emits its characteristic fluorescence. 

Two Types of TaqMan Probes

Applied Biosystems offers two types of TaqMan probes:

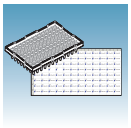
- TaqMan probes with TAMRA™ dye as a quencher
- TaqMan® MGB (minor groove binder) probes with non-fluorescent quencher (NFQ)

For more information about TaqMan probe-based chemistry, refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4348358).

Chemistry Kits for Allelic Discrimination Assays

- TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 200 reactions (PN 4324018)
- TaqMan® Universal PCR Master Mix (PN 4304437, contains AmpErase® UNG)

Notes



Selecting the Probes and Primers

Each allelic discrimination primer/probe set contains two probes, one probe for allele 1 and one probe for allele 2. Applied Biosystems provides three options for selecting primers and probes:

1. **TaqMan® SNP Genotyping Assays** – Provide biologically informative, fully validated, or predesigned TaqMan® MGB-probe-based assays for genotyping single nucleotide polymorphisms (SNPs). For information on available TaqMan® SNP Genotyping Assays:
   a. Go to **http://www.appliedbiosystems.com**.
   b. Click the **myScience** tab at the top of the page to go to the myScience Genomic Products page.
   c. In the Genotyping section, click the TaqMan® SNP Genotyping Assays **Search** option.
   d. Use specific filtering criteria to search for the assay of interest.

2. **Custom TaqMan® SNP Genotyping Assays** – Designs, synthesizes, formulates, and delivers quality-controlled primer and probe sets. Use this service if the assay you need is not currently available. For ordering information:
   a. Go to **http://www.appliedbiosystems.com**.
   b. Click the **myScience** tab at the top of the page to go to the myScience Genomic Products page.
   c. In the Genotyping section, click the Custom TaqMan® SNP Genotyping Assays **Info** option.

3. **TaqMan® Pre-Developed Assay Reagents for Allelic Discrimination (TaqMan® PDARs for Allelic Discrimination)** – Provide optimized assays for the discrimination of specific alleles. For ordering information:
   a. Go to **http://www.appliedbiosystems.com**.
   b. In the Search section, select **All Sections**, type **PDAR**, then click **Go**.
   c. In the Search Results section, click **TaqMan® Pre-Developed Assay Reagents for Allelic Discrimination**.
   d. Click the **Ordering Information** tab.

Notes

4. **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).

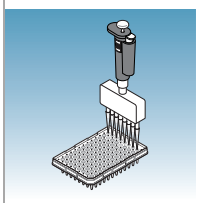
   Applied Biosystems provides Assay Design Guidelines, which have been developed specifically for quantification assays (pertinent to the amplification step in allelic discrimination assays). When used in their entirety, these steps provide a rapid and reliable system for assay design and optimization. For information about the Assay Design Guidelines, refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4348358).

Sample Experiment

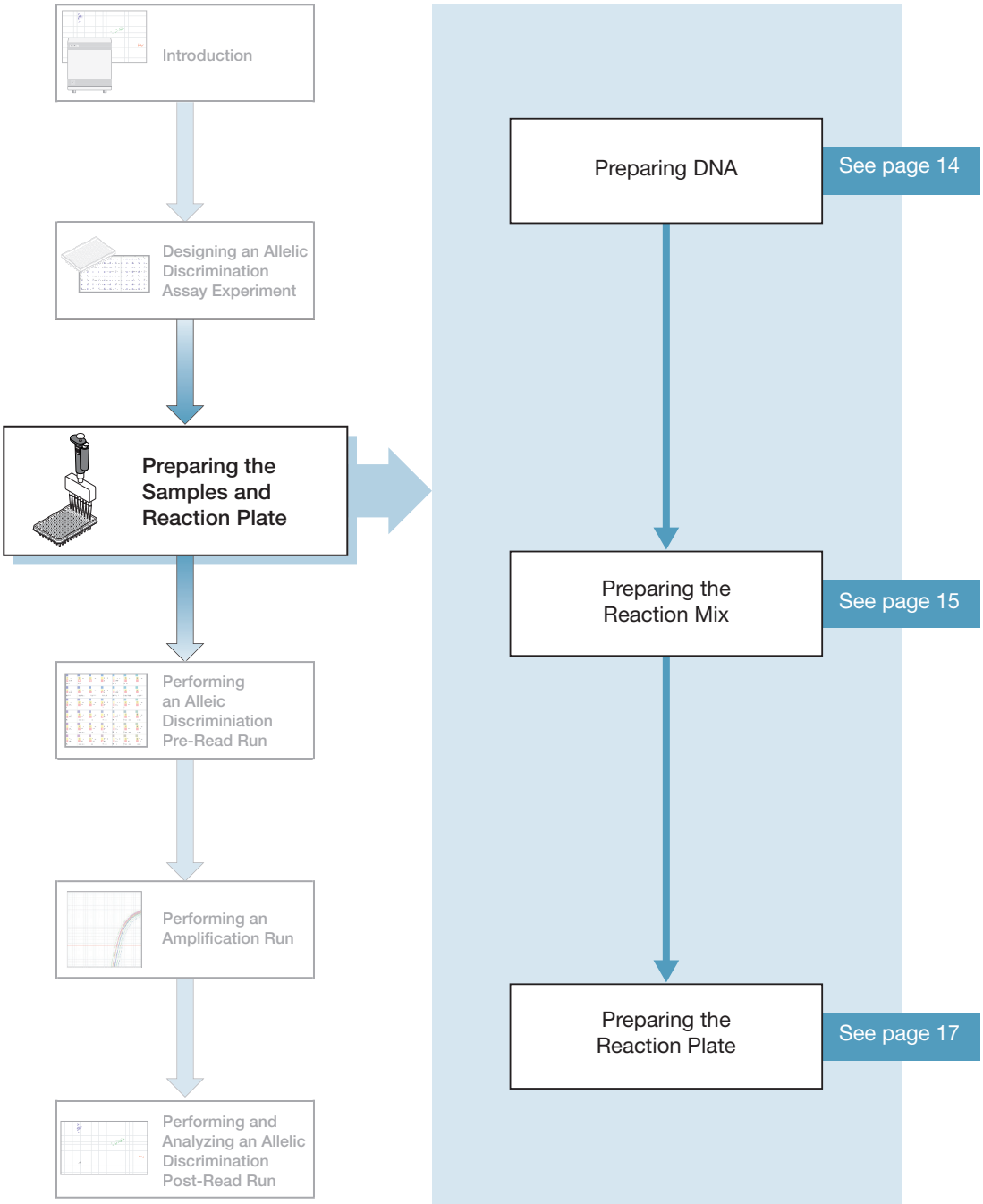
The objective of the sample experiment was to determine the genotype of 92 individuals at the SNP site of a desired target sequence.

The primers and probes were ordered from TaqMan® SNP Genotyping Assays (AB Assay ID C 2984390 10). The probe for allele 1 (AL-1) was labeled with VIC® dye; the probe for allele 2 (AL-2) was labeled with FAM™ dye.

Notes



Preparing the Samples and 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
ABI PRISM® 6100 Nucleic Acid PrepStation	6100-01
ABI PRISM® TransPrep System (purification of gDNA after isolation of RNA from animal and plant tissue)	Applied Biosystems web site
BloodPrep™ Chemistry (genomic DNA from fresh or frozen blood)	4346860
NucPrep® Chemistry (DNA from animal and plant tissue)	4340274

For more information, refer to:

- *ABI PRISM® 6100 Nucleic Acid PrepStation Users Guide* (PN 4326242)
- *TransPrep Chemistry Protocol: Purification of gDNA from Filtrates Obtained After the Isolation of RNA from Homogenized Animal or Plant Tissue Samples* (PN 4326965)
- *DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol* (using BloodPrep™ Chemistry, PN 4343586)
- *NucPrep® Chemistry Protocol: Isolation of Genomic DNA from Animal and Plant Tissue* (PN 4333959)

Quality of DNA

Ensure that the DNA you use for allelic discrimination assay experiments:

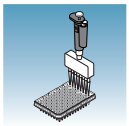
- Is extracted from the raw material you are testing with an optimized protocol
- Does not contain PCR inhibitors
- Has an $A_{260/280}$ ratio greater than 1.7
- Is intact as visualized by gel electrophoresis
- Has not been heated above 60 °C, which can cause degradation

Sample Experiment

In the sample experiment, genomic DNA was isolated from blood using:

- A BloodPrep™ Chemistry Kit
- The recommended template for TaqMan® SNP Genotyping Assays: purified genomic DNA (1 to 20 ng). The final concentration of genomic DNA for all samples in the sample experiment was 10 ng/µL.

Notes



Preparing the Reaction Mix

Reagents and Protocols

Allelic discrimination assays can be run on the 7900HT Fast System using standard reagents and standard protocols.

IMPORTANT! Allelic discrimination assays are not supported using Fast reagents or Fast protocols.

Custom-Designed 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* (PN 4348358) to optimize primer and probe concentrations.

If you obtain your assay from the Custom TaqMan® SNP Genotyping Assays, follow instructions in the protocol for Custom TaqMan® SNP Genotyping Assays: *Assays-by-Design Service for SNP Assays Protocol* (PN 4334431).

TaqMan PDARs for Allelic Discrimination

TaqMan PDARs for Allelic Discrimination require only three components:

- Genomic DNA sample
- Allelic Discrimination Assay Mix (10×), specific for each polymorphism
- TaqMan® Universal PCR Master Mix (2×)

Note: Allele 1 and 2 control DNA is included with each assay, allowing each homozygote signal to be generated on each run.

For instructions on how to use TaqMan PDARs for Allelic Discrimination, refer to the *Pre-Developed TaqMan® Assay Reagents Allelic Discrimination Protocol* (PN 4312214).

TaqMan SNP Genotyping Assays

The allelic discrimination reaction mix contains:

- SNP Genotyping Assay Mix (20×)
- Master mix:
  - TaqMan® Universal PCR Master Mix, No AmpErase® UNG, *or*
  - TaqMan® Universal PCR Master Mix

Notes

The reagents, volumes, and final concentrations provided below are for wet DNA samples and are excerpted from the *TaqMan® SNP Genotyping Assays Protocol* (PN 4332856).

Note: If you are using dried-down DNA samples, refer to the *TaqMan® SNP Genotyping Assays Protocol* (PN 4332856) for instructions on preparing the reaction mix.

Preparing the Reaction Mix

1. Calculate the number of reactions to be performed for each assay.
2. Calculate the volume of components needed for all wells on the reaction plate:

Component	Volume (µL/reaction)	
	Standard 96-Well Reaction Plate	Standard 384-Well Reaction Plate
• TaqMan® Universal PCR Master Mix (2×), No AmpErase® UNG, *or* • TaqMan® Universal PCR Master Mix (2×)	12.50	2.50
SNP Genotyping Assay Mix (20×)	1.25	0.25
Total	13.75	2.75

Note: Prepare extra volume to account for pipetting losses.

3. Swirl the bottle of master mix gently to resuspend.

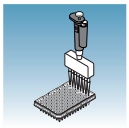
CAUTION **CHEMICAL HAZARD. TaqMan® Universal PCR Master Mix (2×), No AmpErase® UNG,** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

CAUTION **CHEMICAL HAZARD. TaqMan® Universal PCR Master Mix (2×)** 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 SNP Genotyping Assay Mix (20×) briefly.

WARNING **CHEMICAL HAZARD. SNP Genotyping Assay Mix (20×)** 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.

Notes



5. Pipette the volumes required for all wells on the reaction plate (plus extra volume to account for pipetting losses) of master mix and SNP Genotyping Assay Mix (20×) into a microcentrifuge tube. Cap the tube.

Sample Experiment

For the sample experiment:

- The reaction mix was prepared with TaqMan® Universal PCR Master Mix (2×), No AmpErase® UNG.
- Volumes were calculated for a standard 384-well reaction plate. Enough reaction mix was prepared for 4 NTCs and 92 samples/unknowns, plus 10% extra to account for pipetting losses.

Component	Volume (µL) for One Reaction	Volume (µL) for 106 Reactions[a]
TaqMan® Universal PCR Master Mix (2×), No AmpErase® UNG	2.5	265.0
SNP Genotyping Assay Mix (20×)	0.25	26.5
Total	2.75	291.5
a. Extra volume was included to account for pipetting losses.		

Preparing the Reaction Plate

Reaction Plate Formats

Allelic discrimination assays can be run on the 7900HT Fast System using standard 96-well and standard 384-well reaction plates.

IMPORTANT! Allelic discrimination assays are not supported using Fast reaction plates or the TaqMan® Low Density Array.

Reaction Plate Components

A reaction plate for an allelic discrimination assay contains the following:

- Reaction mix
- No Template Controls (NTCs)
- Samples: Unknown genomic DNA
- Nuclease-free water
- Controls (optional): Known genomic DNA

The recommended final reaction volumes are:

- 25 µL for a standard 96-well reaction plate
- 5 µL for a standard 384-well reaction plate

Notes

Preparing the Reaction Plate

1. Invert the reaction mix tube prepared in the previous section to mix.
2. Centrifuge the tube briefly to spin down the contents and to eliminate air bubbles.
3. Pipette the appropriate reaction mix volume into each well of the reaction plate:
   - 13.75 µL per well for a standard 96-well reaction plate
   - 2.75 µL per well for a standard 384-well reaction plate
4. Dilute 1 to 20 ng of each sample into nuclease-free water for a total sample volume of:
   - 11.25 µL per well for a standard 96-well reaction plate
   - 2.25 µL per well for a standard 384-well reaction plate

Sample Experiment

For the sample experiment, the recommended template for TaqMan® SNP Genotyping Assays was used: purified genomic DNA (1 to 20 ng). The final concentration of genomic DNA for all samples in the sample experiment was 10 ng/µL.

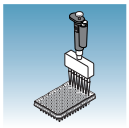
5. Pipette the following volumes of NTC, sample, and (if using) control into the appropriate wells of your reaction plate:

Component	Volume (µL/reaction)	
	Standard 96-Well Reaction Plate	Standard 384-Well Reaction Plate
NTC: Nuclease-free water or TE (Tris-EDTA) buffer	11.25	2.25
Sample: Unknown genomic DNA	11.25	2.25
Optional Control: Known genomic DNA	11.25	2.25

Note: Include NTCs on each reaction plate for both manual evaluation and optimal auto-calling of allelic discrimination assay data. If available, known genomic DNA controls may be included and used to corroborate assay performance.

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

Notes

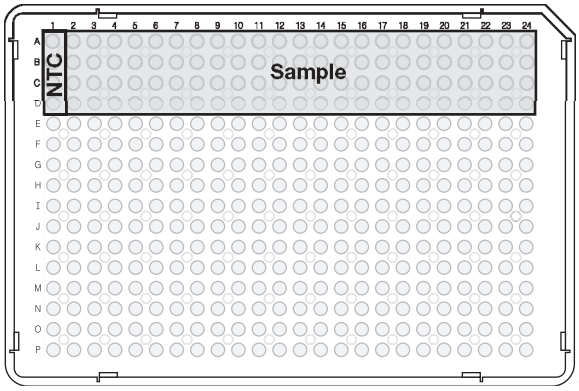


Sample Experiment

To set up the reaction plate for the sample experiment:

- 2.75 µL of reaction mix were pipetted into each well of a standard 384-well reaction plate.
- 2.25 µL of NTC or sample/unknown were added to the wells, as shown below.

To Prepare...	We added...	To wells...
NTC	2.25 µL of nuclease-free water or TE buffer	A1, B1, C1, and D1
Sample or Unknown	2.25 µL of diluted purified genomic DNA	A2 to A24 B2 to B24 C2 to C24 D2 to D24



6. Cover the reaction plate with an optical adhesive cover or Optical Caps.

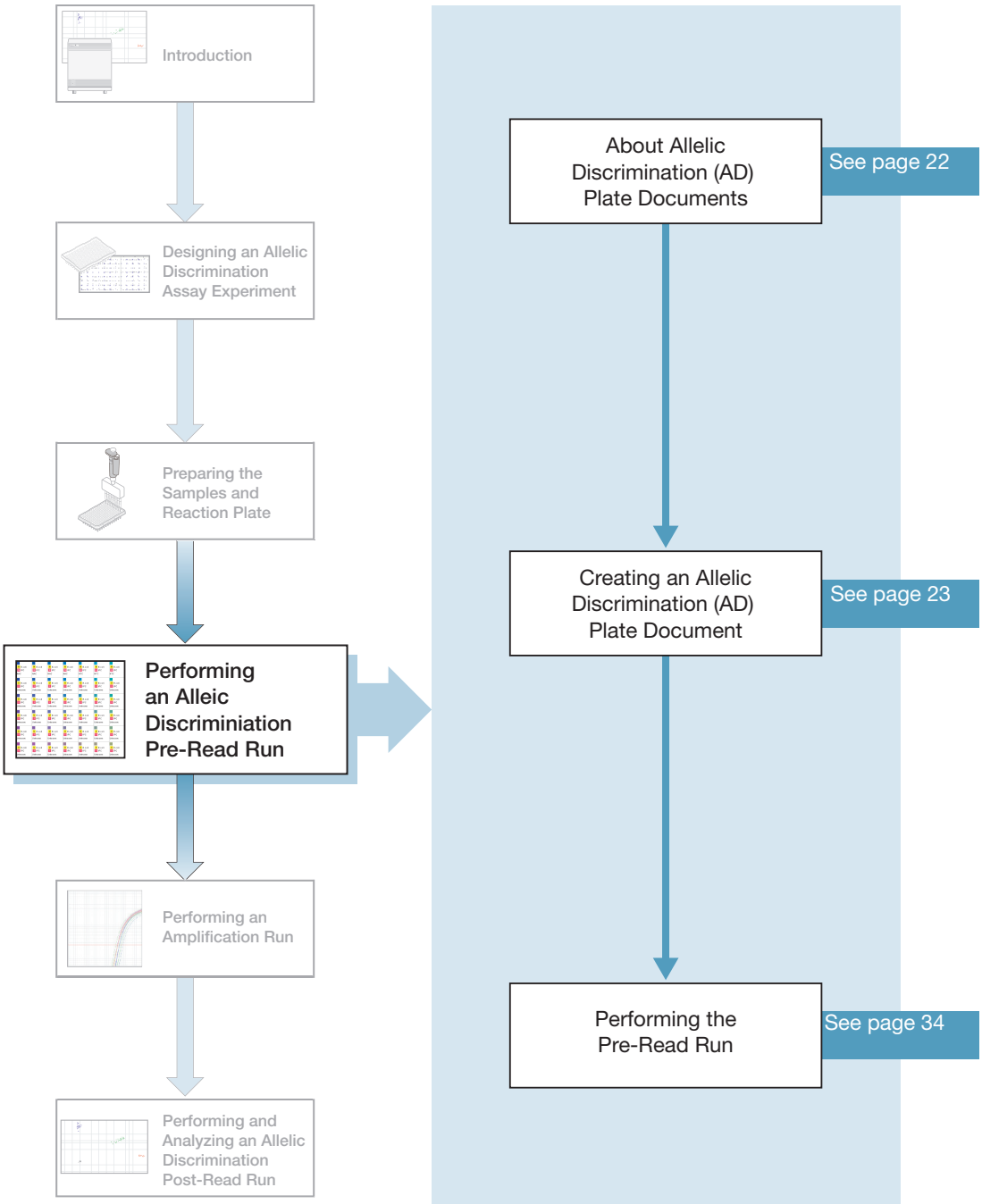
   IMPORTANT! Do not use MicroAmp® caps (domed) or Optical tubes with the 7900HT Fast System. You can use Optical Caps (PN 4323032) *only* on the standard 96-well plates with the 7900HT Fast System.

7. Keep the reaction plate on ice until loading in the 7900HT Fast System.

Notes

Notes

Performing an Allelic Discrimination Pre-Read Run



Notes

About Allelic Discrimination (AD) Plate Documents

An Allelic Discrimination (AD) plate document is an SDS software document that stores data collected from an allelic discrimination assay run for a single reaction plate (standard 96-well or standard 384-well reaction plate). Allelic Discrimination (AD) plate documents also store other information about the run, including sample names and detectors.

Plate Document Parameters

When you create an Allelic Discrimination (AD) plate document, you define specific parameters for each allelic discrimination assay reaction plate:

- **Detectors** – A virtual representation in the SDS software 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™ dye), and allele 2 is detected by the second detector (for example, VIC® dye).
- **Tasks** – A setting that you apply to the markers in a well of a plate document, which determines the way the SDS software uses the data collected from the well during analysis. Allelic Discrimination (AD) plate document markers use two types of tasks:

Task	Symbol	Apply to markers of wells that contain...
Unknown	U	PCR reagents for the amplification of target sequences
No Template Control (NTC)	N	no target template

Options for Creating a Plate Document

This section describes how to create a plate document using the New Plate Document Wizard. For information about other ways to create plate documents, see the *SDS Online Help*.

Reaction Plate Options

The allelic discrimination assay can be run on either a standard 96-well reaction plate or a standard 384-well reaction plate. For clarity, this chapter only illustrates running a standard 384-well reaction plate.

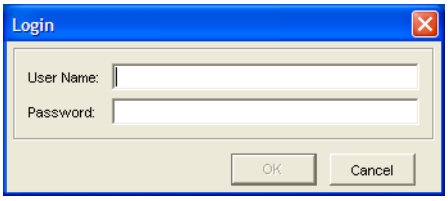
Notes

Creating an Allelic Discrimination (AD) Plate Document

1. If it is not already running, double-click on the desktop (or select **Start > All Programs > Applied Biosystems > SDS 2.3 > SDS 2.3**) to start the SDS software.

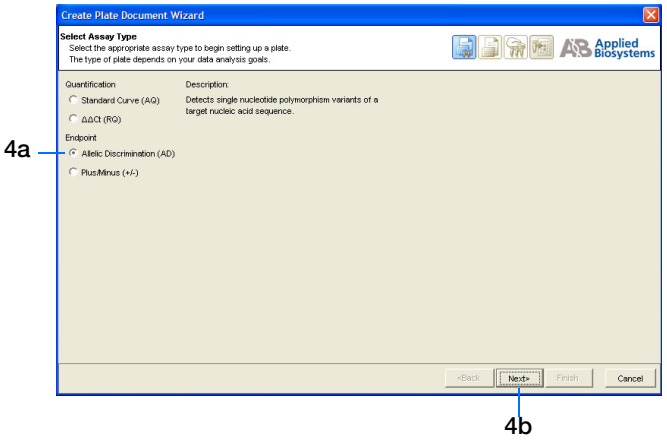
2. If your System Administrator has enabled the login option, the Login dialog box appears. Enter your **User Name** and **Password**, then click **OK**.

   Note: If the login option is not enabled, no Login dialog box appears. Skip to step 3 below.



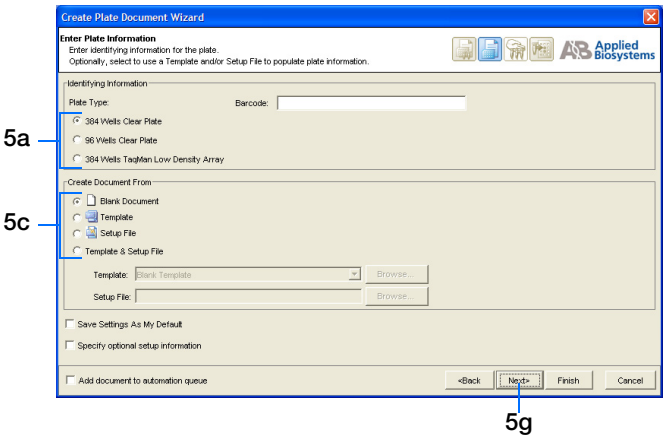
3. In the SDS software menu bar, click (or select **File** > **New Plate Wizard**) to open the Create Plate Document Wizard.

