Pre-Developed TaqMan® Assay Reagents

Allelic Discrimination

Protocol

Applied Biosystems

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FOR PRE-DEVELOPED TAQMAN® ASSAY REAGENTS FOR ALLELIC DISCRIMINATION

NOTICE TO PURCHASER: LIMITED LICENSE

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Contents

Preface

Using the Pre-Developed TaqMan® Assay Reagents – Allelic Discrimination

Preface

This preface contains:

Safety

Safety Alert Words

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

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

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

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

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

Chemical Hazard Warning

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

Chemical Safety Guidelines

To minimize the hazards of chemicals:

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

About MSDSs

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

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

Obtaining MSDSs

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

To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.

3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose
4. To have a copy of a document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.
5. After you enter the required information, click **View/Deliver Selected Documents Now**.

Chemical Waste Hazard

WARNING **CHEMICAL WASTE HAZARD.** Some wastes produced by the operation of the instrument or system are potentially hazardous and can cause injury, illness, or death.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

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

- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

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

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

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

Biological Hazard Safety

BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves. Read and follow the guidelines in these publications:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).

Additional information about biohazard guidelines is available at: **http://www.cdc.gov**

How to Obtain Support

To contact Applied Biosystems Technical Support from North America by telephone, call **1.800.899.5858**.

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

At the Support page, you can:

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

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

Using the Pre-Developed TaqMan® Assay Reagents – Allelic Discrimination

About TaqMan PDARs for AD

Product Overview

Pre-Developed TaqMan® Assay Reagents for Allelic Discrimination (TaqMan® PDARs for AD) are optimized assays for the discrimination of specific alleles. Each TaqMan PDAR for AD allows researchers to genotype individuals for specific alleles.

TaqMan PDARs for AD are easy to perform because the method requires only three components:

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

Allele 1 and Allele 2 Controls are also provided with each assay.

About This Protocol

This protocol describes how to:

- Prepare reaction plates for PCR on an ABI PRISM® 7700 Sequence Detection System (SDS), GeneAmp® PCR System 9700, or GeneAmp® PCR System 9600.
- Perform an endpoint plate read on the 7700 SDS only.
- Analyze endpoint plate reads and call allele types using ABI PRISM® SDS software v. 1.7 or later.

Allelic Discrimination Using the 5´ Nuclease Assay

AD Assay Mix Features

The Allelic Discrimination Assay Mix (AD Assay Mix) contains:

- Sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest
- Two modified TaqMan probes:
  - One probe matches the Allele 1 sequence.
  - One probe matches the Allele 2 sequence.

Modified TaqMan Probes

Each modified TaqMan probe contains:

- A reporter dye at the 5´-end of each probe:
  - VIC® dye is linked to the 5´-end of the Allele 1 probe.
  - FAM™ dye is linked to the 5´-end of the Allele 2 probe.
- A nonfluorescent quencher at the 3´-end of the probe:

  Because the quencher does not fluoresce, the 7700 SDS can measure reporter dye contributions more accurately.
- A minor groove binder:

  This modification increases the melting temperature (T_m) without increasing probe length (Afonina *et al.*, 1997; Kutyavin *et al.*, 1997), permitting the design of shorter probes. This modification results in greater differences in T_m values between matched and mismatched probes, producing more accurate allelic discrimination.

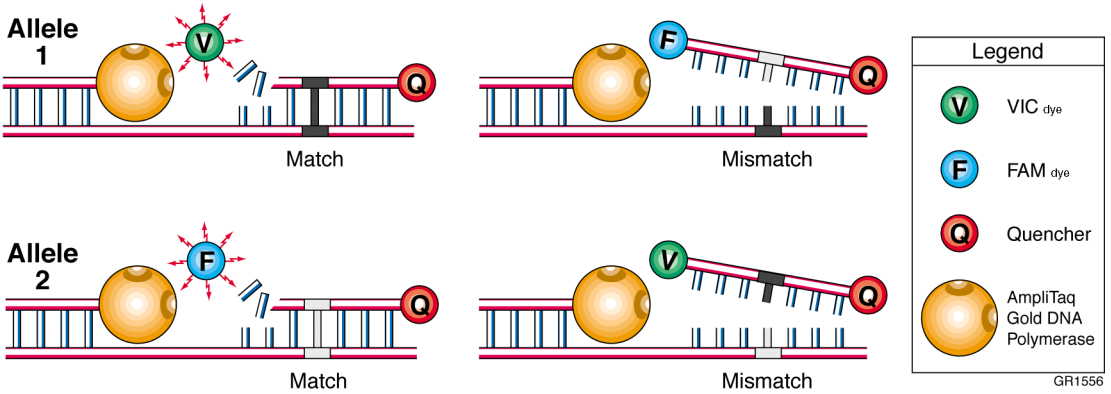
5´ Nuclease Assay

During PCR, each probe anneals specifically to complementary sequences between the forward and reverse primer sites. AmpliTaq Gold® DNA polymerase can cleave only probes that hybridize to the target. Cleavage separates the reporter dye from the quencher dye, increasing fluorescence by the reporter. Thus, the fluorescence signal(s) generated by PCR amplification indicate(s) the sequences that are present in the sample.

Mismatches Between Probe and Target Sequences

Mismatches between a probe and target reduce the efficiency of probe hybridization. Furthermore, AmpliTaq Gold DNA polymerase is more likely to displace the mismatched probe rather than cleave it to release reporter dye.

The next figure illustrates results from matches and mismatches between target and probe sequences in TaqMan PDARs for AD assays (Livak et al., 1995; Livak et al., 1999).



The table below shows the correlation between fluorescence signals and sequences present 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 fluorescent signals	Heterozygosity

Preventing Contamination

Overview

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

About AmpErase UNG

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

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

General PCR Practices

Please follow these recommended procedures:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas, dedicated equipment, and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Use positive-displacement pipettes or aerosol-resistant pipette tips.
- Periodically clean lab benches and equipment with 10% bleach solution.

Materials and Equipment

Assay Contents

This product contains sufficient reagent to perform 400 PCR reactions of 25 µL each. This product also contains sufficient control reagents to perform 16 control reactions for each allele.

Item	Description
Allelic Discrimination (AD) Assay Mix	10X mixture for 400 reactions[a] • Forward and reverse primers flanking a specific polymorphic sequence • VIC dye-labeled probe that binds Allele 1 • FAM dye-labeled probe that binds Allele 2
Allele 1 Control (AL1)	5X solution for 40 reactions[a] Template with the AL1 sequence
Allele 2 Control (AL2)	5X solution for 40 reactions[a] Template with the AL2 sequence
TaqMan® Universal PCR Master Mix	2X solution for 400 reactions[a] • AmpliTaq® Gold DNA polymerase • AmpErase® UNG • dNTPs • Passive reference • Optimized buffer

a. Reaction size is 25 µL.

Visit our web site for a list of available assays (see the "How to Obtain Support" on page ix).

Storage and Stability

All components of the TaqMan PDARs for AD should be stored at 2 to 8 °C.

IMPORTANT! Do not expose the AD Assay Mix to direct light. Excessive exposure to light damages the fluorescent probes.

Required Materials and Equipment Not Included

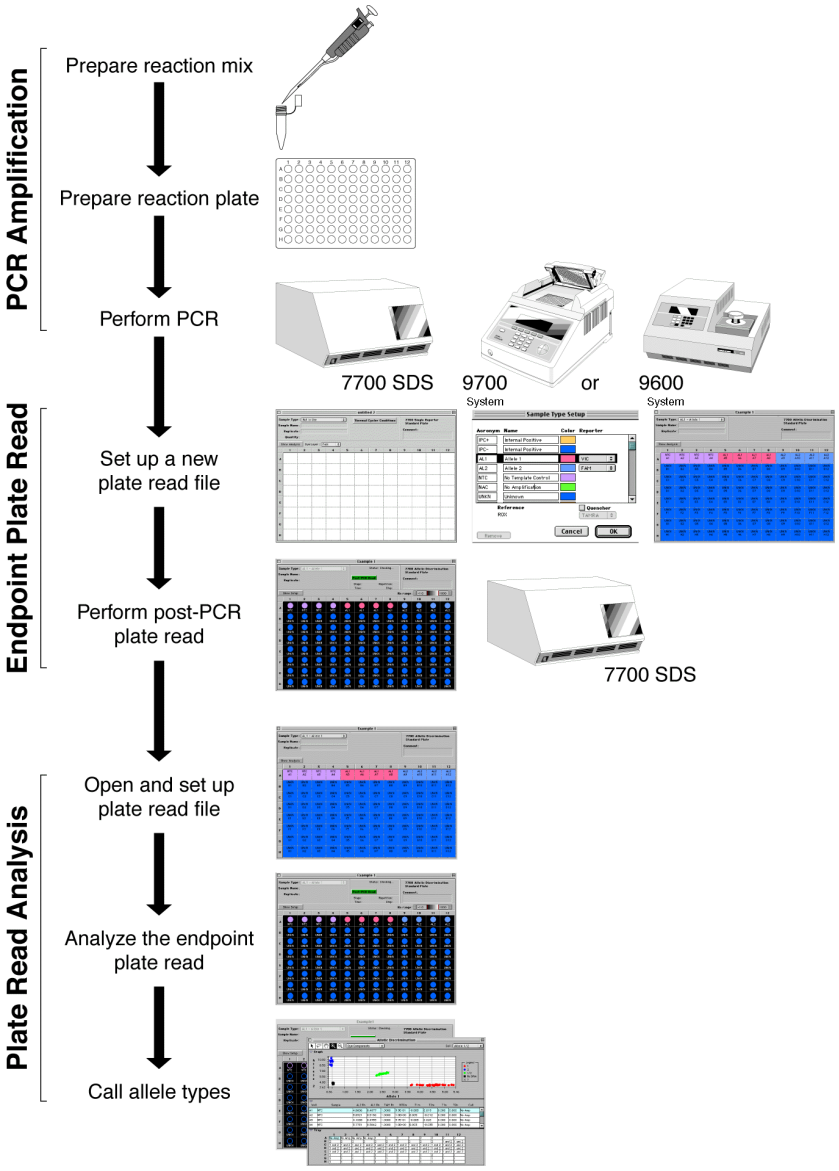
Instruments	Source
ABI PRISM® 7700 Sequence Detection System	Contact your local Applied Biosystems sales office.
GeneAmp® PCR System 9700 thermal cycler	
GeneAmp® PCR System 9600 thermal cycler	
Software	
Sequence Detector Software v.1.7	