4. Select the assay type:
   a. Select **Allelic Discrimination (AD)**.
   b. Click **Next>**.



5. Enter the plate information:
   a. Select a Plate Type: **384 Wells Clear Plate** or **96 Wells Clear Plate**.
   b. *Optional.* In the Barcode field, scan or enter the barcode for the reaction plate.

   Note: The Automation Accessory must be installed in order to use barcodes. A barcode is required if you are adding the plate document to the automation queue. See step f on page 24.



Notes

c. In the Create Document From pane, select your preferred source.

Note: You can create a plate document from scratch (select **Blank Document**) or you can create a plate document from a template and/or an existing plate document (select **Template**, **Setup File**, or **Template & Setup File**). For more information, see the *SDS Online Help*.

---

d. *Optional.* Check **Save Settings As My Default**. Check this option if you want the SDS software to automatically apply the current page settings every time you use the Create Plate Document Wizard.

e. *Optional.* Check **Specify optional setup information**. Check this option if you want to enter a name, study, or comments for the plate document.

f. *Optional.* Check **Add document to automation queue**. Check this option if you want to add the finished plate document to the Automation Controller software plate queue.

Note: This checkbox is only enabled if you are connected to an Automation Accessory. For more information, see Appendix B on page 83.

Note: This checkbox appears on subsequent pages of the Create Plate Document Wizard. You only need to select this option on one page to enable it.

---

g. Click **Next>**.

Notes

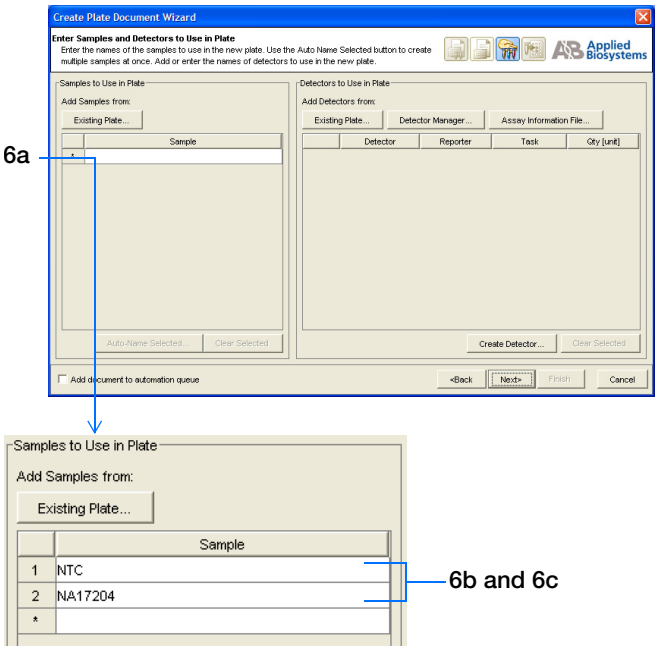
6. Enter the samples to use in the plate:

a. Double-click in the Sample field to activate the cursor.

b. Type a sample name (for example, **NTC**), then press **Enter**. The sample row is numbered and another row appears.

c. Repeat steps a through b for your remaining samples.

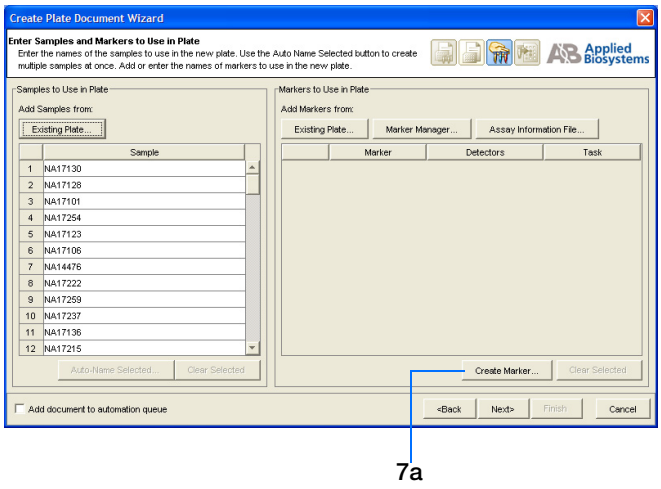
Note: You may also copy sample names from an existing plate document (click **Existing Plate**) or enter multiple sample names at once (click **Auto-Name Selected**). For more information, see the *SDS Online Help*.



7. Enter markers to use in the plate:

a. Click **Create Marker** to open the Marker Information dialog box.

Note: You can also add existing markers from other sources (click **Existing Plate**, **Marker Manager**, or **Assay Information File**). For more information, see the *SDS Online Help*.



Notes

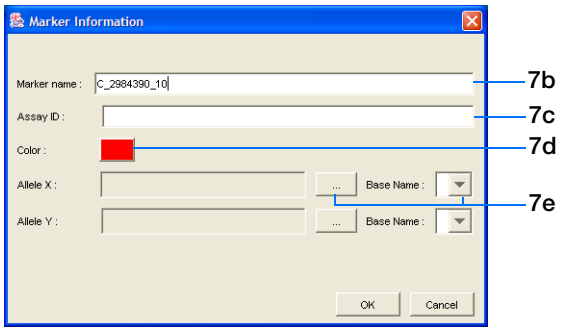
b. Enter a **Marker name** (for example, **C_2984390_10**).

Note: The marker name must be unique and it should reflect the locus or polymorphism it targets.

c. Leave the Assay ID field blank.

d. Click the **Color** box to open the Marker Color dialog box, select a color to represent the marker, then click **OK**.

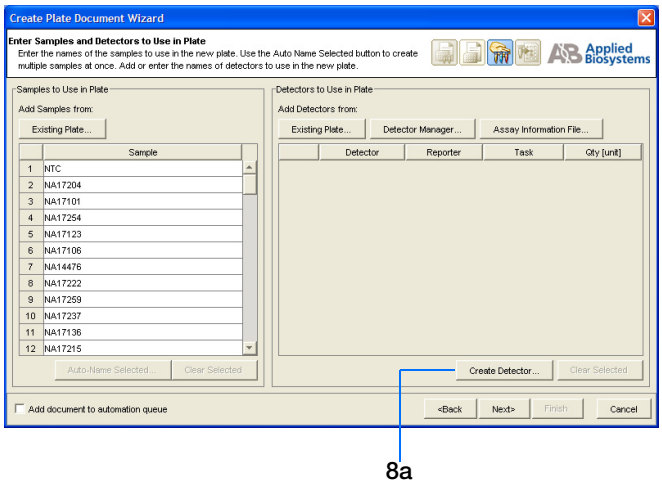
e. Click the Allele X **Browse** button (...) to open the Detector Manager.



8. Enter detectors to use in the plate:

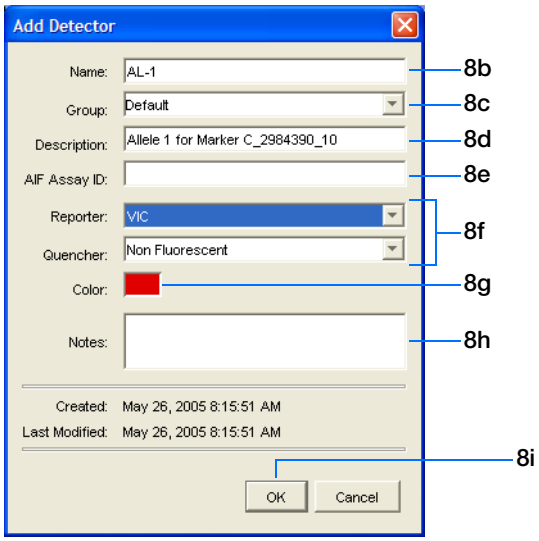
a. Click **Create Detector** to open the Add Detector dialog box.

Note: You may also add detectors from other sources (click **Existing Plate**, **Detector Manager**, or **Assay Information File**). For more information, see the *SDS Online Help*.



b. Enter a **Name** for the detector (for example, **AL-1**).

Note: The name of the detector must be unique and it should reflect the target locus of the assay. Do not use the same name for multiple detectors. The SDS software does not distinguish between detectors of the same name, even if they use a different dye set.



Notes

c. *Optional*. In the Group field, enter or select a group for the detector.

Note: A detector group is an optional feature of the SDS software designed to help you organize your detectors.

d. *Optional*. In the Description field, enter a brief description of the assay (up to 32 characters).

e. Leave the AIF Assay ID field blank.

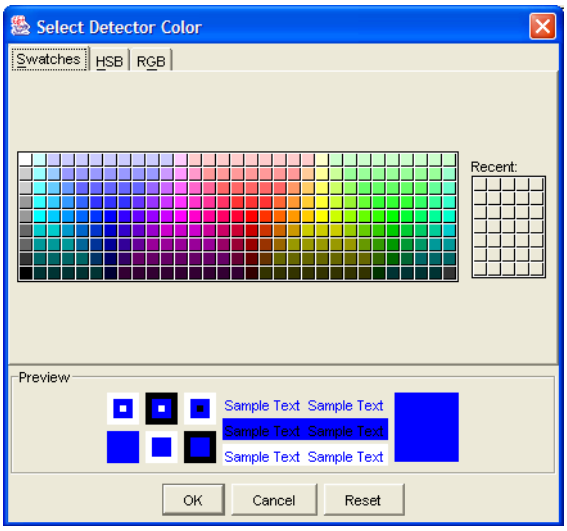
Note: For more information on Assay Information Files (AIF), see the *SDS Online Help*.

f. From the Reporter/Quencher drop-down lists, select the appropriate dyes for the detector.

Note: For TaqMan® probes, select **TAMRA** as the quencher; for TaqMan® MGB probes select **None** as the quencher.

Note: If you are using a custom dye not manufactured by Applied Biosystems, you must create and run a pure dye plate for the dye before applying it to a detector. For more information, see the *SDS Online Help*.

g. Click the **Color** box to open the Select Detector Color dialog box, select a color to represent the detector, then click **OK**.



Notes

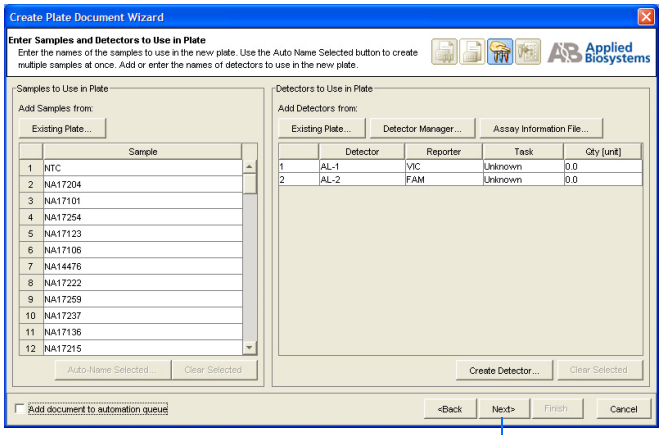
h. *Optional*. In the Notes field, enter any additional comments for the detector (up to 200 characters).

i. Click **OK**. The software saves the new detector and displays it in the Create Plate Document Wizard.

j. Repeat steps 8a through 8e for Allele Y, then skip to step 8k.

IMPORTANT! The detector for Allele X and the detector for Allele Y should have different reporter dyes (for example, VIC® dye for detector AL-1 and FAM™ dye for detector AL-2).

k. Click **Next>**.



Sample Experiment

In the sample experiment, there were two detectors: AL-1 and AL-2.

For your own experiment, enter the appropriate number of detectors. Use detector names that represent the detectors in your experiment.

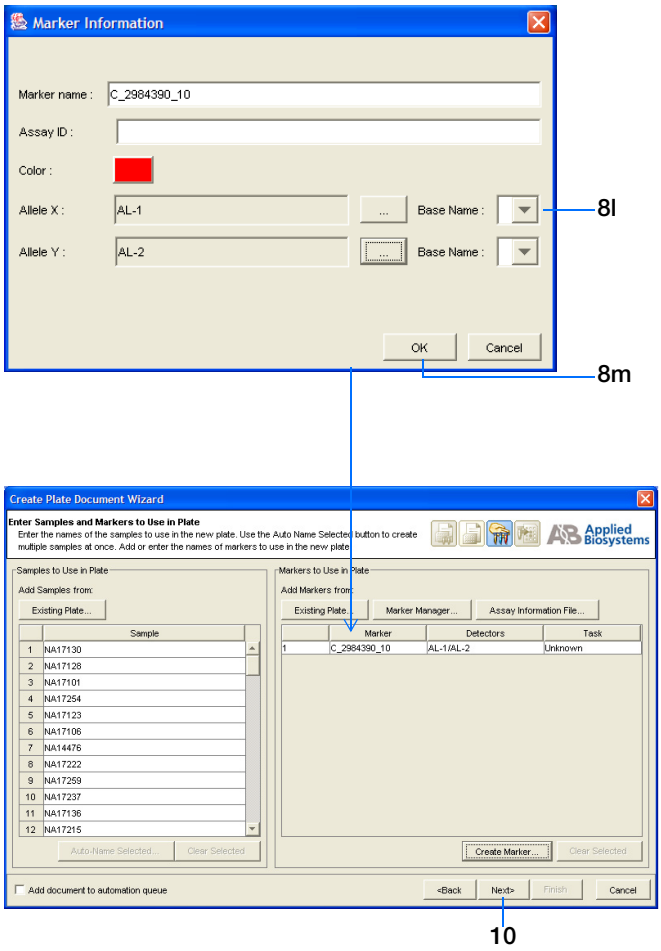
Notes

l. *Optional*. Select a base (**A**, **C**, **G**, or **T**) from the Allele X and Allele Y Base Name drop-down lists.

m. Click **OK**. The Marker Information dialog box closes and the new marker is displayed in the Create Plate Document Wizard.

9. Repeat step 7 to create markers for any remaining assays on the plate.

10. Click **Next>**.



Sample Experiment

In the sample experiment, only one marker was used: C_2984390_10. The detectors assigned to it were AL-1 and AL-2.

For your own experiment, enter the appropriate number of markers with the appropriate detectors assigned. Use marker and detector names that represent the markers and detectors in your experiment.

Notes

11. Assign samples and markers to the wells:

a. From the Plate Layout drop-down list, select **Individual Wells**.

IMPORTANT! If you change the plate layout *after* assigning samples or markers to the wells, all assignments are cleared from the Plate Grid.

b. In the Plate Grid, select the well(s) containing the first sample.

Note: To select more than one well at a time, hold down the **ctrl** key or **shift** key while selecting the wells.

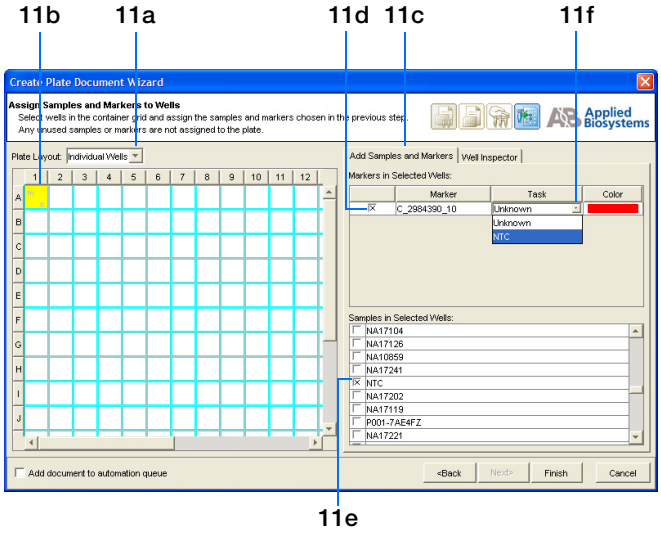
c. Select the **Add Samples and Markers** tab.

d. In the Markers in Selected Wells pane, check the appropriate marker for the selected well(s).

e. In the Samples in Selected Wells pane, check the appropriate sample for the selected well(s).

f. Click in the Task field and select a task for the marker from the drop-down list: **Unknown** or **NTC**.

g. Repeat steps 11b through 11f for the remaining samples, then go on to step 12.



Notes

12. Verify the sample setup information for each well:

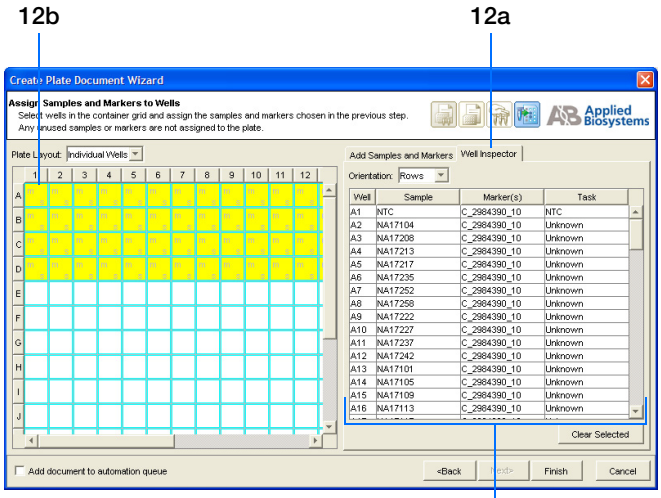
a. Select the **Well Inspector** tab.

b. Select the desired well(s) in the Plate Grid.

Note: To select more than one well at a time, hold down the **ctrl** key or **shift** key while selecting the wells.

c. View the sample setup information for the selected well(s) in the Well Inspector table.

Note: If you need to correct the sample setup information for a well, select the well in the Well Inspector table, click **Clear Selected**, then assign the correct setup information.



12c. The sample setup information for the selected well(s) is displayed here.

13. Click **Finish**. The Create Plate Document Wizard closes and the new plate document is displayed.

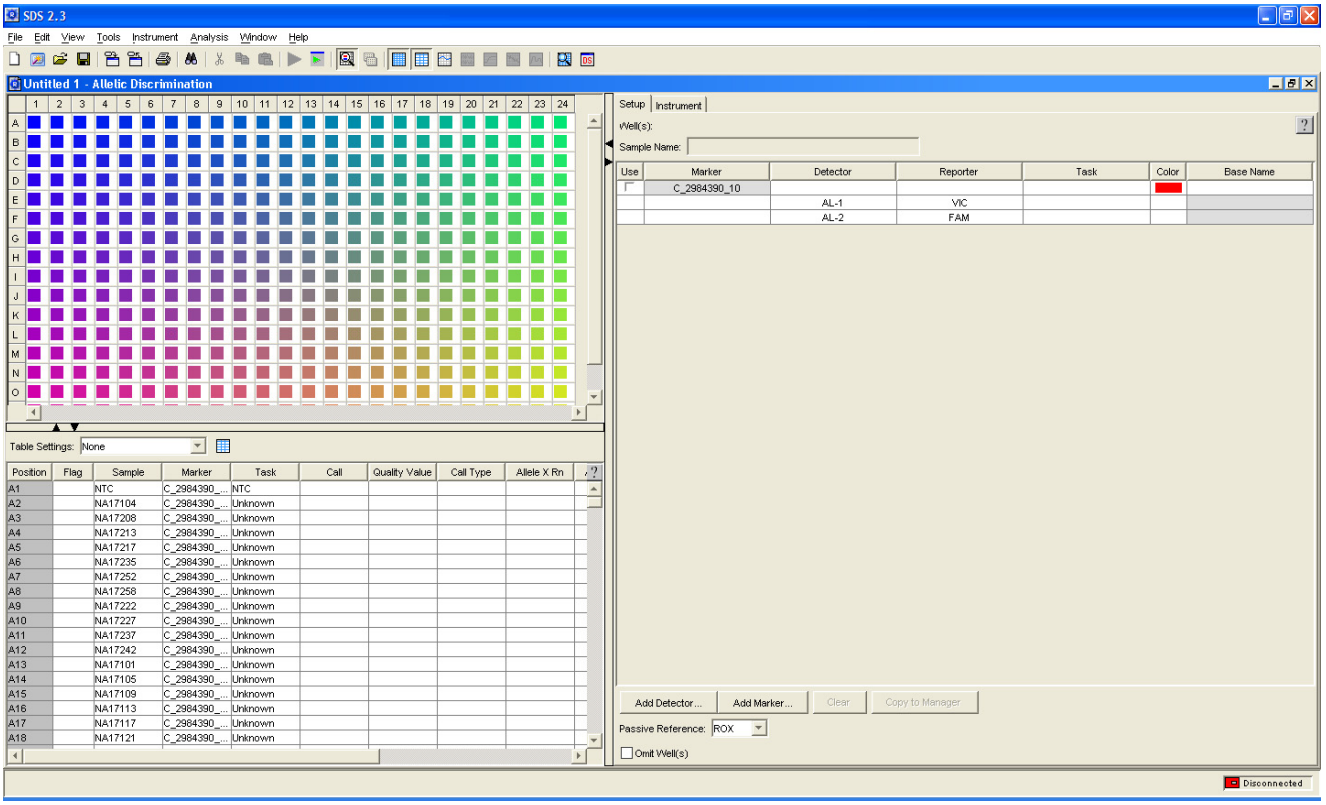
Note: If your experiment does not use all the wells in a reaction plate, do not omit the wells from use at this time. You can omit unused wells after the run. For information about omitting unused wells, see the *SDS Online Help*.

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

Notes

Sample Experiment

For the sample experiment, the Allelic Discrimination (AD) plate document looked like this:



14. Save the new plate document:

> **IMPORTANT!** Before the plate document can be run, you must save it as an SDS 7900HT Document (*.sds). If you close the plate document without first saving it, your information will be lost.

a. Select **File** > **Save** to open the Save As dialog box.

Notes

b. In the Save in field, navigate to and select a directory for the new plate document.

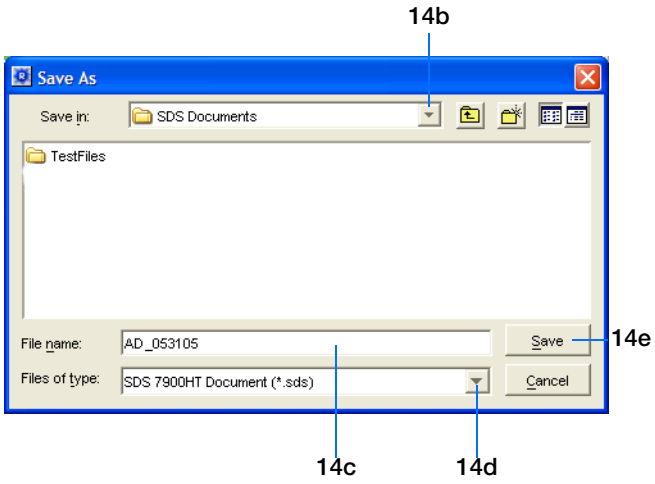
c. In the File name field, either:

- Enter a file name for the plate document, *or*
- Enter or scan the barcode number for the reaction plate

Note: The SDS software does not require that the plate document name match the barcode of the corresponding reaction plate.

d. From the Files of type drop-down list, select **SDS 7900HT Document (*.sds)**.

e. Click **Save**. The software saves the plate document to the specified directory.



4

Notes

Performing the Pre-Read Run

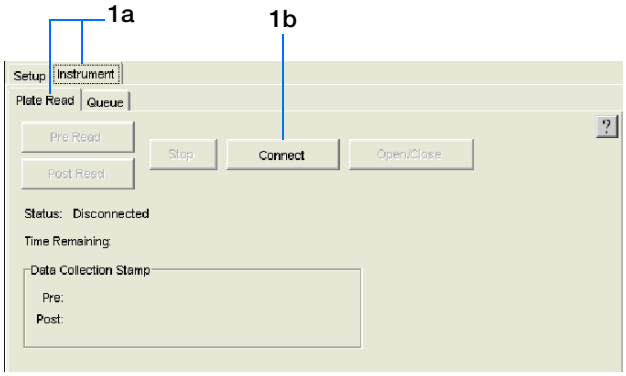
About the Pre-Read Run

A pre-read run records the background fluorescence of each well of the allelic discrimination plate before performing PCR amplification (see Chapter 5). Then, during the post-read run (see Chapter 6), the pre-read fluorescence is subtracted from the post-read fluorescence to ensure that fluorescence due only to amplification is recorded.

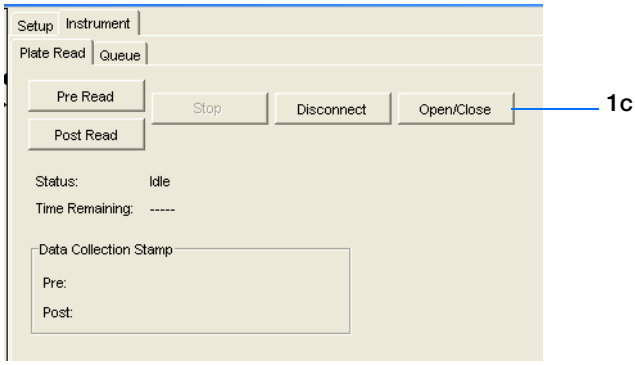
1. To run an individual plate:

   a. In the Allelic Discrimination (AD) plate document, select the **Instrument** > **Plate Read** tabs.

   b. Click **Connect** to connect the plate document to the instrument.



   c. Click **Open/Close**. The instrument tray rotates to the OUT position.

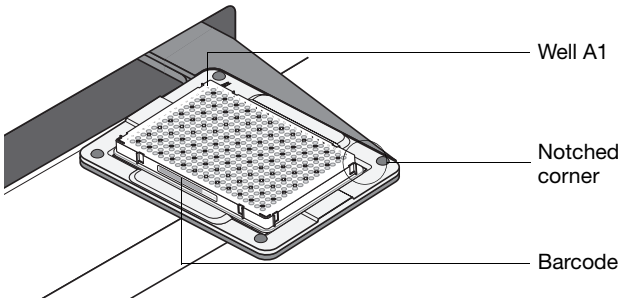


Notes

d. Place the prepared reaction plate into the instrument tray as shown.

IMPORTANT! For the standard 384-well and standard 96-well reaction plates, the A1 position is located in the top-left side of the instrument.

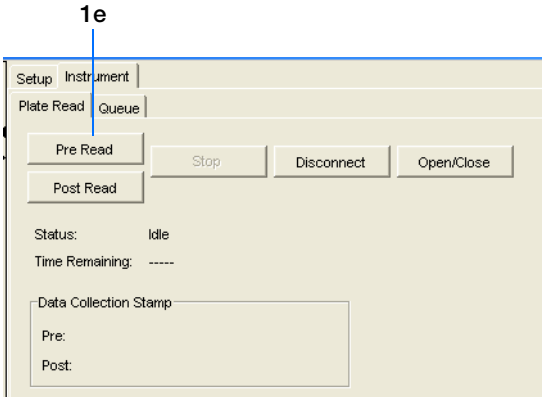
Standard 384-well reaction plate



e. Click **Pre Read**. The instrument tray rotates to the IN position.

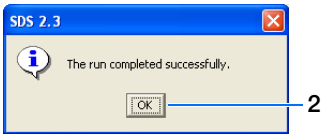
During the pre-read run, the instrument collects one fluorescence scan per well.

As the instrument performs the run, it displays status information in the Plate Read tab.

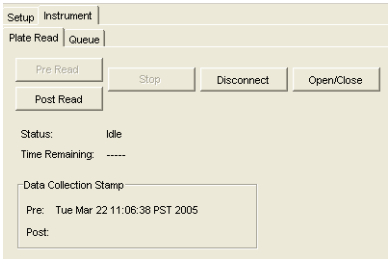


2. After the pre-read run is finished:

- A message indicates whether or not the run was successful. Click **OK** to close the dialog box. The instrument tray rotates to the OUT position.



- The date and time of completion is recorded in the Date Collection Stamp pane and the Pre Read button is disabled.



4

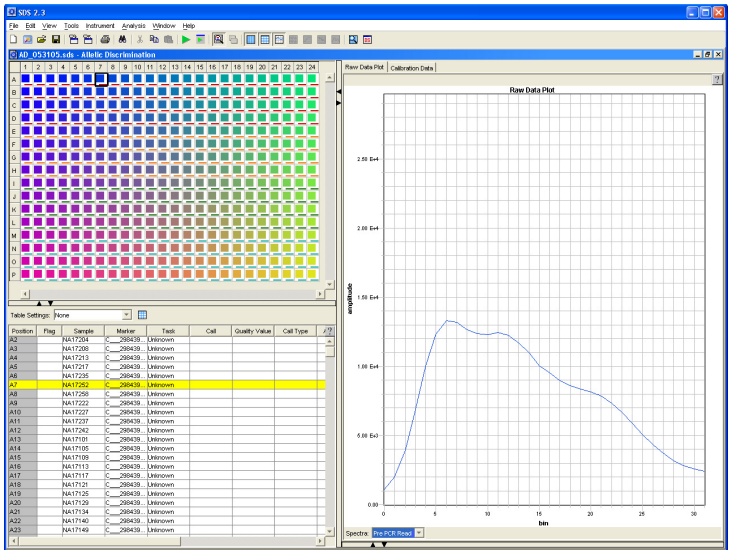
Notes

3. *Optional*. Review the fluorescence data generated during the pre-read run:

   a. Click (Hide/Show System Raw Data Pane).

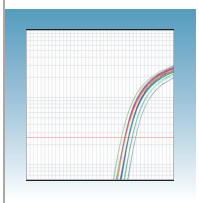
   b. In the Plate Grid, select the well(s) you want to view.

   c. Select the **Raw Data Plot** tab, then select **Pre PCR Read** from the Spectra drop-down list to view the pre-read run data.

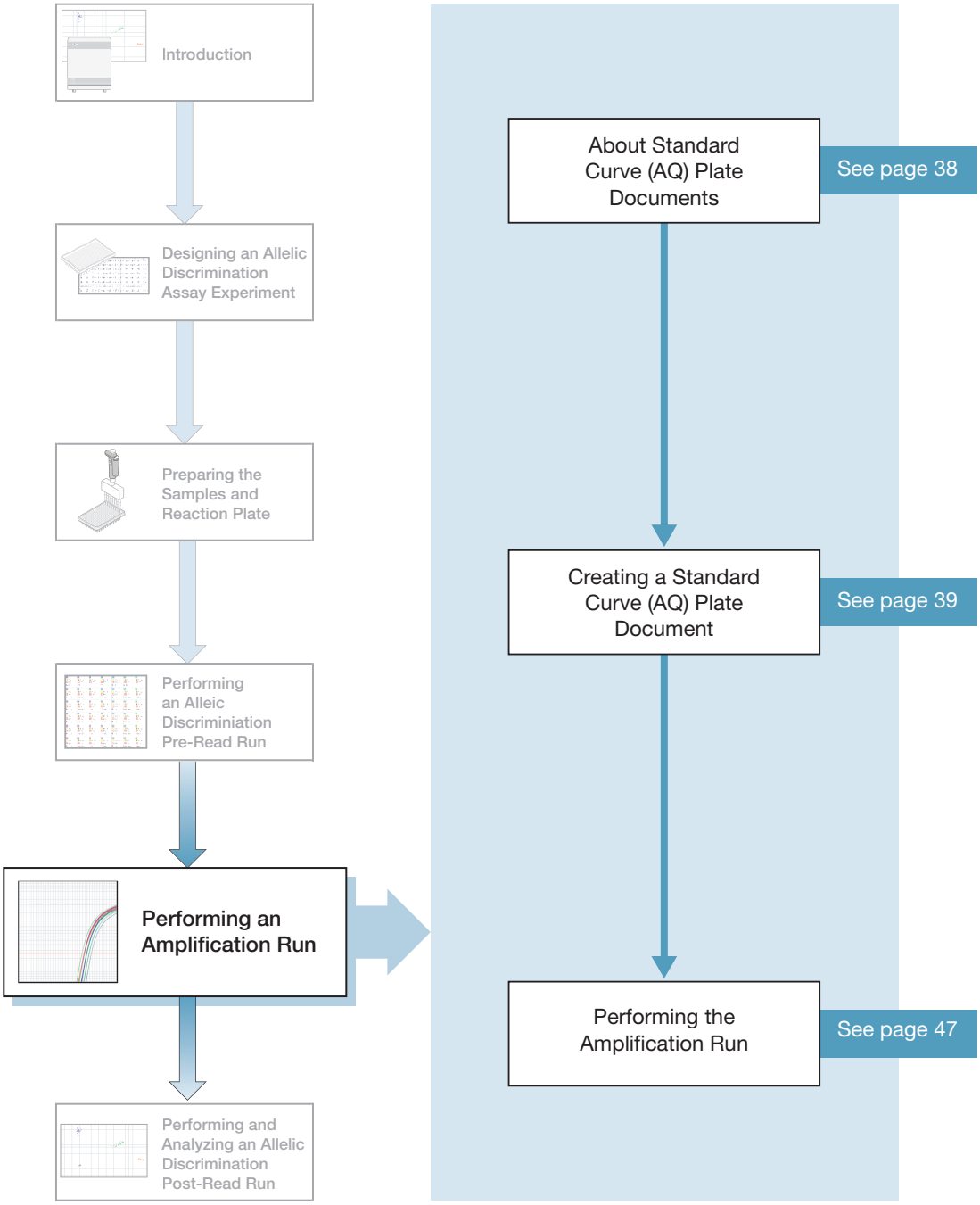


4. Select **File > Close**. All raw data generated during the pre-read run and any changes to the plate document settings are saved to the file that you specified in step 14 on page 32.

Notes



Performing an Amplification Run



Notes

About Standard Curve (AQ) Plate Documents

Using Standard Curve (AQ) Plate Documents for Amplification

You create and use Standard Curve (AQ) plate documents to store real-time data for allelic discrimination assays. Because the Standard Curve (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 plate.

Benefits of Real-Time Amplification

Because the allelic discrimination assay is an end-point assay, you can amplify the target sequences offline using any thermal cycler. However, using the 7900HT Fast System to amplify the target sequences provides real-time PCR data. When you perform allele-calling (described in "Assigning Calls" on page 62), you can study the amplification plots if you observe questionable calls or do not observe data for a well.

Options for Creating a Plate Document

This section describes how to create a plate document using the New Plate Document Wizard. For information about other ways to create plate documents, see the *SDS Online Help*.

Reaction Plate Options

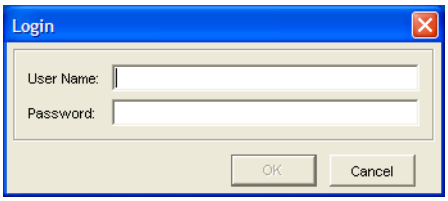
The allelic discrimination assay can be run on either a standard 96-well reaction plate or a standard 384-well reaction plate. For clarity, this chapter only illustrates running a standard 384-well reaction plate.

Notes

Creating a Standard Curve (AQ) Plate Document

1. Double-click on the desktop (or select **Start > All Programs > Applied Biosystems > SDS 2.3 > SDS 2.3**) to start the SDS software.

2. If your System Administrator has enabled the login option, the Login dialog box appears. Enter your **User Name** and **Password**, then click **OK**.



   Note: If the login option is not enabled, no Login dialog box appears. Skip to step 3 below.

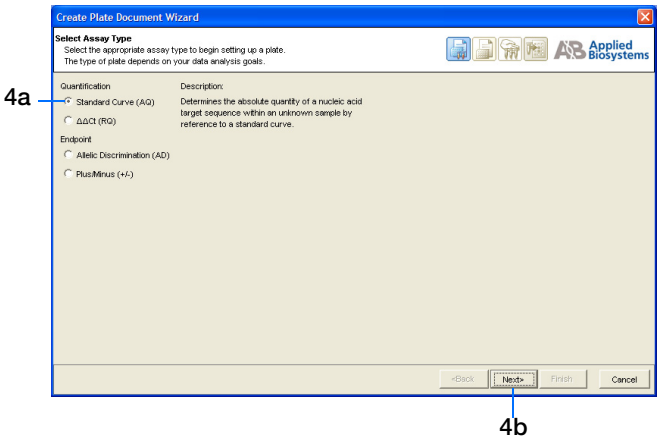
3. In the SDS software menu bar, click (or select **File** > **New Plate Wizard**) to open the Create Plate Document Wizard.

4. Select the assay type:

   a. Select **Standard Curve (AQ)**.

   Note: A standard curve is not necessary for a non-quantitation amplification run.

   b. Click **Next>**.

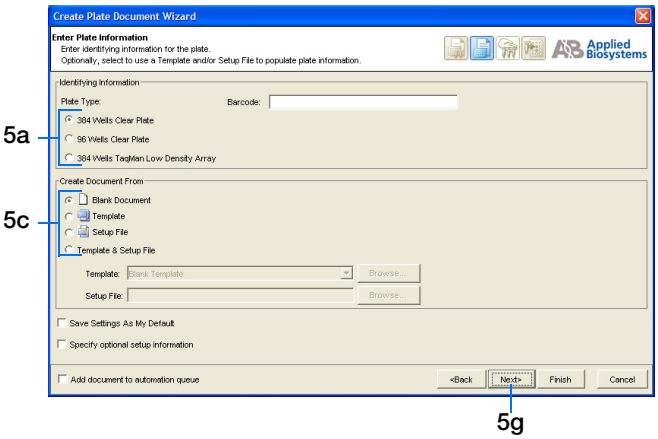


5. Enter the plate information:

   a. Select a Plate Type: **384 Wells Clear Plate** or **96 Wells Clear Plate**.

   b. *Optional.* In the Barcode field, scan or enter the barcode for the reaction plate.

   Note: The Automation Accessory must be installed in order to use barcodes. A barcode is required if you are adding the plate document to the automation queue. See step f on page 40.



Notes

c. In the Create Document From pane, select your preferred source.

Note: You can create a plate document from scratch (select **Blank Document**) or you can create a plate document from a template and/or an existing plate document (select **Template**, **Setup File**, or **Template & Setup File**). For more information, see the *SDS Online Help*.

---

d. *Optional.* Check **Save Settings As My Default**. Check this option if you want the SDS software to automatically apply the current page settings every time you use the Create Plate Document Wizard.

e. *Optional.* Check **Specify optional setup information**. Check this option if you want to enter a name, study, or comments for the plate document.

f. *Optional.* Check **Add document to automation queue**. Check this option if you want to add the finished plate document to the Automation Controller software plate queue.

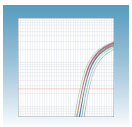
Note: This checkbox is only enabled if you are connected to an Automation Accessory. For more information, see Appendix B on page 83.

Note: This checkbox appears on subsequent pages of the Create Plate Document Wizard. You only need to select this option on one page to enable it.

---

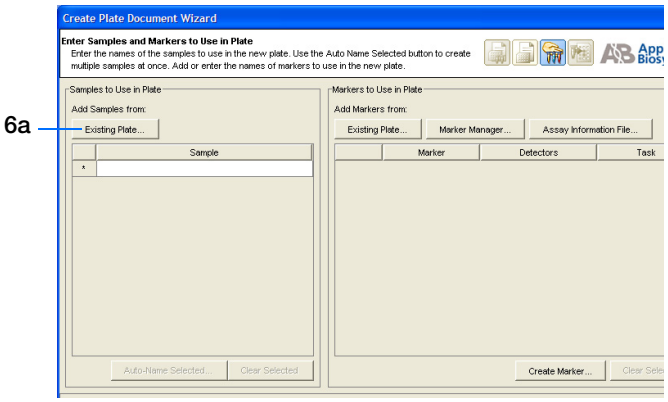
g. Click **Next>**.

Notes



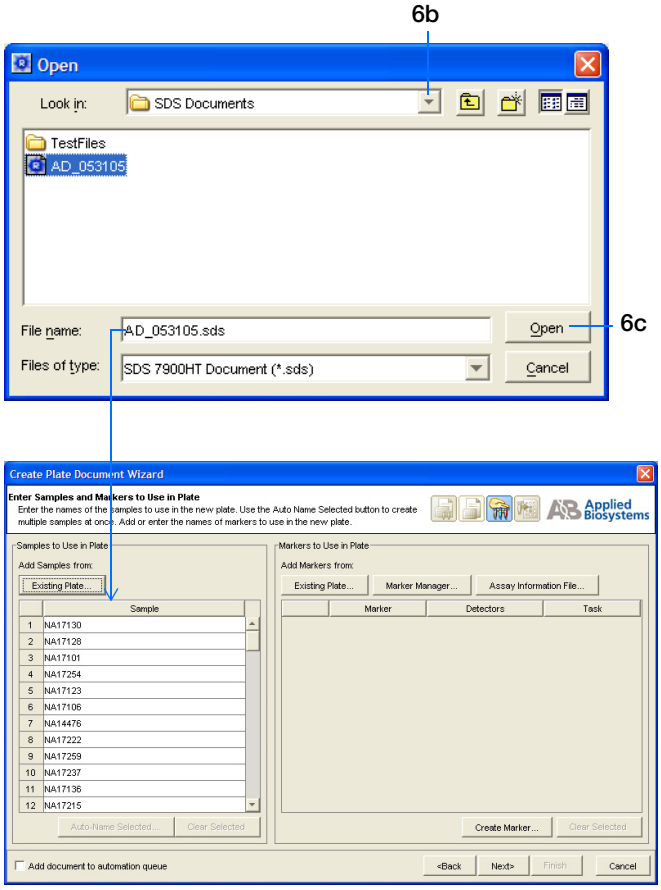
6. Import the sample names from the Allelic Discrimination (AD) plate document you created in Chapter 4 (see step 6 on page 25).

a. Click **Existing Plate**.



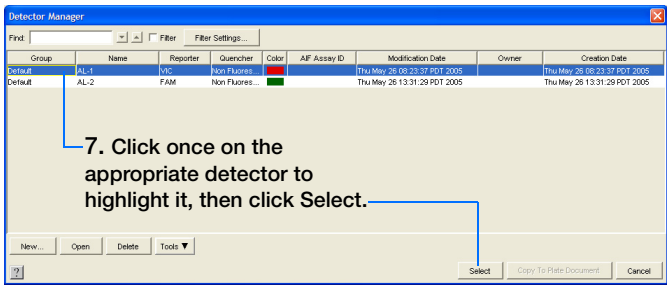
b. In the Open dialog box, navigate to and select the Standard Curve (AQ) plate document.

c. Click **Open**. The sample names appear in the Create Plate Document Wizard.

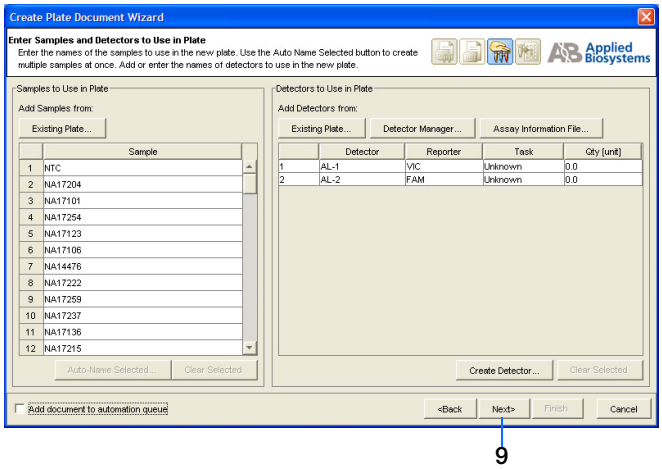


Notes

7. In the Detector Manager, locate the appropriate detector from the Allelic Discrimination (AD) plate document you created in Chapter 4 (see step 8 on page 26), click once on the detector to highlight it, then click **Select**.



8. Repeat step 7 for any remaining detectors.
9. Click **Next>**.



Sample Experiment

In the sample experiment, there were two detectors: AL-1 and AL-2.

For your own experiment, enter the appropriate number of detectors. Use detector names that represent the detectors in your experiment.

Notes

10. Assign samples and detectors to the wells, using the same assignments you used for the Allelic Discrimination (AD) plate document (see step 11 on page 30):

a. From the Plate Layout drop-down list, select **Individual Wells**.

IMPORTANT! If you change the plate layout *after* assigning samples or detectors to the wells, all assignments are cleared from the Plate Grid.

---

b. In the Plate Grid, select the well(s) containing the first sample.

Note: To select more than one well at a time, hold down the **ctrl** key or **shift** key while selecting the wells.

---

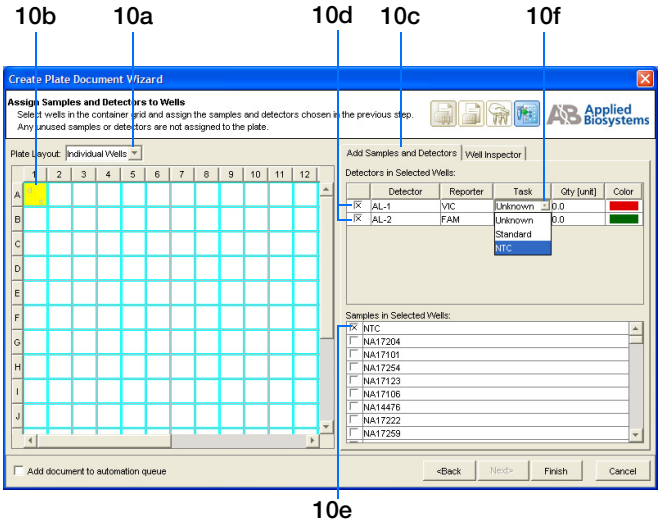
c. Select the **Add Samples and Detectors** tab.

d. In the Detectors in Selected Wells pane, check the appropriate detector(s) for the selected well(s).

e. In the Samples in Selected Wells pane, check the appropriate sample for the selected well(s).

f. Click in the Task field and select a task for the detector from the drop-down list: **Unknown** or **NTC**. Do not select Standard.

g. Repeat steps 10b through 10d for the remaining samples.



Notes

11. *Optional*. Verify the sample setup information for each well:

a. Select the **Well Inspector** tab.

b. Select the desired well(s) in the Plate Grid.

Note: To select more than one well at a time, hold down the **ctrl** key or **shift** key while selecting the wells.

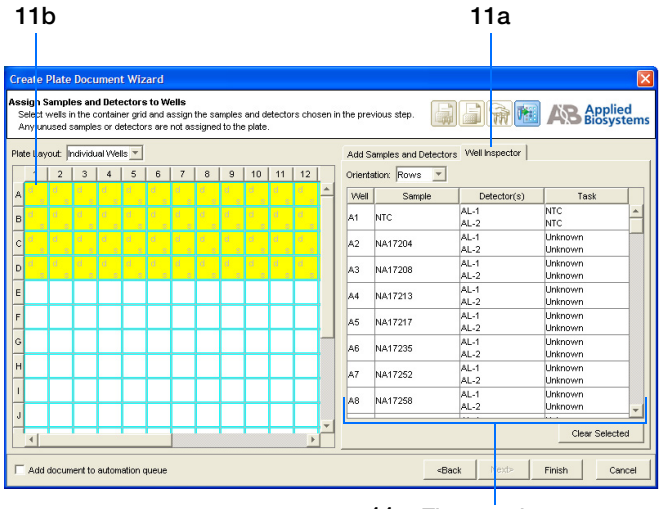
c. View the sample setup information for the selected well(s) in the Well Inspector table.

Note: If you need to correct the sample setup information for a well, select the well in the Well Inspector table, click **Clear Selected**, then assign the correct setup information.

12. Click **Finish**. The Create Plate Document Wizard closes and the new plate document is displayed.

Note: If your experiment does not use all the wells in a reaction plate, do not omit the wells from use at this time. You can omit unused wells after the run. For information about omitting unused wells, see the *SDS Online Help*.

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

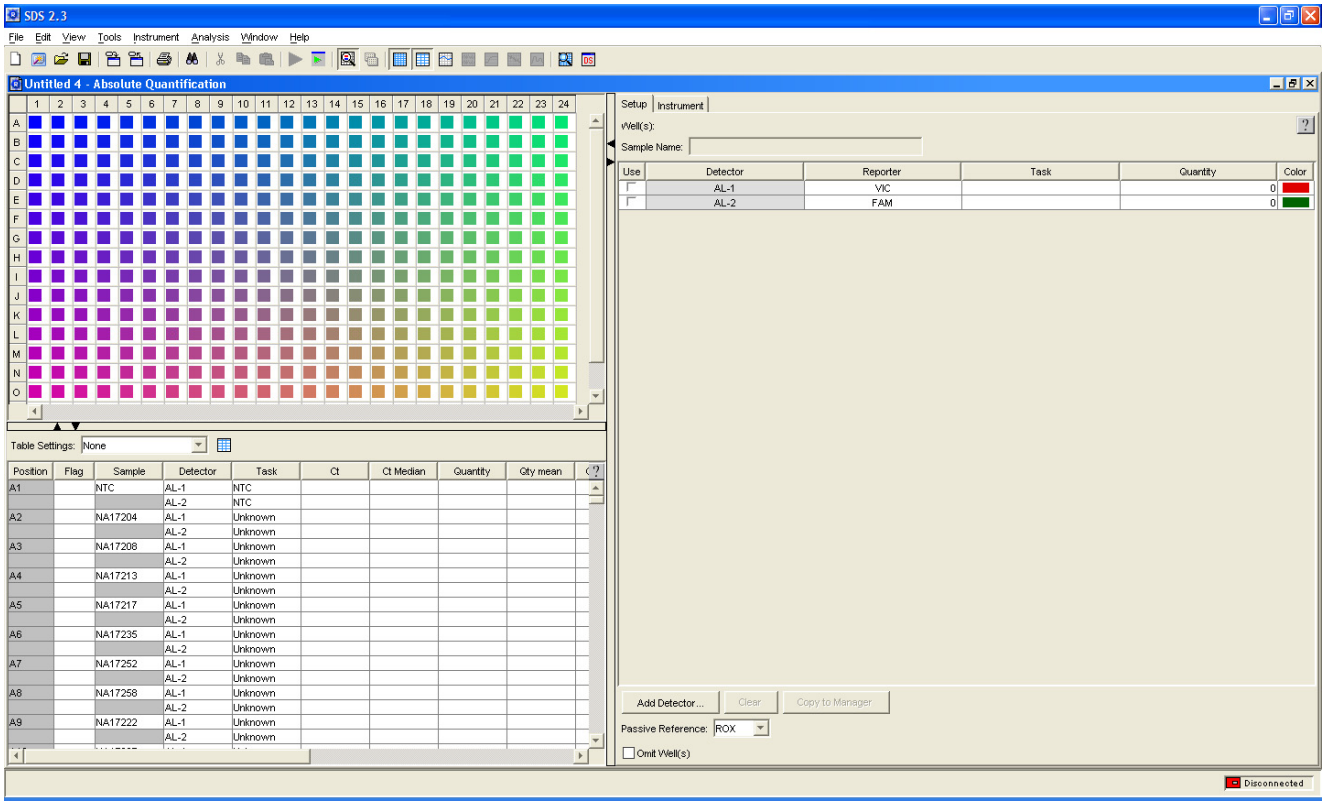


11c. The sample setup information for the selected wells is displayed here.

Notes

Sample Experiment

For the sample experiment, the Standard Curve (AQ) plate document looked like this:



Notes

13. Save the new plate document:

IMPORTANT! Before the plate document can be run, you must save it as an SDS 7900HT Document (*.sds). If you close the plate document without first saving it, your information will be lost.

a. Select **File** > **Save** to open the Save As dialog box.

b. In the Save in field, navigate to and select a directory for the new plate document.

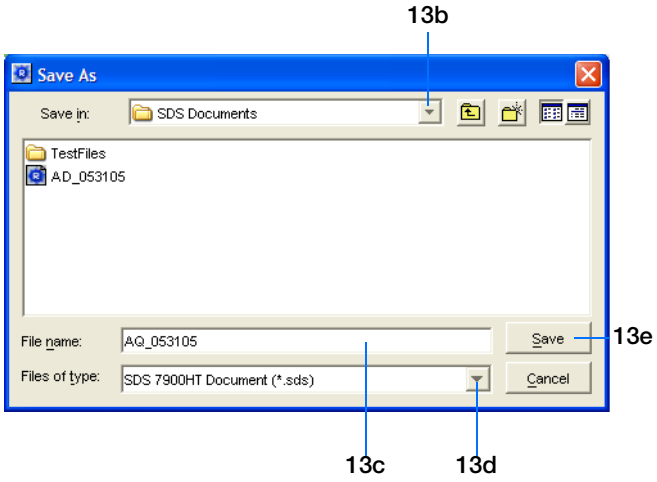
c. In the File name field, either:

- Enter a file name for the plate document, *or*
- Enter or scan the barcode number for the reaction plate

Note: The SDS software does not require that the plate document name match the barcode of the corresponding reaction plate.

d. From the Files of type drop-down list, select **SDS 7900HT Document (*.sds)**.

e. Click **Save**. The software saves the plate document to the specified directory.



Notes

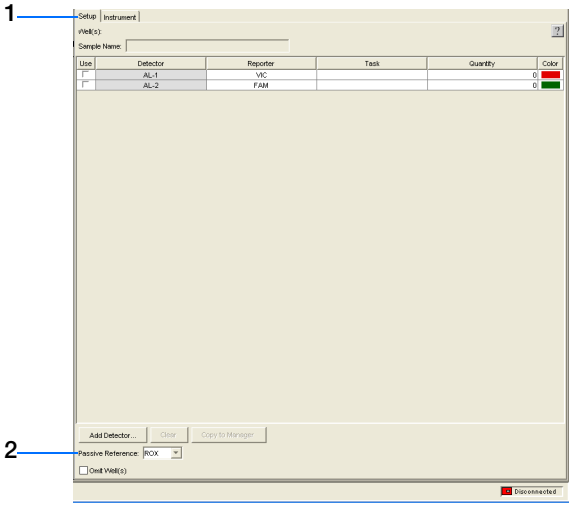
Performing the Amplification Run

1. In the Standard Curve (AQ) plate document you just created, select the **Setup** tab.

2. Verify that the Passive Reference setting is **ROX** dye. (ROX™ dye is the default.)

   Note: If your experiment does not use all the wells in a reaction plate, do not omit the wells from use at this time. You can omit unused wells after the run. For information about omitting unused wells, see the *SDS Online Help*.

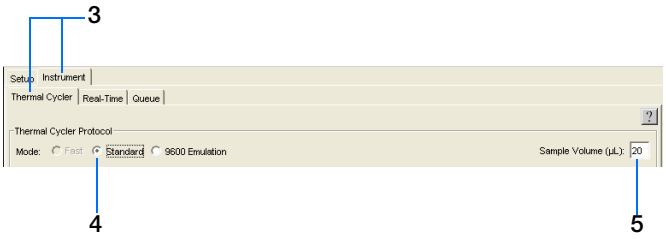
   Note: If necessary, you can change the sample setup information (sample name, detector, task) after a run is complete.



3. Select the **Instrument > Thermal Cycler** tabs.

4. Select a Mode: **Standard** or **9600 Emulation**.

   Note: When Standard mode is selected, the SDS software uses the 9700 thermal cycler ramp rate in the 7900HT Fast System for standard PCR reactions. When the 9600 Emulation mode is selected, the SDS software reduces the 7900HT Fast System ramp rate to match that of the 9600 thermal cycler in the ABI PRISM® 7700 Sequence Detection System.

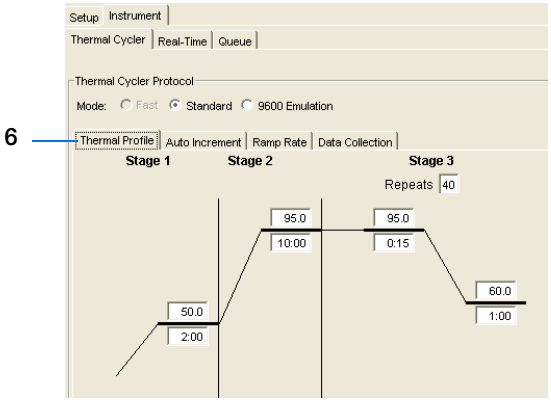


5. Enter a Sample Volume.

   Note: The default sample volumes are 50 µL for a standard 96-well reaction plate and 20 µL for a standard 384-well reaction plate.

Notes

6. Select the **Thermal Profile** tab.

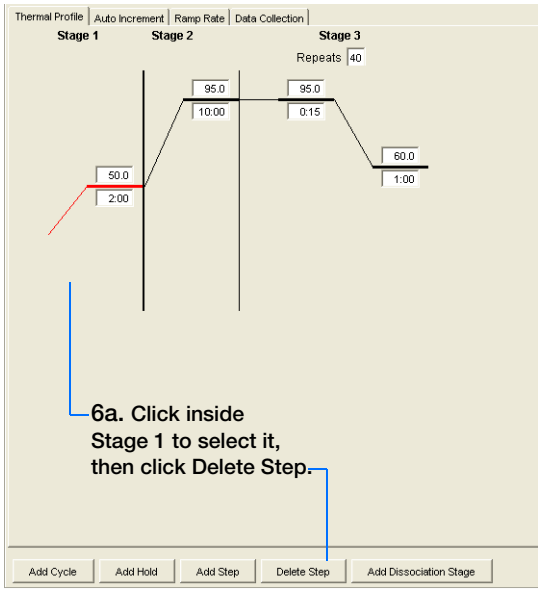


- If you are using TaqMan® Universal PCR Master Mix (PN 4304437, which contains AmpErase® UNG), accept the default times and temperatures (shown at right).

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

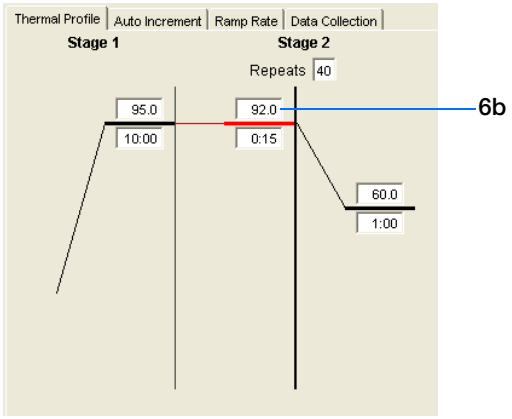
- If you are using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (PN 4324018):

  a. Delete the default Stage 1 by clicking inside the stage to select it (the ramp line turns red), then clicking **Delete Step**.



Notes

b. Change the temperature for the new Stage 2 from 95.0 to 92.0 by double-clicking inside the top box, then typing **92.0**.



c. Accept the remaining default times and temperatures.

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

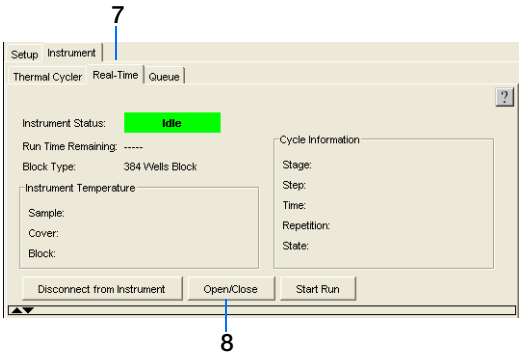
Sample Experiment

For the sample experiment, the following parameters were selected for the amplification run:

- Mode: **Standard**.
- Sample Volume: **5 µL** (This is the recommended volume for TaqMan® SNP Genotyping Assays run on a standard 384-well reaction plate.)
- Thermal Profile: The thermal profile was changed per steps 6a through 6c above for TaqMan® Universal PCR Master Mix, No AmpErase® UNG.

7. Select the **Real-Time** tab.

8. Click **Open/Close**. The instrument tray rotates to the OUT position.

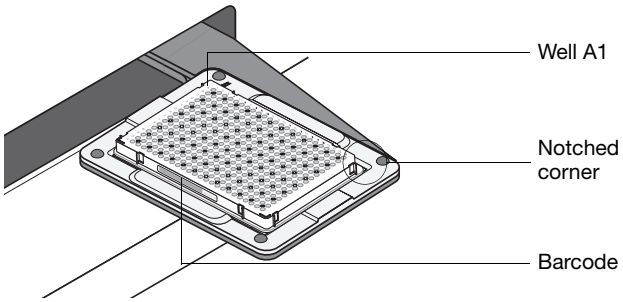


Notes

9. Place the prepared reaction plate into the instrument tray as shown.

IMPORTANT! For standard 384-well and standard 96-well reaction plates, the A1 position is located in the top-left side of the instrument.

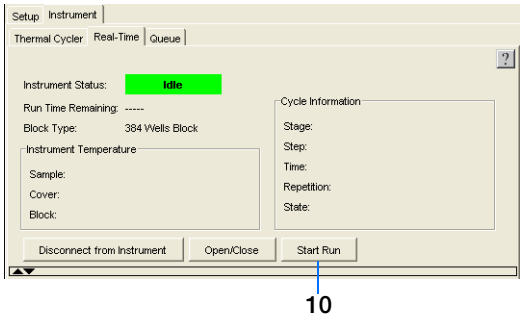
Standard 384-well reaction plate



10. Click **Start Run**. The instrument tray rotates to the IN position and the instrument performs the amplification run.

    As the instrument performs the amplification run, it displays real-time status information in the Real-Time 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.

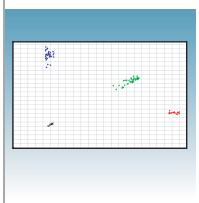


11. Select **File** > **Save**. All data generated during the run are saved to the plate document.

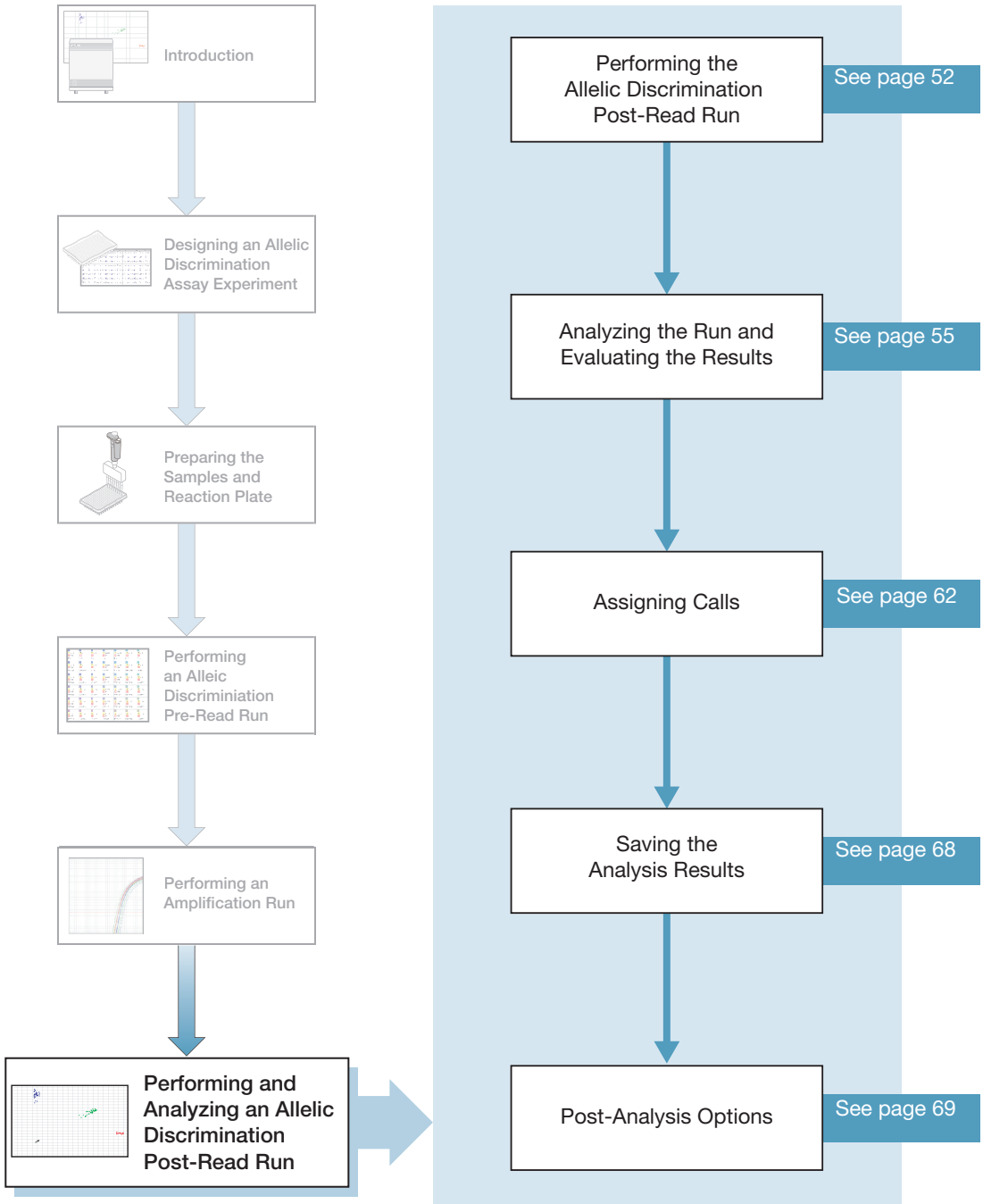
Note: If necessary, the amplification run data from the Standard Curve (AQ) plate document can be analyzed later to help troubleshoot the allelic discrimination assay results. For more information, see Appendix C on page 89.

12. Close the plate document, then proceed to Chapter 6 on page 51 to complete the allelic discrimination assay workflow.

Notes



Performing and Analyzing an Allelic Discrimination Post-Read Run



Notes

Performing the Allelic Discrimination Post-Read Run

About the Post-Read Run

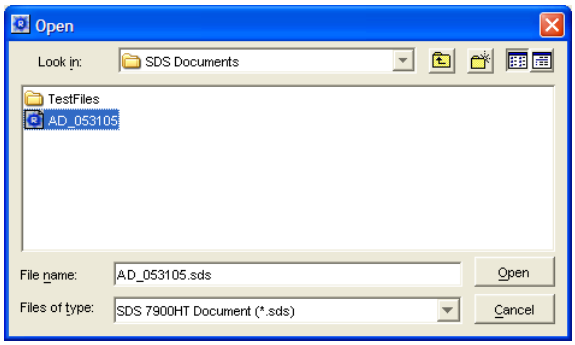
A post-read run records the fluorescence of each well of the allelic discrimination plate after performing PCR amplification (see Chapter 5). During the post-read run, the pre-read background fluorescence (see Chapter 4) is subtracted from the post-read fluorescence to ensure that fluorescence due only to amplification is recorded.

Reaction Plate Options

The plus/minus assay can be run on both standard 96-well and standard 384-well reaction plates. For clarity, this chapter only illustrates the running of a standard 96-well reaction plate.

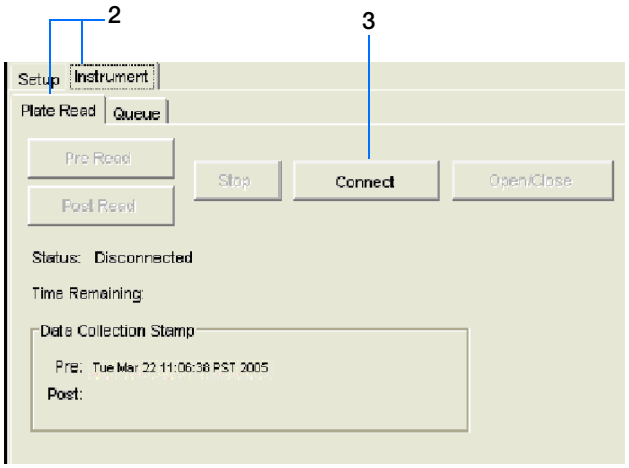
To perform the post-read run:

1. Select **File > Open**, browse to open the previously saved pre-read plate document, then click **Open**. The allelic discrimination plate document opens in the main SDS software window.

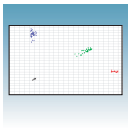


2. In the Allelic Discrimination (AD) plate document, select the **Instrument** > **Plate Read** tabs.

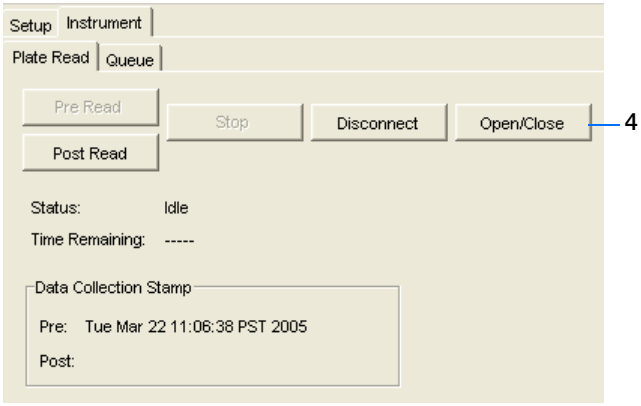
3. Click **Connect** to connect the plate document to the instrument.



Notes



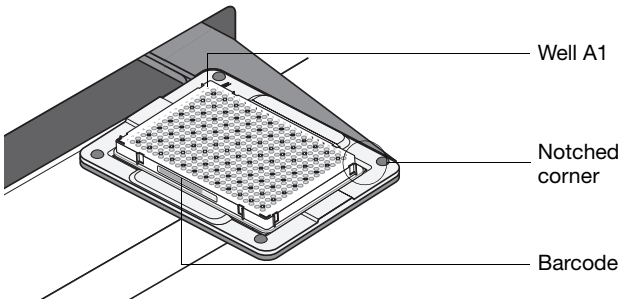
4. Click **Open/Close**. The instrument tray rotates to the OUT position.



5. Place the prepared reaction plate into the instrument tray as shown.

IMPORTANT! For the standard 384-well and standard 96-well reaction plates, the A1 position is located in the top-left side of the instrument.

Standard 384-well reaction plate

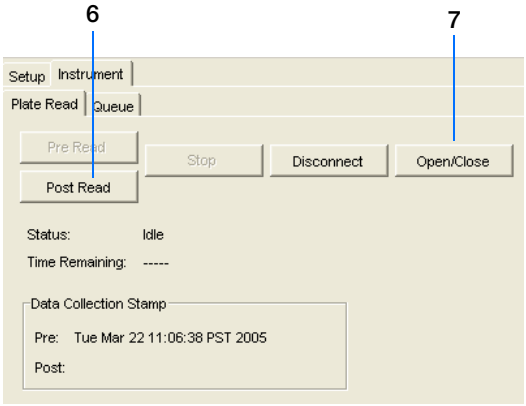


6. Click **Post Read**. The instrument tray rotates to the IN position and the instrument performs the run.

   As the instrument performs the run, it displays status information in the Plate Read tab.

   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.

7. When the run is complete, click **Open/Close** to eject the reaction plate.



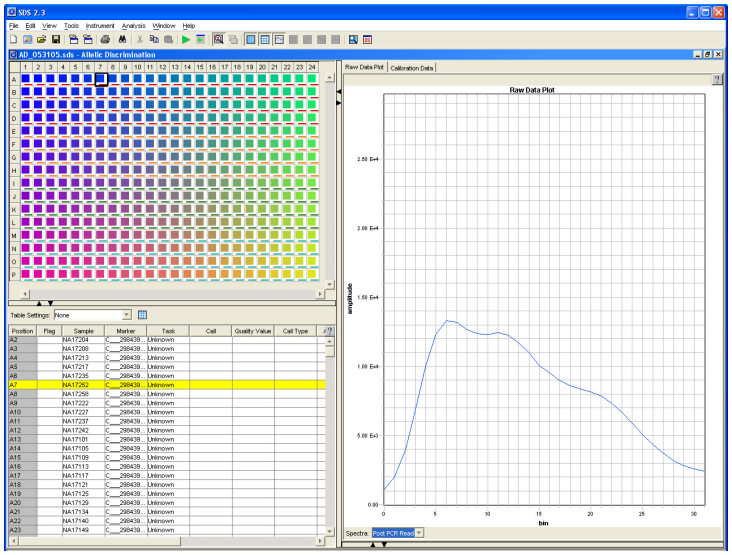
Notes

8. *Optional*. Review the fluorescence data generated during the post-read run:

   a. Click (Hide/Show System Raw Data Pane).

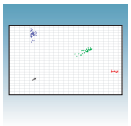
   b. In the Plate Grid, select the well(s) you want to view.

   c. Select the **Raw Data Plot** tab, then select **Post PCR Read** from the Spectra drop-down list to view the post-read run data.



9. Select **File > Close**. All raw data generated during the post-read run and any changes to the plate document settings are saved to the file that you specified in step 1 on page 52.

Notes



Analyzing the Run and Evaluating the Results

Analyzing the Run

1. In the Allelic Discrimination (AD) plate document, click ▶ (or select **Analysis** > **Analyze**). The SDS software analyzes the run data.

Tip: Reanalyzing Data

Once you click the Analyze button, it becomes disabled. To reanalyze the data, you can:

- Reenable the Analyze button by changing the setup information in the plate document (for example, removing a well or omitting a marker assignment), then click ▶ (or select **Analysis** > **Analyze**) again.
- Change analysis settings in the Analysis Settings dialog box, then click **OK**. The SDS software automatically reanalyzes the data when you click OK. (For information on using the Analysis Settings dialog box, see "Assigning Calls" on page 62.)

  Note: In order for the SDS software to automatically reanalyze the data via the Analysis Settings dialog box, you must have already analyzed the data once by clicking the Analyze button.

2. Select the **Results** tab, then proceed to "Evaluating the Results" below.

Evaluating the Results

To evaluate the analysis results, you can use the:

- Allelic Discrimination Plot (page 57)
- Results Grid (page 60)
- Results Table (page 61)

Note: The results displays are actively synchronized. For example, selecting a well in the Results Grid also selects the corresponding well in the Results Table and Allelic Discrimination Plot.

After evaluating the initial analysis results:

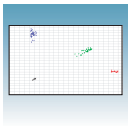
- If you are satisfied with the initial analysis results, proceed to "Saving the Analysis Results" on page 68.

Notes

- If you are not satisfied with the initial analysis results, you can assign calls (see page 62) or scrutinize the allele calls.

  Note: For information on scrutinizing the allele calls, see the *SDS Online Help*.

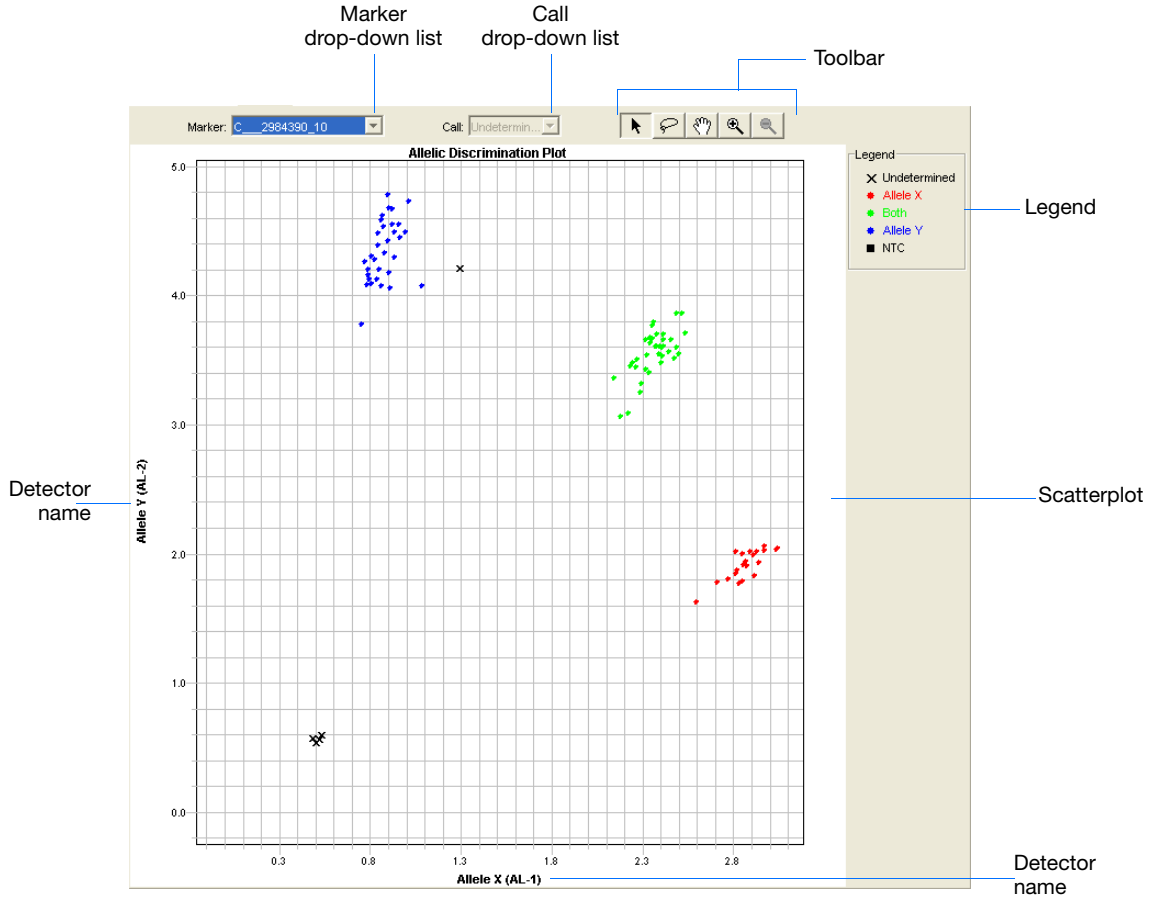
Notes



Using the Allelic Discrimination Plot

The SDS software graphs the results of allelic discrimination runs on a scatterplot (the Allelic Discrimination Plot) that contrasts reporter dye fluorescence. After signal normalization and multicomponent analysis, the SDS software graphs the normalized data from each well as a single datapoint on the scatterplot.

The figure below illustrates the components of the Allelic Discrimination Plot.



- **Marker drop-down list** – Determines the marker data that the SDS software displays in the scatterplot.
- **Call drop-down list** – When a datapoint is selected, this menu allows you to assign an allele call to the datapoint within the scatterplot.
- **Toolbar** – Contains the following tools for manipulating the scatterplot:

Icon	Description
	Selects: • Individual datapoints by clicking, or • Groups of datapoints by clicking and dragging a box across a group of datapoints.
	Selects groups of datapoints by encircling them with the tool.

Notes

Icon	Description
	Repositions the view within the scatterplot by clicking and dragging the screen.
	Zooms in on the scatterplot by: • Clicking the mouse button within the scatterplot, or • Clicking and dragging a section of the scatterplot to view.
	Zooms out on the scatterplot by clicking the mouse button within the scatterplot.

- **Scatterplot** – A scatterplot of datapoints from the run (also called the Allelic Discrimination Plot).
- **Legend** – An explanation of the symbols in the scatterplot.
- **Detector names** – The names you assigned to the detectors are displayed on the axes of the scatterplot.

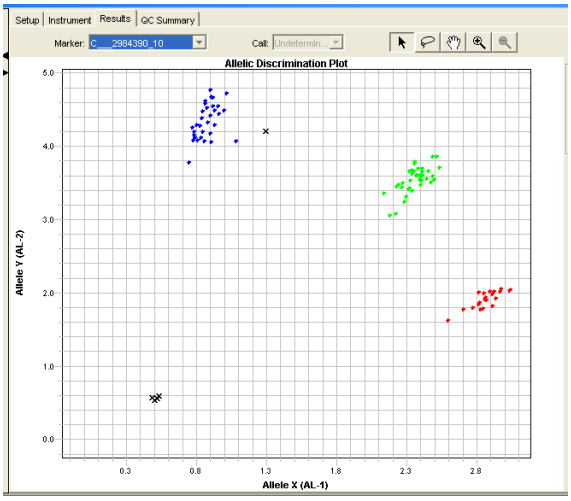
Note: You can adjust the appearance of the Allelic Discrimination Plot or the datapoints it contains using the Display Settings dialog box (**View > Display Setting**).

Note: For more information on the Allelic Discrimination Plot tools or the Display Settings dialog box, see the *SDS Online Help*.

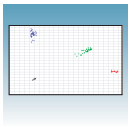
To evaluate results using the Allelic Discrimination Plot:

In the Results tab, select a marker from the Marker drop-down list.

- If the auto caller is enabled:
  – Alleles are identified for the selected marker and displayed in the Allelic Discrimination Plot:



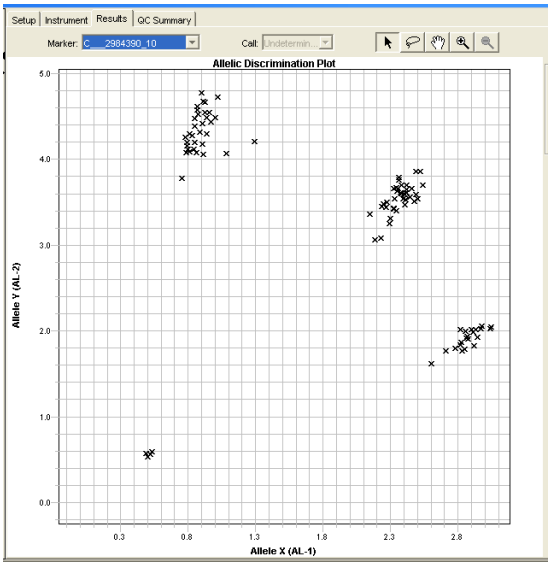
Notes



– The samples are grouped as follows:

Samples Containing...	Are Grouped In...	Symbol
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 (Not shown in the example.)	■
Undetermined	Anywhere on plot	×

- If the auto caller is not enabled, crossmarks (× – Undetermined) representing the selected marker are displayed in the Allelic Discrimination Plot.



Notes

Using the Results Grid

The Results Grid displays the assay-specific setup and analysis properties for the plate document in a well format corresponding to the type of reaction plate used for the run. The following parameters can be displayed for each well in the plate document:

- Well color
- Flag
- Sample
- Marker Color
- Marker
- Number of markers
- Call: Allele Y, Allele X, Both, Undetermined. The call symbols are as follows:

Wells Containing...	Symbol
Allele X	●
Allele Y	●
Both (Allele X and Allele Y – heterozygote)	●
NTC or Undetermined	×

Note: You can configure which parameters are displayed in the Results Grid by selecting **View > Display Setting**, then selecting **Results Grid** in the Display Settings dialog box. For more information, see the *SDS Online Help*.

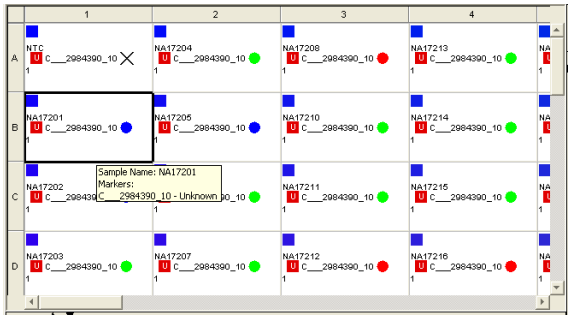
To evaluate results using the Results Grid:

1. Right-click inside the Plate Grid, then select **Result View**.

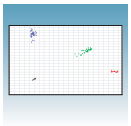
   Note: The default view for the Plate Grid is the Setup View.

2. If desired, select **View > Grid Zoom** to zoom in on individual wells.

3. Pass the cursor over a well to see the information for that well.



Notes



Using the Results Table

The Results table displays the assay-specific setup and analysis properties for the plate document in a table format. The following parameters can be displayed for each well in the plate document:

- Well position
- Flag
- Sample
- Marker
- Task: Unknown, NTC
- Call: Allele Y, Allele X, Both, NTC, Undetermined
- Quality Value
- Call Type
- Allele X R_n
- Allele Y R_n
- Passive Reference R_n

  Note: See Appendix C for an explanation of terms used in quantitation analysis.

- Flags: HMD (the well has missing data), FOS (fluorescence is off-scale), LME (large mean squared error), EW (the well is empty), BPR (bad passive reference)

Note: You can configure which parameters are displayed in the Results Table by double-clicking on a column header, by selecting a user-defined Table Settings profile from the drop-down list, or by clicking (**Table Settings**) to open the Table Settings dialog box. For more information, see the *SDS Online Help*.

Notes

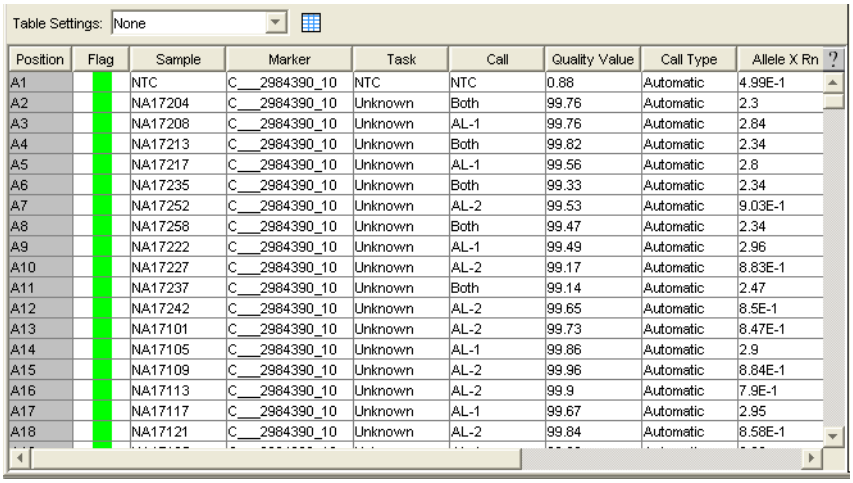


Table Settings: None

Position	Flag	Sample	Marker	Task	Call	Quality Value	Call Type	Allele X Rn
A1		NTC	C___2984390_10	NTC	NTC	0.88	Automatic	4.99E-1
A2		NA17204	C___2984390_10	Unknown	Both	99.76	Automatic	2.3
A3		NA17208	C___2984390_10	Unknown	AL-1	99.76	Automatic	2.84
A4		NA17213	C___2984390_10	Unknown	Both	99.82	Automatic	2.34
A5		NA17217	C___2984390_10	Unknown	AL-1	99.56	Automatic	2.8
A6		NA17235	C___2984390_10	Unknown	Both	99.33	Automatic	2.34
A7		NA17252	C___2984390_10	Unknown	AL-2	99.53	Automatic	9.03E-1
A8		NA17258	C___2984390_10	Unknown	Both	99.47	Automatic	2.34
A9		NA17222	C___2984390_10	Unknown	AL-1	99.49	Automatic	2.96
A10		NA17227	C___2984390_10	Unknown	AL-2	99.17	Automatic	8.83E-1
A11		NA17237	C___2984390_10	Unknown	Both	99.14	Automatic	2.47
A12		NA17242	C___2984390_10	Unknown	AL-2	99.65	Automatic	8.5E-1
A13		NA17101	C___2984390_10	Unknown	AL-2	99.73	Automatic	8.47E-1
A14		NA17105	C___2984390_10	Unknown	AL-1	99.86	Automatic	2.9
A15		NA17109	C___2984390_10	Unknown	AL-2	99.96	Automatic	8.84E-1
A16		NA17113	C___2984390_10	Unknown	AL-2	99.9	Automatic	7.9E-1
A17		NA17117	C___2984390_10	Unknown	AL-1	99.67	Automatic	2.95
A18		NA17121	C___2984390_10	Unknown	AL-2	99.84	Automatic	8.58E-1

Assigning Calls

Note: Assigning calls is optional. If you are satisfied with the initial analysis results (page 55), you do not need to assign calls.

The analysis of SNP (or genotyping) data involves the automatic or manual “calling” of sample data for each marker. The “calls” are data labels assigned to individual samples to reflect their genomic content.

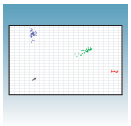
You can call sample data:

- Automatically, using the auto caller (see page 62)
- Manually, using the toolbar and scatterplot (see page 65)

Assigning Calls Automatically

1. In the Allelic Discrimination (AD) plate document, click (or select **Analysis** > **Analysis Settings**) to open the Analysis Settings dialog box.

Notes



2. Set the marker analysis settings:

   a. Select the **Marker** tab.

   b. Select **All Markers** from the Marker drop-down list.

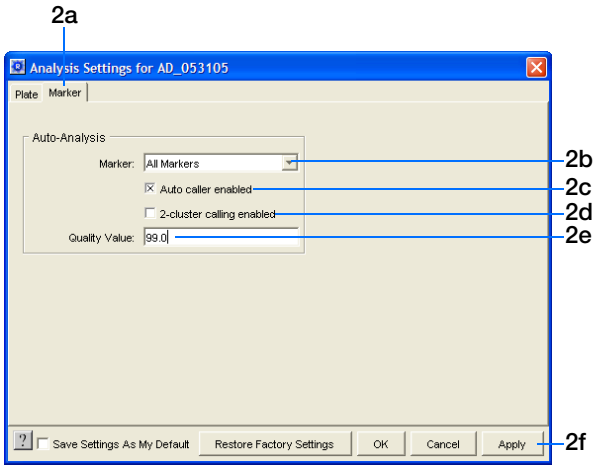
   Note: You can also select individual markers from the Marker drop-down list.

   c. Select the **Auto caller enabled** check box to activate automatic analysis.

   d. If you expect the analyzed data to consist of only two clusters, select the **2-cluster calling enabled** check box.

   e. In the Quality Value field, enter a percentage value to apply as the quality interval for auto-calling samples. (The greater the value, the more stringent the allele calling.)

   f. Click **Apply** to save your settings in the Marker tab.



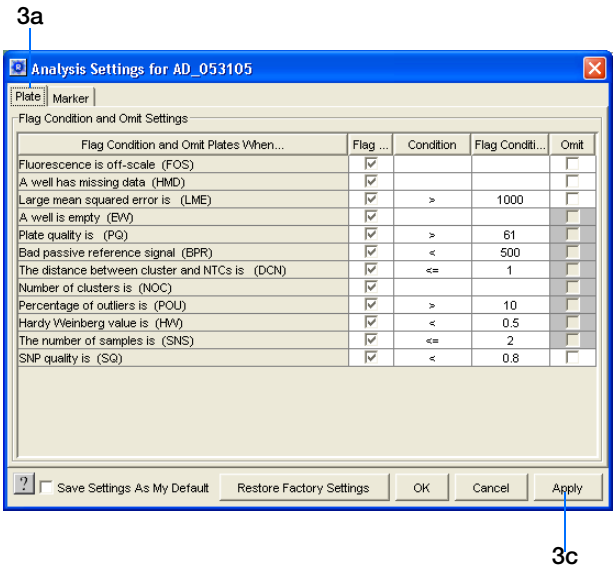
3. *Optional.* Assign flags:

   a. Select the **Plate** tab.

   b. Assign flags as desired.

   Note: When you assign flags, you can flag conditions and omit wells when certain criteria are met (for example, when a well has missing data). For more information, see Appendix D on page 99.

   c. Click **Apply** to save your settings in the Plate tab.



4. Click **OK** to close the Analysis Settings dialog box. The SDS software automatically reanalyzes the data using your new analysis settings.

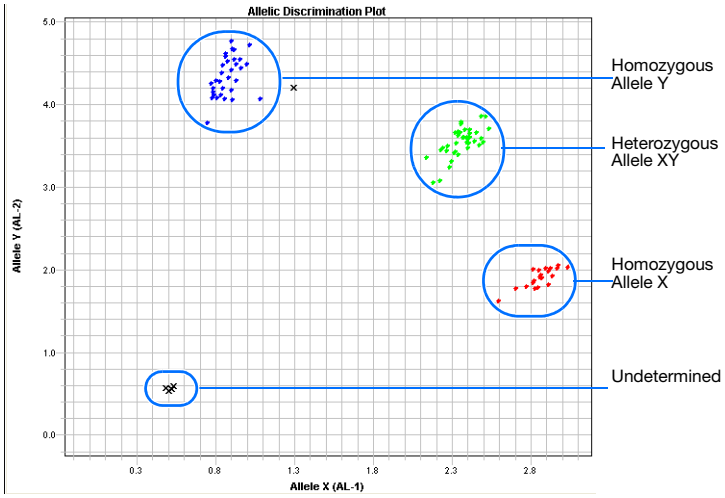
   Note: In order for the SDS software to automatically reanalyze the data via the Analysis Settings dialog box, you must have already analyzed the data once by clicking the Analyze button (see step 1 on page 55).

Notes

5. Select the **Results** tab to display the Allelic Discrimination Plot.

6. Select a marker from the Marker drop-down list. For the selected marker:
   - Alleles are identified in the Allelic Discrimination Plot
   - Samples are grouped as follows:

Samples Containing...	Are Grouped In...	Symbol
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 (Not shown in the example.)	■
Undetermined	Anywhere on plot	×

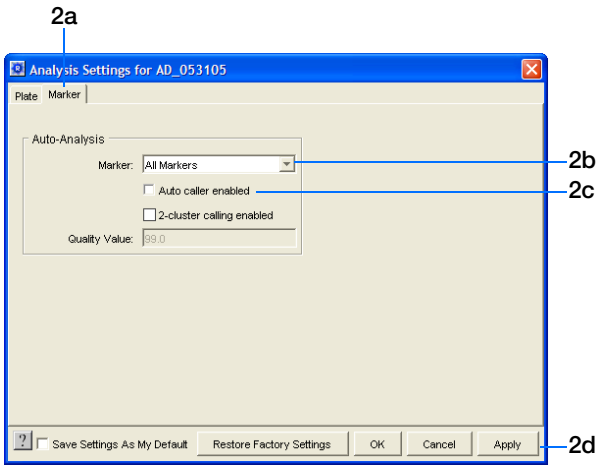


Notes

Assigning Calls Manually

1. In the Allelic Discrimination (AD) plate document, click (or select **Analysis** > **Analysis Settings**) to open the Analysis Settings dialog box.

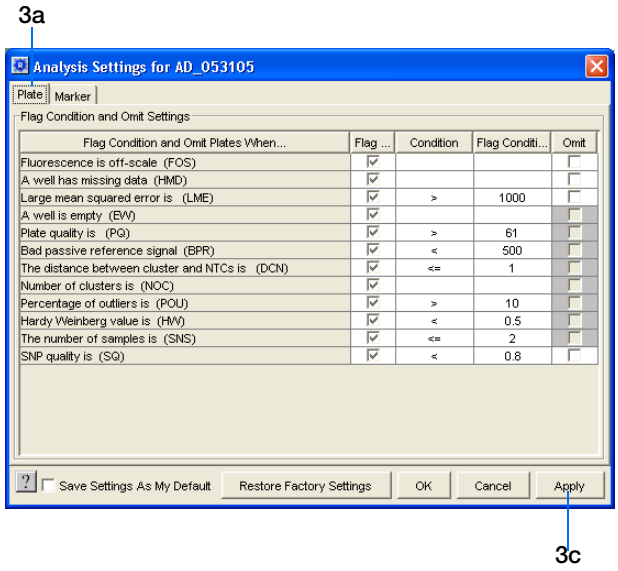
2. Set the marker analysis settings:
   a. Select the **Marker** tab.
   b. Select **All Markers** from the **Marker** drop-down list.
   c. Deselect the **Auto caller enabled** check box.
   d. Click **Apply** to save your settings in the Marker tab.



3. *Optional.* Assign flags:
   a. Select the **Plate** tab.
   b. Assign flags as desired.

   Note: When you assign flags, you can flag conditions and omit wells when certain criteria are met (for example, when a well has missing data). For more information, see Appendix D on page 99.

   c. Click **Apply** to save your settings in the Plate tab.



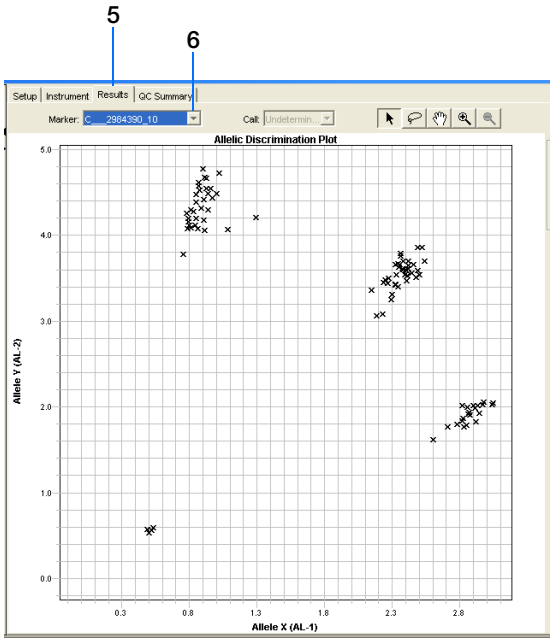
Notes

4. Click **OK** to close the Analysis Settings dialog box. The SDS software automatically reanalyzes the data using your new analysis settings.

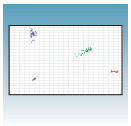
   Note: In order for the SDS software to automatically reanalyze the data via the Analysis Settings dialog box, you must have already analyzed the data once by clicking the Analyze button (see step 1 on page 55).

5. Select the **Results** tab to display the Allelic Discrimination Plot.

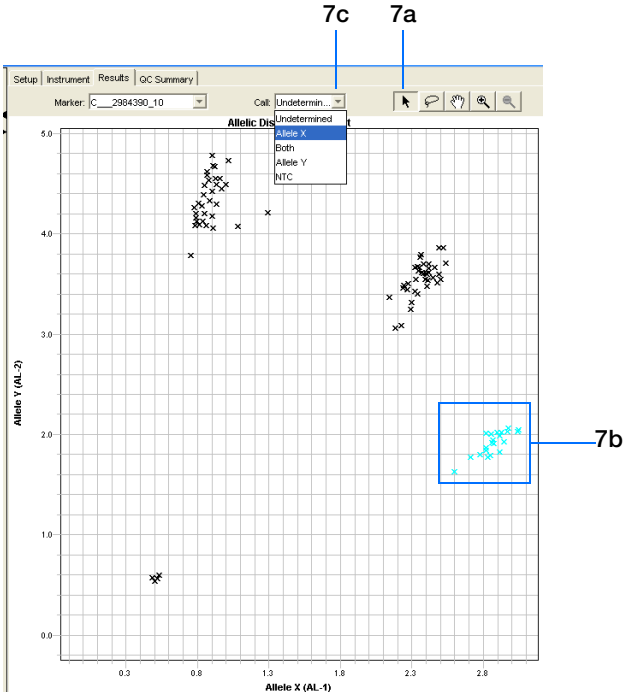
6. Select a marker from the Marker drop-down list. Crossmarks (× – Undetermined) representing the selected marker are displayed in the Allelic Discrimination Plot.



Notes



7. To assign calls:
   a. Click the selection tool.
   b. Click-drag a box around the allele data points in the lower-right of the plot.
   c. In the Call drop-down list, select **Allele X**.
   d. Click-drag a box around the allele data points in the upper-left of the plot.
   e. In the Call drop-down list, select **Allele Y**.
   f. Click-drag a box around the allele data points in the center of the plot.
   g. In the Call drop-down list, select **Both**.
   h. Click-drag a box around any allele data points that are not included in any of the grouped data points.
   i. In the Call drop-down list, select **Undetermined**.
8. If you are assigning calls for multiple markers, select a different marker from the Marker drop-down list and repeat step 7.



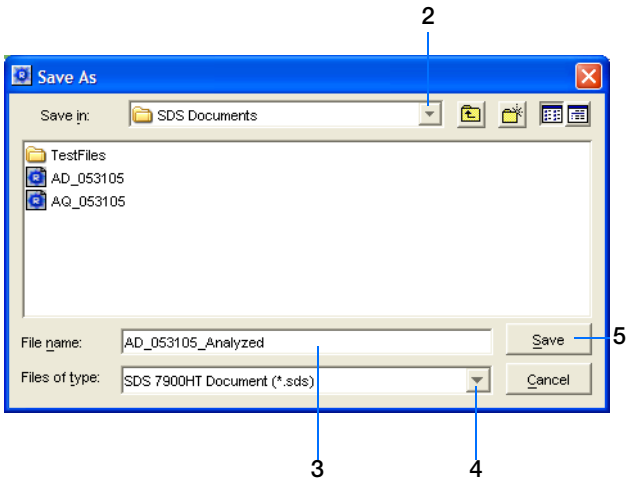
Notes

Saving the Analysis Results

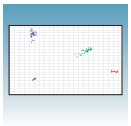
IMPORTANT! Although the SDS software saves any changes made to the appearance of a plate document and to the analysis settings, it does not save the calls made during the analysis.

1. Select **File** > **Save As** to open the Save As dialog box.
2. In the Save in field, navigate to and select a directory for the analyzed plate document.
3. In the File name field, either:
   - Enter a file name for the plate document, *or*
   - Enter or scan the barcode number for the reaction plate

   Note: The SDS software does not require that the plate document name match the barcode of the corresponding reaction plate.
4. From the Files of type drop-down list, select **SDS 7900HT Document (*.sds)**.
5. Click **Save**. The software saves the plate document to the specified directory.



Notes



Post-Analysis Options

The following options are available after the analysis:

- Exporting data as text files (below)
- Exporting data as graphic files (page 70)
- Printing a data report (page 72)

Exporting Data as Text Files

You can export raw or analyzed data from plate documents into tab-delimited text files (*.txt). The text files can then be imported into spreadsheet software, such as Microsoft® Excel software.

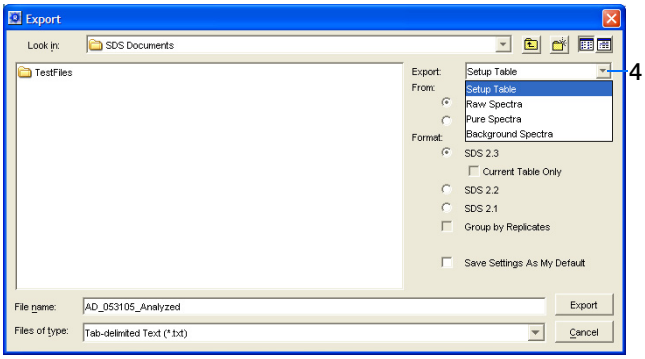
1. Open the plate document from which you wish to export data.

2. Select **File** > **Export** to open the Export dialog box.

3. In the Look in field, navigate to and select a directory for the new file.

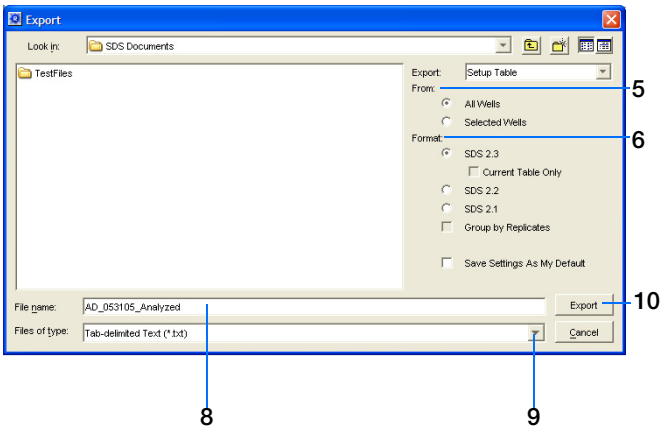
4. From the Export drop-down list, select the data type to export.

   Note: For more information about the data type options, see the *SDS Online Help*.



5. Select to export data from **All Wells** or a select group of wells (**Selected Wells**).

   Note: To use the Selected Wells option, you must select a subset of wells in the plate document before opening the Export dialog box.



6. From the Format options, select the appropriate software version.

7. *Optional.* Check **Save Settings As My Default**. Check this option if you want the SDS software to automatically apply the current settings every time you export data as a text file.

Notes

8. In the File name field, type a name for the new file.

9. From the Files of type drop-down list, select **Tab-delimited Text (*.txt)**.

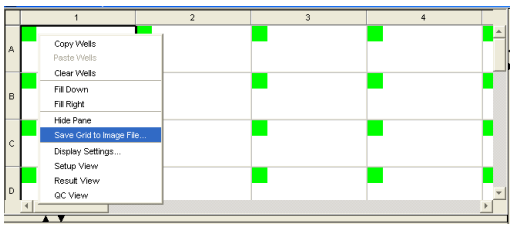
10. Click **Export**. The software exports the new file to the specified directory.

Exporting Data as Graphic Files

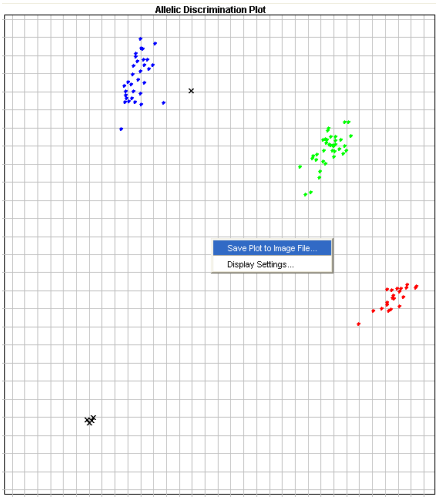
You can export the Plate Grid and plot images from plate documents as Joint Photographic Experts Group (JPEG) graphic files (*.jpeg). The JPEG files can then be viewed in most common word processing, spreadsheet, and HTML-based software.

1. Open the plate document from which you wish to export data.

2. To export the:
   - Plate Grid, right-click in the Plate Grid, then select **Save Grid to Image File**. The Save dialog box appears.



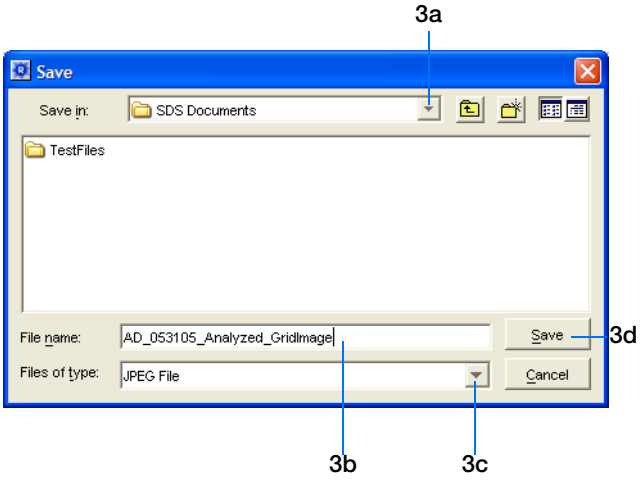
   - Plot, right-click in the plot, then select **Save Plot to Image File**. The Save dialog box appears.



Note: If desired, adjust the plot dimensions (length and width) as you want them to appear in the exported file. The exported file retains the dimensions of the original screen element.

Notes

3. Complete the Save dialog box:
   a. In the Save in field, navigate to and select a directory for the new file.
   b. In the File name field, type a name for the new file.
   c. From the Files of type drop-down list, select **JPEG File**.
   d. Click **Save**. The software saves the new file to the specified directory.



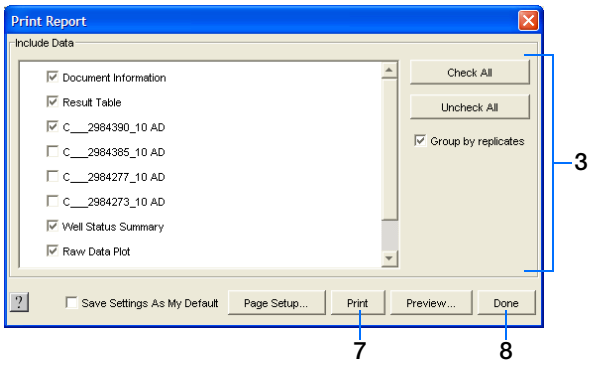
Notes

Printing a Data Report

You can use the SDS software to print a report of the analyzed data containing individual or multiple elements of the plate document.

Note: For more information on printing reports, see the *SDS Online Help*.

1. Open the plate document from which you wish to print a report.
2. Click (or select **File** > **Print Report**) to open the Print Report dialog box.
3. In the Include Data pane, select the plate document element(s) to print.
4. *Optional.* To format the display of the report and how the report is printed, click **Page Setup**.
5. *Optional.* Check **Save Settings As My Default**. Check this option if you want the SDS software to automatically apply the current settings every time you print a report.
6. *Optional.* Click **Preview** to view the report before printing it.
7. Click **Print** to open the Print dialog box and print the report.
8. When the report has finished printing, click **Done** to close the Print Report dialog box.



Notes

Sample Experiment

About the Sample Experiment

Overview

To better illustrate how to design, perform, and analyze allelic discrimination experiments, this appendix guides you through a sample experiment. The sample experiment represents a typical allelic discrimination experiment setup that you can use as a quick-start procedure to familiarize yourself with the allelic discrimination workflow. Detailed steps in the allelic discrimination workflow are described in the preceding chapters of this guide. Also in the preceding chapters are Sample Experiment boxes, which provide details for some of the related steps in the sample experiment.

Description

The objective of the sample experiment was to determine the genotype of 92 individuals at the SNP site of a desired target sequence.

The experiment used multiplex PCR. The primers and probes were ordered from TaqMan® SNP Genotyping Assays (AB Assay ID C 2984390 10).

Reactions were set up for PCR using the TaqMan® Universal PCR Master Mix (2×), No AmpErase® UNG, and appropriate primers and probes.

The sample experiment data and results were generated using a 7900HT Fast System by performing:

- **An amplification run** using a Standard Curve (AQ) plate document to generate real-time PCR data. The real-time PCR data could then be used to analyze and troubleshoot the PCR data for the allelic discrimination assay, if needed.
- **An allelic discrimination run** using an Allelic Discrimination (AD) plate document. The SDS software analyzed the data, then we assigned allele calls (automatically or manually).

Notes

Sample Allelic Discrimination Experiment Procedure

1. Design the experiment (for more detail, see Chapter 2 on page 9):
   a. Order the appropriate master mix.
      The sample experiment used TaqMan® Universal PCR Master Mix, No AmpErase® UNG.
   b. Select and order the probes and primers.
2. Extract the DNA from samples (for more detail, see "Preparing DNA" on page 14).
   The sample DNA for the sample 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 (for more detail, see "Preparing the Reaction Mix" on page 15).
   The sample experiment was run on a standard 384-well reaction plate. As shown below, enough reaction mix was prepared for 4 NTCs and 92 samples/unknowns, plus 10% extra to account for pipetting losses.

Component	Volume (µL)	
	One Reaction	106 Reactions‡
TaqMan® Universal PCR Master Mix (2×), No AmpErase® UNG	2.50	265
SNP Genotyping Assay Mix (20×)	0.25	26.5
Total	2.75	291.5

‡ Extra volume was included to account for pipetting losses.

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.

CAUTION **CHEMICAL HAZARD.** **TaqMan® Universal PCR Master Mix (2×)** 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.

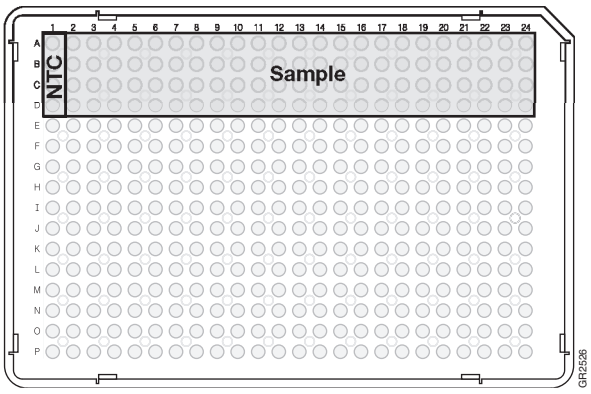
WARNING **CHEMICAL HAZARD. SNP Genotyping Assay Mix (20×)** contains **formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and

Notes

4. Prepare the reaction plate (for more detail, see "Preparing the Reaction Plate" on page 17). A reaction plate for an allelic discrimination assay contains the following:
   - Reaction mix
   - No Template Controls (NTCs)
   - Unknown genomic DNA samples
   - Known genomic DNA controls (optional, not included in the sample experiment)

For the sample experiment, a standard 384-well reaction plate was used. 2.75 µL of reaction mix were pipetted into each well. 2.25 µL of the following solutions were pipetted into the indicated wells:

To wells...	We added...
A1, B1, C1, and D1 (NTC, No Template Control)	Nuclease-free water or TE (Tris-EDTA) buffer
A2 through A24 B2 through B24 C2 through C24 D2 through D24 (Sample or Unknown)	Diluted purified genomic DNA

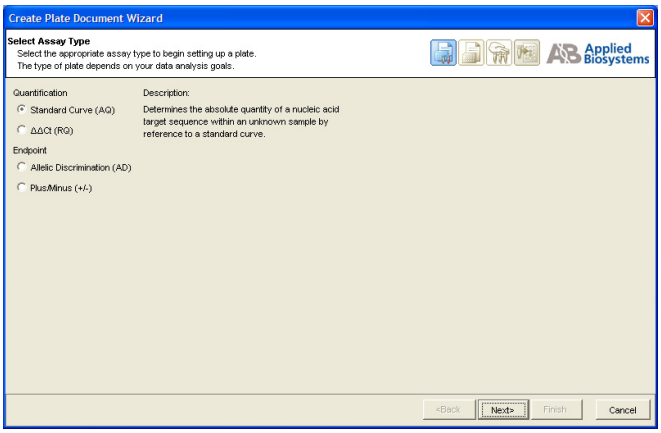


5. Create a Standard Curve (AQ) plate document for amplifying samples (for more detail, see "Creating a Standard Curve (AQ) Plate Document" on page 39):

Notes

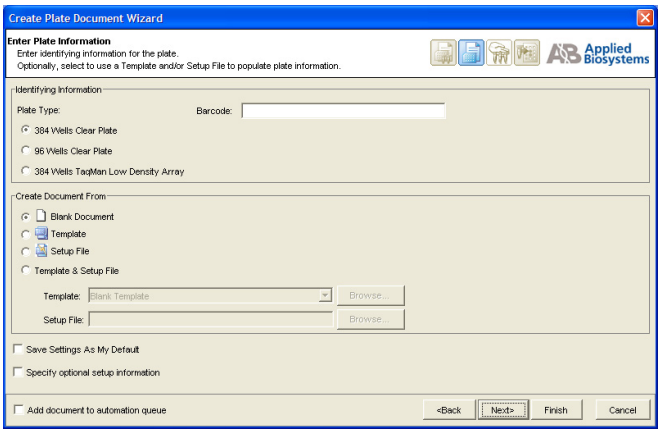
a. Select **File > New Plate Wizard**.

b. For the assay type, select **Standard Curve (AQ)**, then click **Next**.

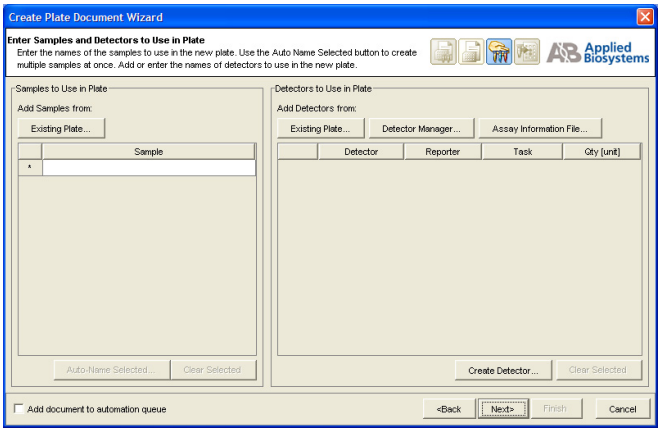


Note: A standard curve is not necessary for a non-quantitation amplification run.

c. Select **96 Wells Clear Plate** or **384 Wells Clear Plate**, select **Blank Document**, then click **Next**.

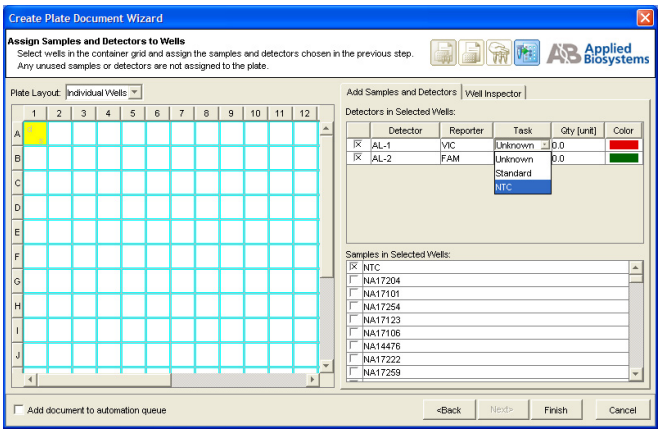


d. Enter the samples and detectors to use in the plate, then click **Next**.

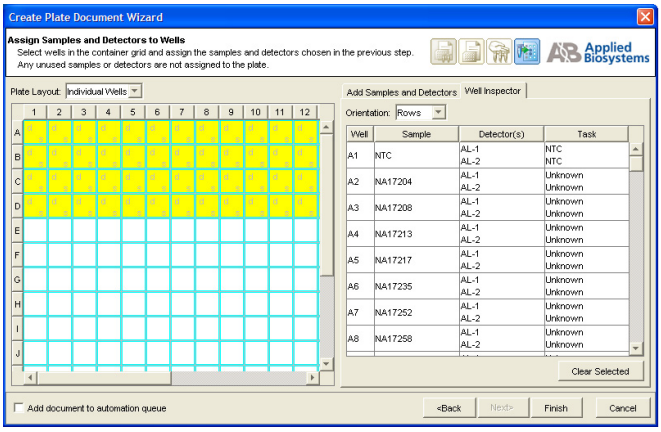


Notes

e. Assign detectors, samples, and tasks to the wells.



f. Select the Well Inspector tab to verify the sample setup information for each well, then click **Finish**.



g. Save the new plate document.

6. Perform the amplification run (for more detail, see "Performing the Amplification Run" on page 47):

   a. In the Standard Curve (AQ) plate document, select the **Instrument > Thermal Cycler** tabs.

   b. For the Mode, select **Standard** or **9600 Emulation**.

   c. Enter a Sample Volume.

Notes

d. Select the **Thermal Profile** tab; accept the default stages (shown at right) or modify the thermal profile as needed.

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

For the sample experiment (using TaqMan® Universal PCR Master Mix, No AmpErase® UNG), the thermal profile was modified as shown at right.

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

e. Select the **Real-Time** tab, click **Open/Close**, then load the reaction plate into the instrument

f. Click **Start Run**. As the instrument performs the amplification run, it displays real-time status information in the Real-Time tab.

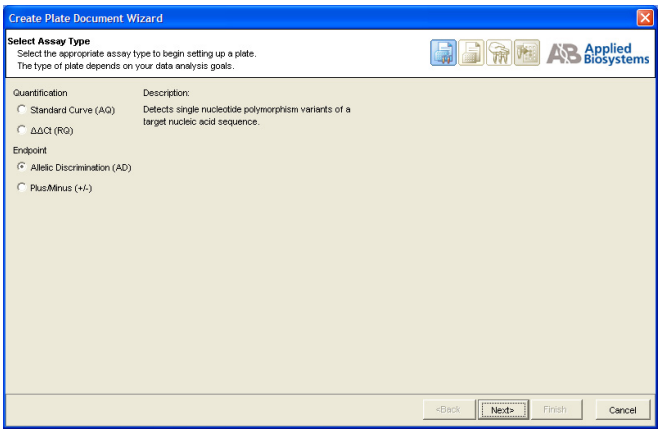
g. When the amplification run is complete, select **File > Save**, then close the plate document.

7. Create an Allelic Discrimination (AD) plate document (for more detail, see "Creating an Allelic Discrimination (AD) Plate Document" on page 23):

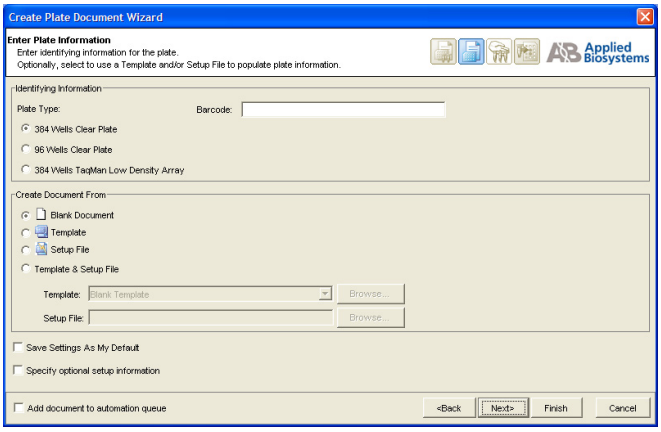
Notes

a. Select **File > New Plate Wizard**.

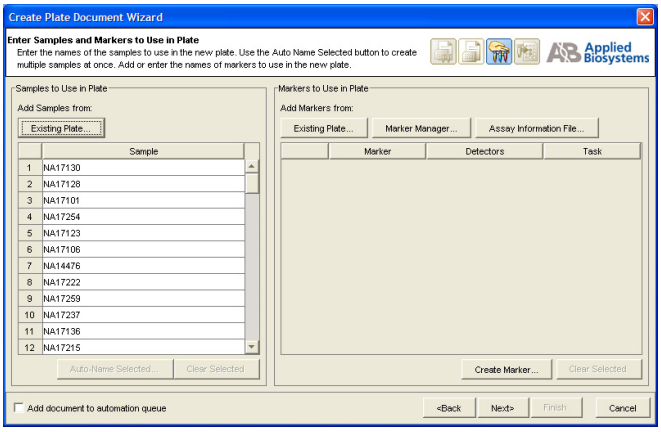
b. For the assay type, select **Allelic Discrimination (AD)**, then click **Next**.



c. Select **96 Wells Clear Plate** or **384 Wells Clear Plate**, select **Blank Document**, then click **Next**.

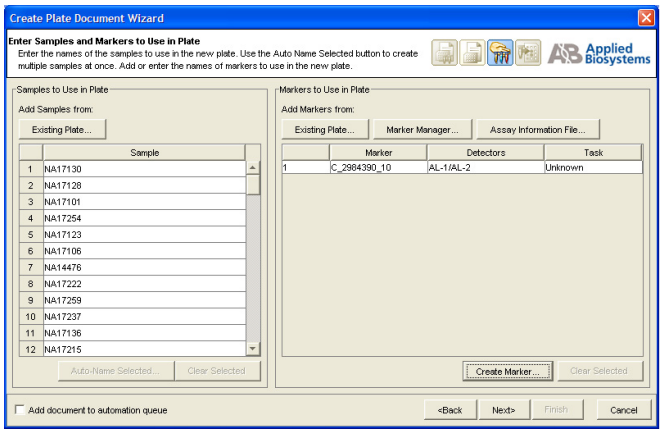


d. Import the sample names from the Standard Curve (AQ) plate document you just created.

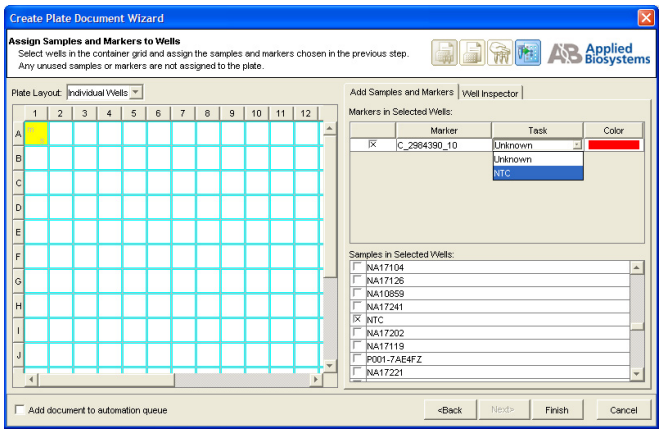


Notes

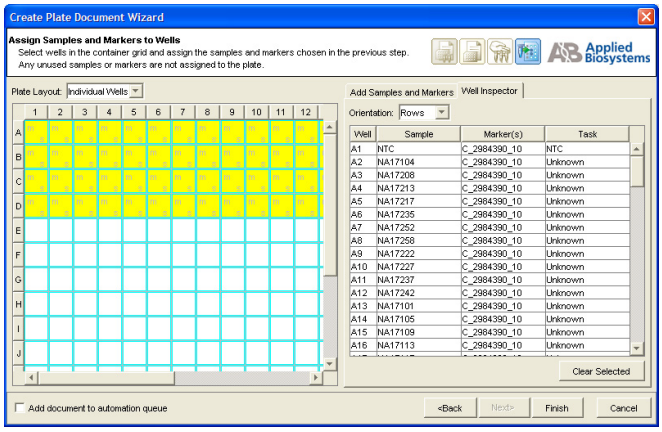
e. Enter the markers to use in the plate, then click **Next**.



f. Assign markers, samples, and tasks to the wells.



g. Select the Well Inspector tab to verify the sample setup information for each well, then click **Finish**.



h. Save the new plate document.

Notes

8. Perform the allelic discrimination run (for more detail, see "Performing the Allelic Discrimination Post-Read Run" on page 52):
   a. In the Allelic Discrimination (AD) plate document, select the **Instrument** > **Plate Read** tabs.
   b. Click **Open/Close**, then load the reaction plate into the instrument.
   c. Click **Post Read**. As the instrument performs the run, it displays status information in the Plate Read tab.

9. Analyze and evaluate the allelic discrimination run (for more detail, see "Analyzing the Run and Evaluating the Results" on page 55):
   a. Click (or select **Analysis** > **Analyze**). The SDS software analyzes the run data.
   b. Select the **Results** tab.
   c. View the analyzed run data in the Plate Grid, Results Table, and Allelic Discrimination Plot.

10. If desired, assign calls automatically or manually (for more detail, see "Assigning Calls" on page 62).

11. Save the plate document (for more detail, see "Saving the Analysis Results" on page 68).

12. If desired, export or print the results data (for more detail, see "Post-Analysis Options" on page 69).

Notes

Notes

SDS Automation Controller Software

Overview

The Sequence Detection Systems (SDS) Automation Controller Software v2.3 provides an interface between the Zymark® Twister Microplate Handler, the 7900HT Fast System, the fixed-position bar code reader, and the plate documents created in main Sequence Detection Systems Software v2.3.

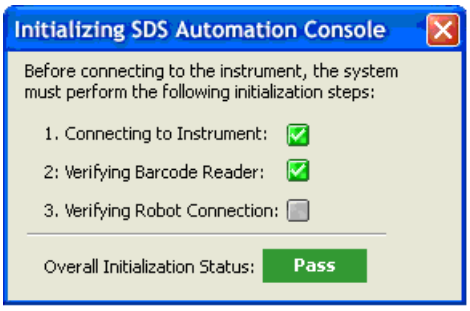
This Automation Controller software controls and coordinates the action of the 7900HT instrument and the automation module. It initiates and controls the sequence detection run and acquires data during the run.

This option allows for plates to be run as part of a group or batch allowing for high-throughput unattended operation. For more information about the Automation Controller, refer to the *SDS Online Help*.

Using the Automation Controller Software

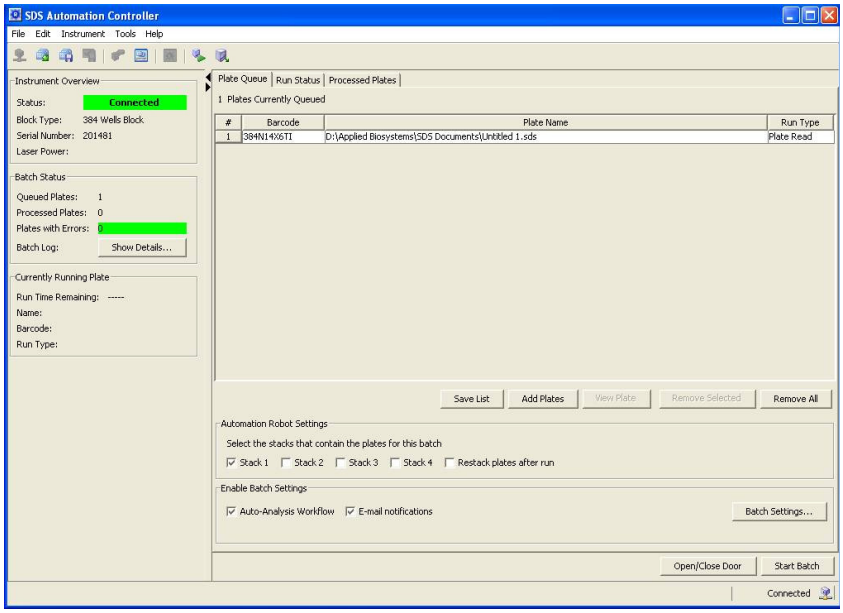
Note: Before launching the Automation Controller software, you must close the main Sequence Detection Systems Software v2.3 application. Failure to do so will result in an instrument connection failure.

1. Double click the Automation Controller shortcut icon. An initialization pop-up will appear displaying the status of instrument connection and other verifications. If you encounter an error, refer to the *SDS Online Help*.



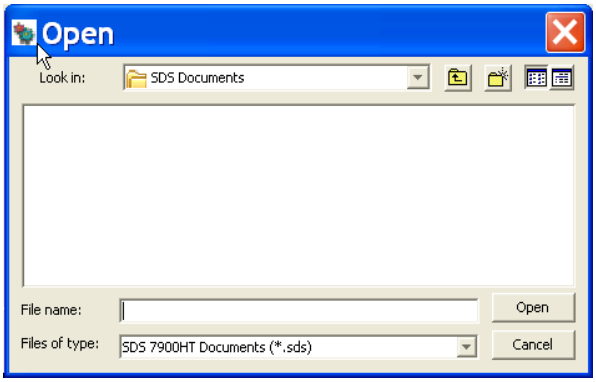
Notes

2. If you have already created and saved the desired plates (sent them to the queue) in the main SDS application, then the plates will appear in the Plate Queue tab. For more information about creating plate documents and sending them to the queue, refer to "Creating a Standard Curve (AQ) Plate Document" on page 39 or "Creating an Allelic Discrimination (AD) Plate Document" on page 23.



3. You can add plates by clicking **Add Plates** or (or click).

   a. At the Open dialog box, navigate to the location where the plate files are located.

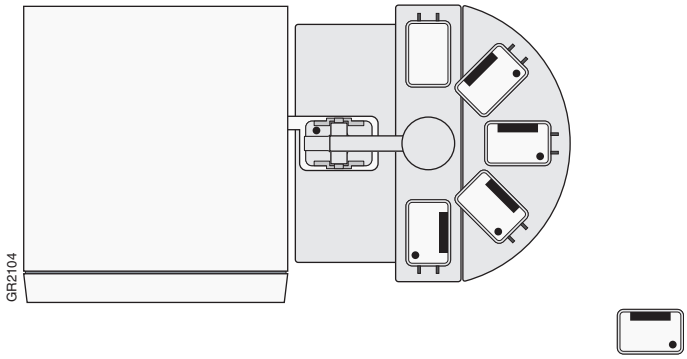


   b. Use the barcode reader to scan the Plate IDs. Note that you can automatically scan the next plate without having to select Add Plates again. You can also save the list of queued plates, by clicking **Save List**.

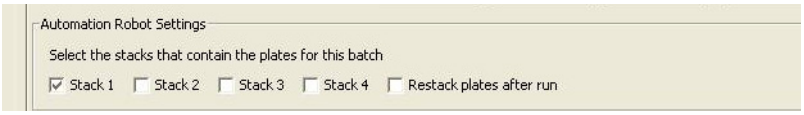
Note: To remove a plate, click on the plate name then click **Remove**. To remove all plates, click **Remove All**.

Notes

4. Place the plates in the input stack of the Zymark® Twister Microplate Handler. The order of the plates may vary from the order they were scanned in (the Handler's fixed barcode reader will verify the location of each plate). The software matches the plate document and the method before it starts the run. Orient the plates inside the stacks so that the well A1 of each plate corresponds to the locations shown in the figure below.

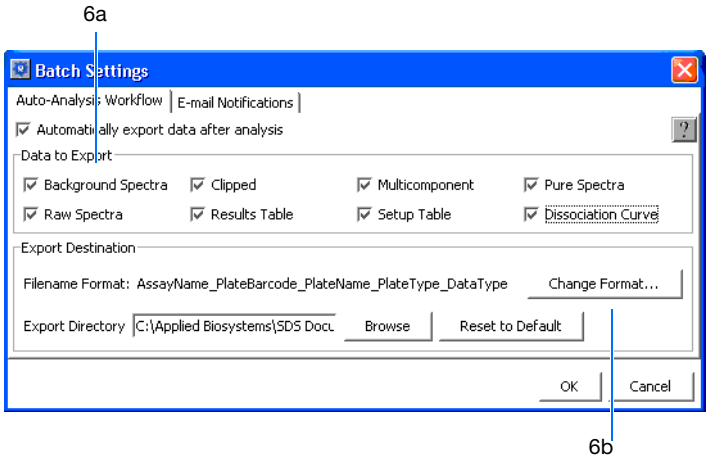


5. Click into the boxes next to each stack location where the plates are located.



6. Select **Tools > Batch Settings** to specify the desired output settings.

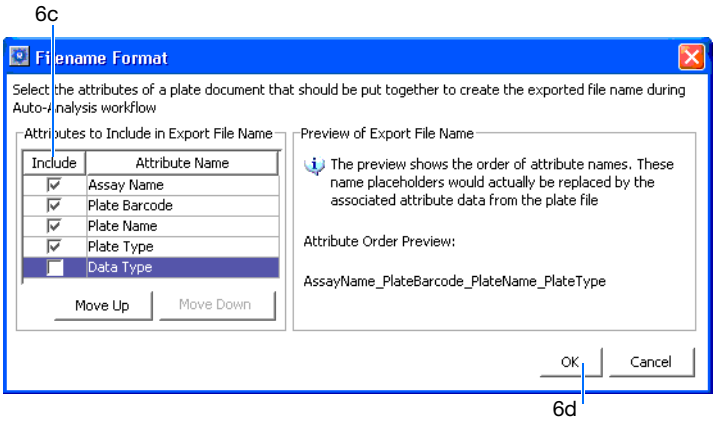
   a. In the Batch Settings dialog box, at the Auto-Analysis Workflow tab, click into a box to select that export option. You can also change the export directory by clicking the Browse button (located in the Export Destination box) and specifying a new location for the file data to be exported to. Note that after analysis exported data will be saved as *.txt files.



   b. Click on **Change Format** to assign attributes to be included in the export file name.

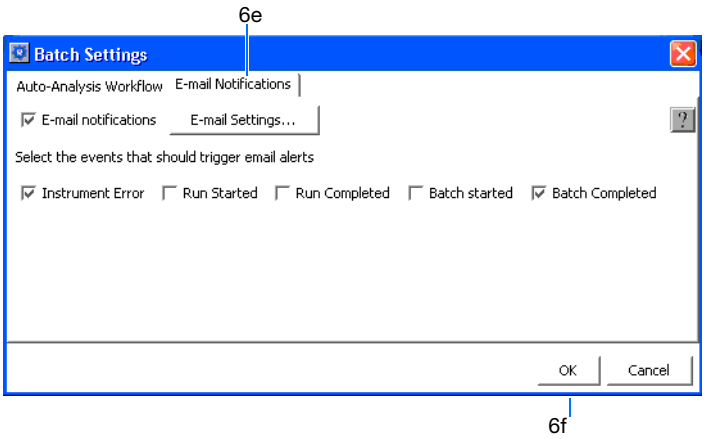
Notes

c. At the Filename dialog box, click into a field box (under the Include column) to deselect an attribute.



d. Click **OK**.

e. Back at the Batch Settings dialog box, click on the **Email Notifications** tab to specify desired email notification options. For more information on email settings, see your network administrator.

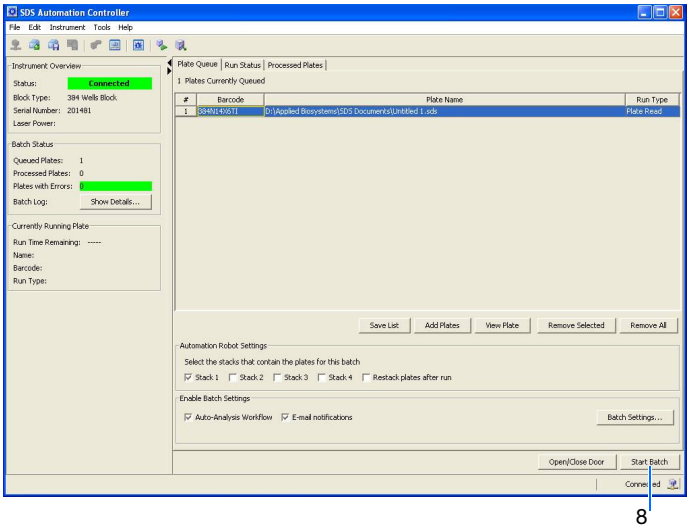


f. Click **OK**.

7. Verify that the plate adapter and the output stack of the Handler are empty.

Notes

8. Click **Start Batch**.

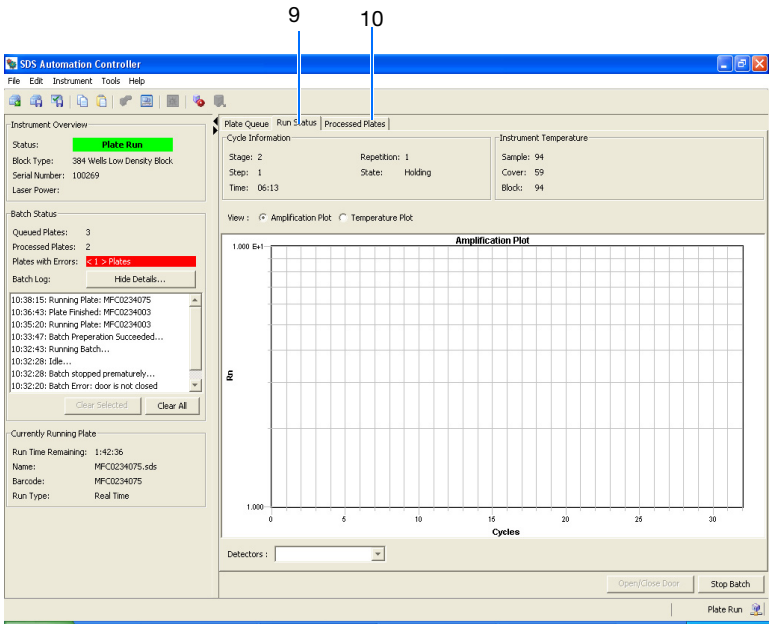


Note: Note that if the plate type does not match the plate block installed on the instrument, the Automation Controller will not start. You can assign plate types by selecting **Tools > Options** and selecting the desired plate format. The handler loads the plates and the instrument starts the run.

Note: To stop the run, click **Stop Batch**. You will see a warning message asking you to verify stopping the run.

Notes

9. Click on the **Run Status** tab to view the plate run status. Note that once a plate is being processed, it will not appear in the Processed Plates tab or be listed in the Queued Plates section of the Batch Status box. The plate information will be available under the Currently Running Plate section.



Note: For standard curve (AQ) and ddCt (RQ) plates, a view of the amplification and temperature plot will be available and can be selected by clicking on the radial button.

10. Click on **Processed Plates** to view information about the processed plates.

Notes

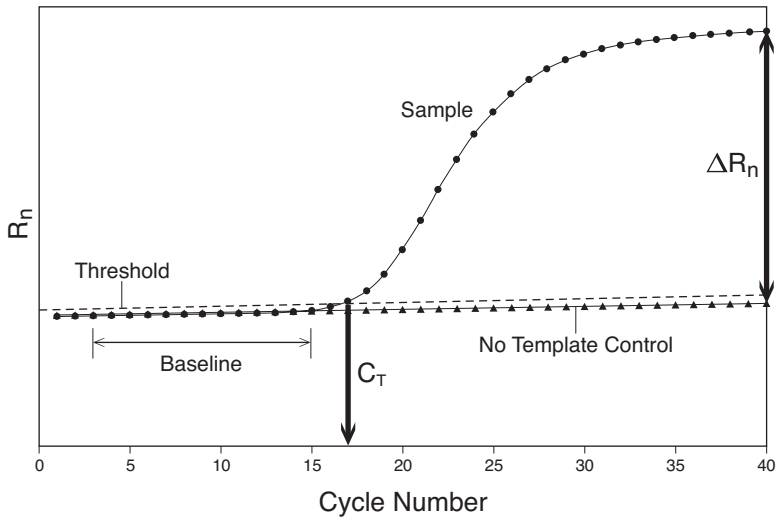
Analyzing and Viewing Amplification Data

Terms Used in Quantitation Analysis

The following are terms commonly used in quantitation analysis.

Term	Definition
Baseline	A line fit to fluorescence intensity values during the initial cycles of PCR, in which there is little change in 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 volume.
Reporter dye	The dye attached to the 5′ end of a TaqMan® probe. The dye provides a signal that indicates 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 is a representative DNA amplification plot and includes some of the terms defined above.



Notes

Analyzing the Amplification Data

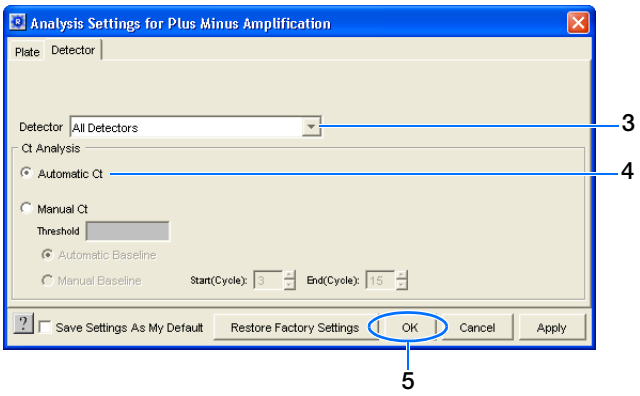
Before you can analyze the amplification data from the standard curve (AQ) plate document you generated in Chapter 5, you must specify parameter values (baseline and threshold settings or C_T) for the analysis.

Unless you have already determined the optimal baseline and threshold settings (C_T) for your experiment, use the automatic baseline and threshold feature of the SDS software (Automatic C_T) to analyze the amplification run. If the baseline and threshold are called correctly for each well, you can proceed to view the results (see "Reviewing the Automatic C_T Results" on page 91). Otherwise, you must manually set the baseline and threshold (see "Reviewing the Automatic C_T Results" on page 91).

For more information about determining C_T, refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4348358). For more information about manually adjusting C_T, refer to the *SDS Online Help*.

Starting the Analysis

1. Open the AQ plate document you want to analyze.
2. Click (or select **Analysis** > **Analysis Settings**). The Analysis Settings dialog box opens.



3. In the Detector drop-down list of the Detector tab, select **All Detectors**.
4. In the Ct Analysis window, select **Automatic Ct**. The SDS software will automatically generate baseline values for each well and threshold values for each detector.
5. Click **OK** to apply any changes and close the Analysis Settings dialog box.
6. Click (or select **Analysis** > **Analyze**). The Results and QC Summary tabs are available after analysis completes.

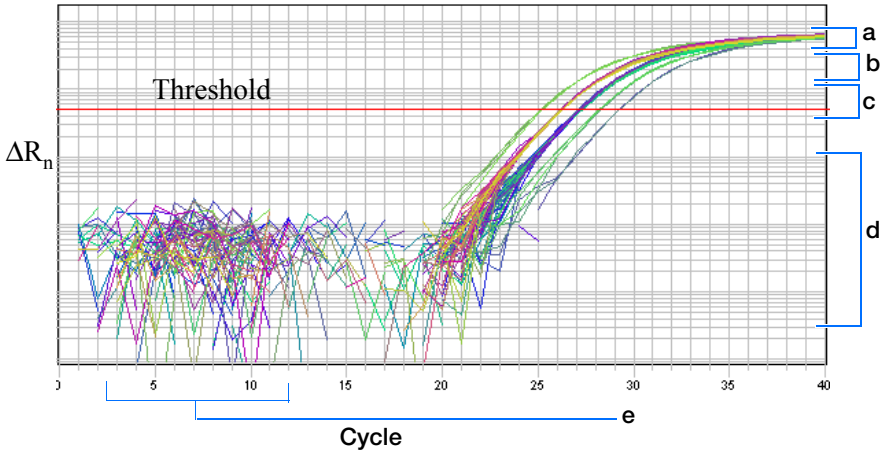
Notes

Reviewing the Automatic C_T Results

Click the **Results** tab to view results of the amplification run for each well. The SDS software Automatic C_T analysis process calculates baseline and threshold values for a detector based on the assumption that the data exhibits a "typical" amplification curve.

A typical amplification curve has a:

- Plateau phase (a)
- Linear phase (b)
- Exponential (geometric) phase (c)
- Background (d)
- Baseline (e)



IMPORTANT! Experimental error (such as contamination, pipetting errors, etc.) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the SDS software. Therefore, Applied Biosystems recommends you examine the amplification plot and review the assigned baseline and threshold parameter values for each well after analysis completes, and if necessary manually adjust the baseline and threshold. For more information, see the *SDS Online Help*.

C

Notes

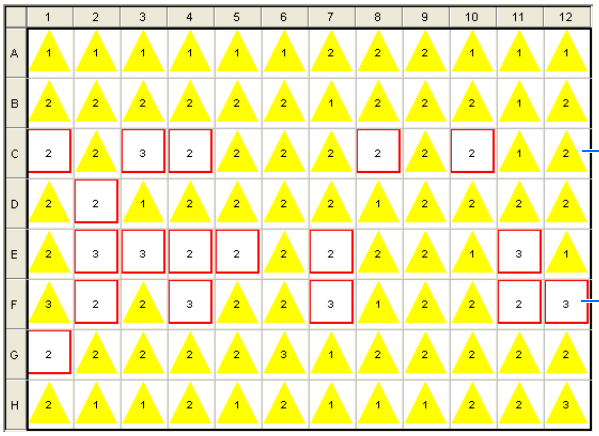
Viewing the Amplification Data

The following options are available for further determination of the analysis results:

- Using the Plate Grid Results View (below)
- Using the Raw Data Plot (page 93)
- Using the Multicomponent Data Plot (page 93)
- Using the Amplification Plot Views (page 94)

Using the Plate Grid Results View

Before analysis, the plate grid displays the setup information for each well, including: detector and sample information. After analysis, the plate grid displays the category and number of data flags generated for each well of the plate, as illustrated below.

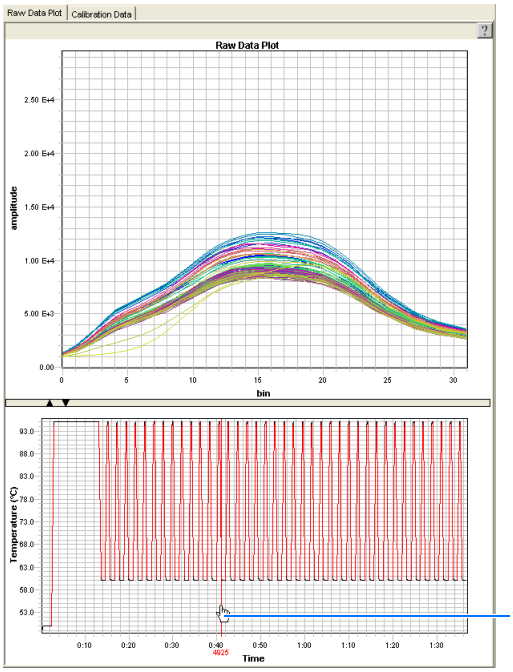


Note: A list of the data flags generated for the plate document is also displayed in both the QC Summary tab and Results Table. See the *SDS Online Help* for more information about viewing and configuring the plate data flags.

Notes

Using the Raw Data Plot

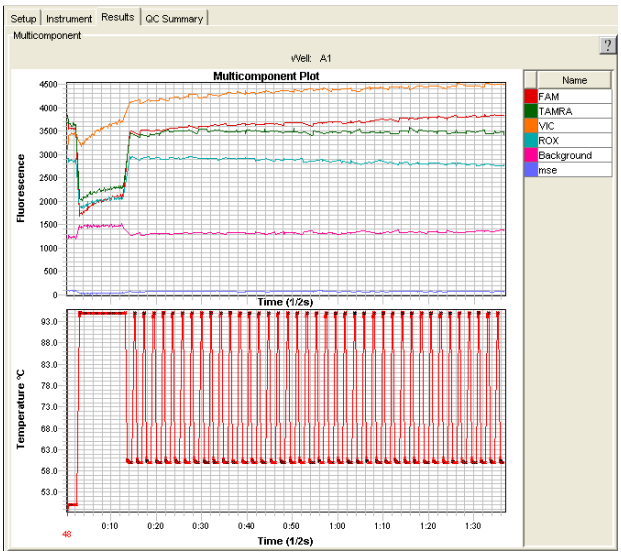
The Raw Data Plot (click) displays the fluorescence spectra and temperature profile of selected wells. The vertical slider in the Temperature plot allows you to see the spectra for each run cycle by dragging it with the pointer.



Click and drag the slider to view temporal changes in the raw data profile

Using the Multicomponent Data Plot

The Multicomponent Data Plot (click) displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.



Note: Only one well can be displayed at a time.

Notes

Using the Amplification Plot Views

The Amplification Plot views allow you to review the post-run amplification results for selected wells from the plate grid.

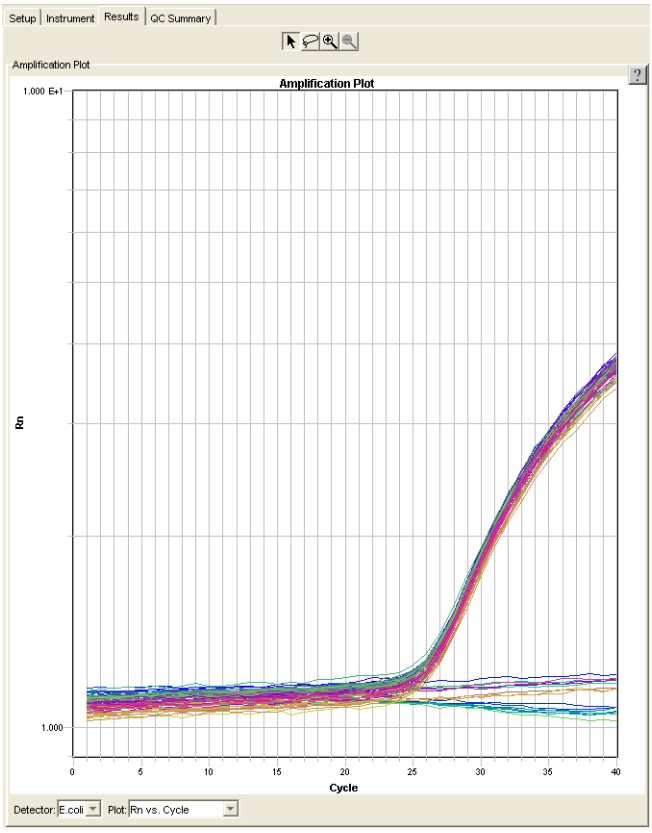
Several different views are available from the **Plot** drop-down list:

- **R_n vs. Cycle** (below)
- **ΔR_n vs. Cycle** (page 95)
- **C_T vs. Well Position** (page 96)

Note: For more information on using the Amplification Plot views to evaluate amplification results, see the *SDS Online Help*.

R_n vs. Cycle (linear scale)

This plot displays normalized reporter dye fluorescence signal (as R_n) in linear scale as a function of run cycle. You can use this plot to identify and examine irregular amplification.

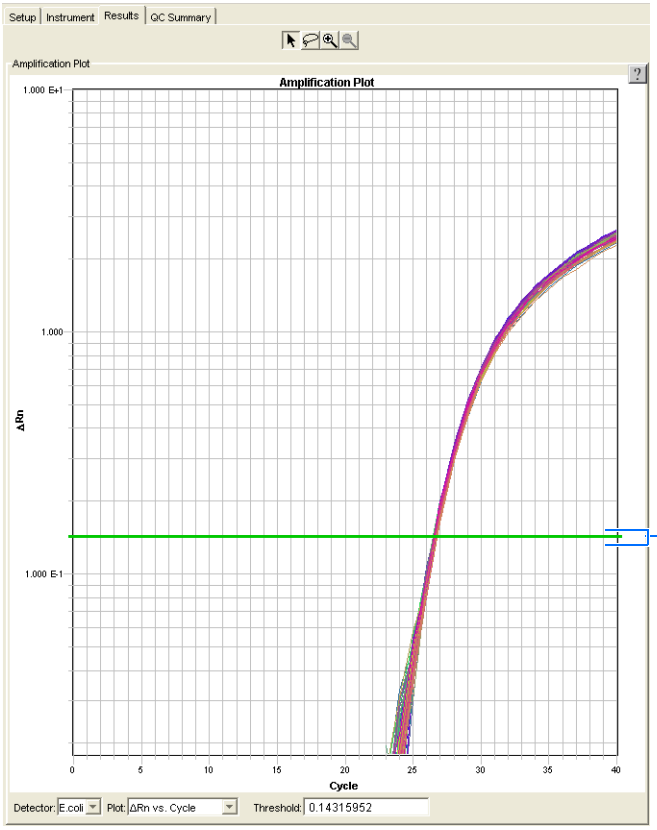


Note: For more information about R_n, refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4348358).

Notes

ΔR_n vs.Cycle (log scale)

This plot displays the reporter dye fluorescence signal (as ΔR_n) in log scale as a function of run cycle, and is the default view after analysis completes. You can use this plot to identify and examine irregular amplification and to manually set the threshold and baseline parameters for the run.

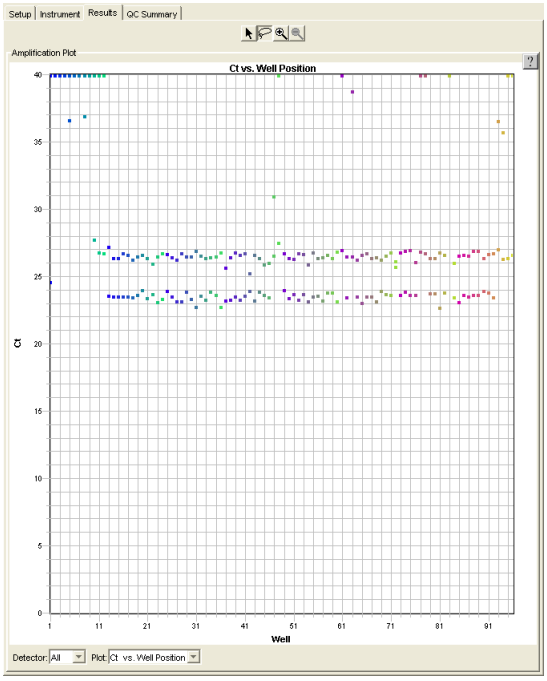


IMPORTANT! Applied Biosystems recommends verifying that the baseline and threshold were called correctly for each well. If necessary, adjust the values manually as described in the *SDS Online Help*.

Notes

Ct vs. Well Position Plot

This plot displays threshold run cycle (C_T) as a function of well position. You can use this plot to locate outliers from detector data sets. See "Omitting Samples" below.



Omitting Samples

Experimental error (such as contamination, pipetting errors, etc.) can produce atypical amplification data for some wells. These wells typically produce C_T values that differ significantly from the average. If included in the baseline and threshold value calculations, these outlying data (outliers) can result in erroneous threshold run cycle (C_T) measurements.

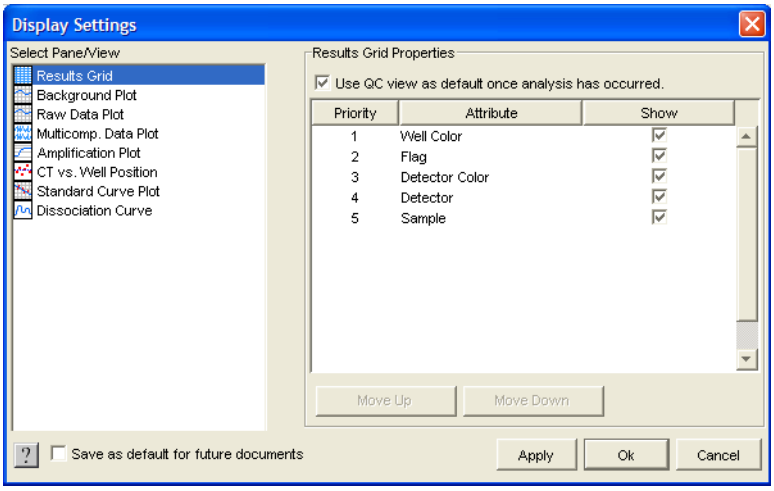
To ensure precision, carefully review your data set for outliers. You can remove outliers manually using the Ct vs. Well Position plot and the plate grid. For more information about omitting wells, refer to the *SDS Online Help*.

Note: You will need to re-analyze the data each time you remove samples from the data set.

Notes

Adjusting Plot Settings

Click or select **View > Display Setting** to open the Display Settings dialog box and adjust the selected plot settings.



Note: The adjustable settings depend on which plot you are viewing. Refer to the *SDS Online Help* for more information.

C

Notes

Notes

Flags and Filtering for Allelic Discrimination (AD) Plate Documents

Overview

In Sequence Detection Systems (SDS) Software v2.3, you can automatically flag and filter results data to meet specified criteria. Flags and filters are assay-specific and are assigned at either the system level or are user-configured.

- System level flags are not user-configurable. These flags are displayed in the Results Table and the QC Summary tab.
- User-configured flags are set/defined by the user. These flags are also displayed in the Results Table and QC Summary tab.

For more information about flags and filters, see the *SDS Online Help*.

Using the Analysis Settings Dialog Box to Assign Flags

You assign flags when you configure the analysis settings for an individual plate document or study. When you open the Analysis Settings dialog box for your current plate document, a list of the flags applicable to that plate document appears in the Plate tab.

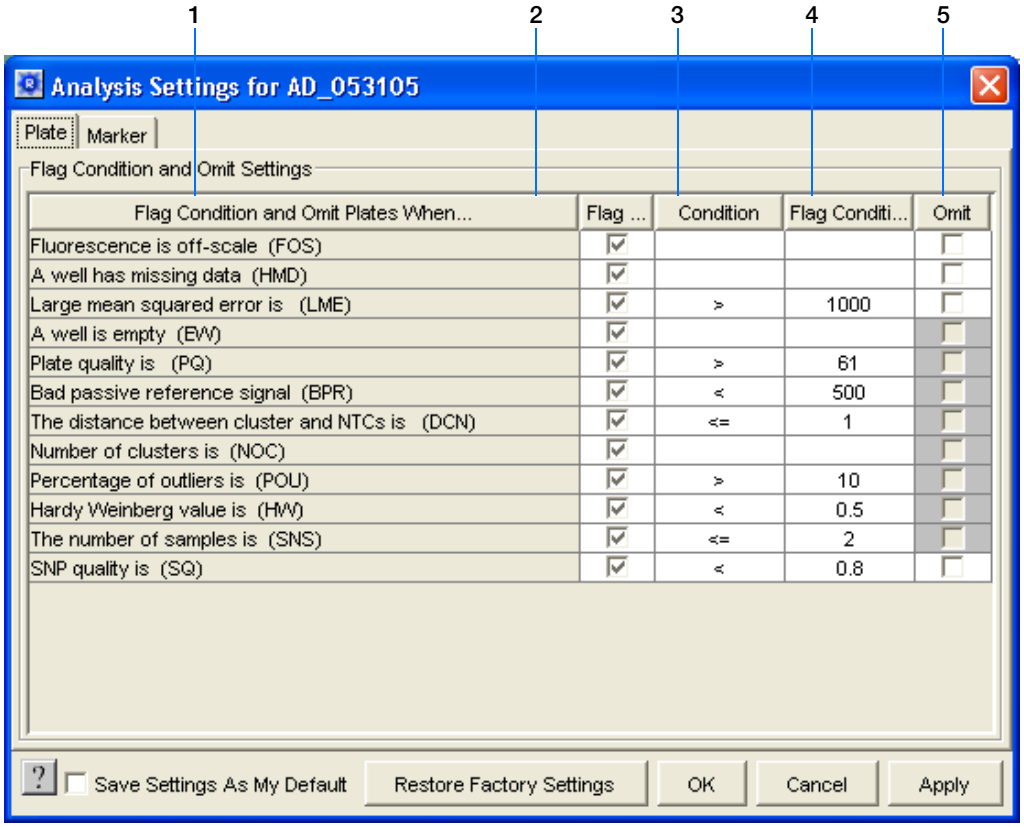
1. Click (or select **Analysis** > **Analysis Settings**) to open the Analysis Settings dialog box.
2. Select the **Plate** tab. A list of all applicable flags for the open plate document is displayed.

For more information about assigning flags, see the *SDS Online Help*.

Plate Tab

The contents of the Plate tab are described on page 100.

Notes



No.	Column	Description
1	Flag Condition and Omit Plates When...	Name of the flag setting.
2	Flag Condition	Indicates whether or not to flag a condition. If the checkbox is checked, the selected condition will be flagged.
3	Condition	Allows you to select conditions for the flag from a drop-down list. The drop-down list contains conditions such as <, >, =, etc. **Note:** The Condition drop-down lists vary per flag. If conditions are not applicable to a flag, no drop-down list is available.
4	Flag Condition Value	Allows you to enter a numeric range or threshold for a flag. If you specify a value outside the allowed range/threshold, an error message is displayed. **Note:** If ranges/thresholds are not applicable to a flag, you will not be able to enter a value.
5	Omit	Determines whether or not to omit a well if the flag condition is met. If the checkbox is checked, the well will be omitted if the condition is met. **Note:** Well omission behavior is different depending on whether they are omitted manually or by the flag and filter settings. Wells omitted here will contain result values that were generated until the wells were omitted. You will be able to view the flag data/condition for omitted wells. However, when wells are omitted manually, results data for these wells are lost.

Notes

Viewing Flags

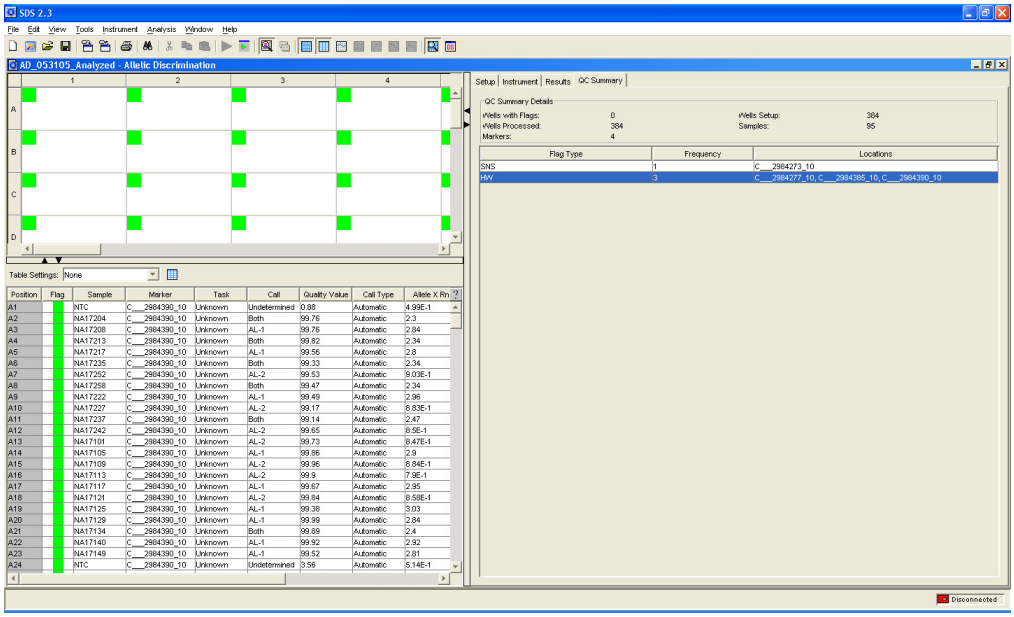
In the plate document, you can view flags in the:

- QC View of the Plate Grid

  For Allelic Discrimination (AD) plate documents, the Plate Grid automatically switches to QC View after analysis. (To zoom in on the flags, click **View > Grid Zoom**.) Only flags are displayed in the QC view; all other display items (sample name, detector color, etc.) are turned off.
- QC Summary Tab

  The QC Summary tab provides an overview of the plate document's quality.
- Results Table

  View flags, filter the results based on flags, and sort the table by flags.



Flag Icons

The following flag icons are used in the Results Table and QC view of the Plate Grid:

Icon	Description
	Passing well (no flags are assigned)
	Flagged well
	Well omitted by the algorithm
	Well omitted manually **Note:** In the Results Table, the Flag column is blank for manually omitted wells.

Notes

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.

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.

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