Materials	Source
ABI PRISM® Sequence Detection Systems Software v. 1.7 Upgrade Kit	Applied Biosystems PN 4313011[a]
MicroAmp® Optical 96-Well Reaction Plates and Optical Caps	Applied Biosystems PN 403012
Sequence Detection Systems Spectral Calibration Kit	Applied Biosystems PN 4305822
ABI PRISM® 7700 Sequence Detection System User Bulletin: Generating New Spectra Components[b]	Applied Biosystems PN 4306234
Centrifuge with 96-well plate adapter	Major Laboratory Supplier (MLS)
DNase-free water	MLS
Disposable gloves	MLS
Microcentrifuge	MLS
Pipette tips, aerosol-resistant	MLS
Pipettors, positive-displacement, air-displacement	MLS
Polypropylene tubes	MLS
TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)	MLS

a. Required to run allelic discrimination assays using TaqMan PDARs for AD. Contact Applied Biosystems technical support to obtain this version.

b. Necessary only for 7700 instruments not calibrated with the VIC dye. See "Calibrating the 7700 System" on page 13 for more information.

Allelic Discrimination Assay Overview



PCR Amplification

PCR Amplification Overview

In this step, you:

- Prepare the reaction mix
- Prepare an optical 96-well reaction plate containing:
  - No Template Controls
  - Allele 1 Controls
  - Allele 2 Controls
  - Genomic DNA samples
- Perform PCR

Recommended Template

The recommended template for TaqMan PDARs for AD assays is genomic DNA (2 to 20 ng/µL).

Reagent Preparation Guidelines

The following guidelines ensure optimal PCR performance:

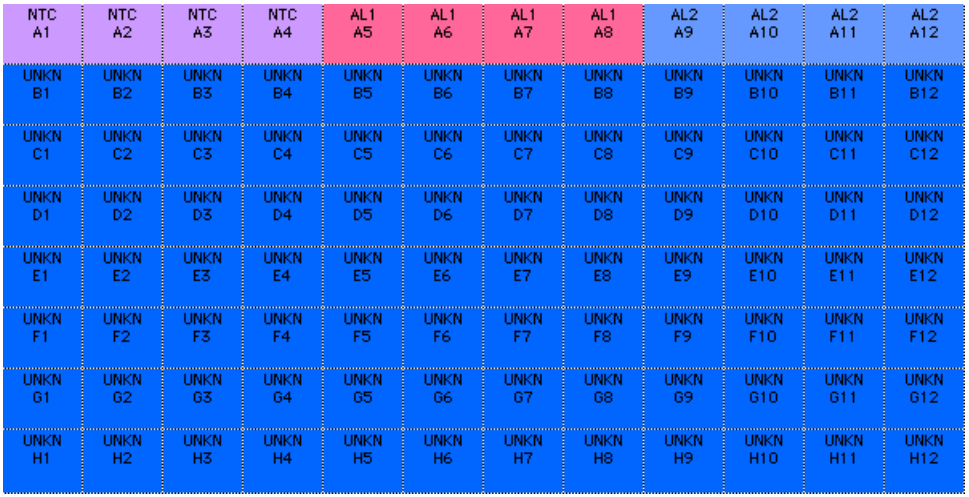
- Keep all TaqMan PDAR mixes protected from light, in the refrigerator, until you are ready to use them. Excessive exposure to light damages the fluorescent probes.
- Before using TaqMan PDARs for AD, resuspend the 10X AD Assay Mix by vortexing and briefly centrifuge the tube. Resuspend the 2X TaqMan Universal PCR Master Mix by swirling the bottle.
- Prior to use, thaw any frozen genomic DNA samples by placing them on ice. When thawed, resuspend the samples by vortexing and then briefly centrifuge the tubes.
- Prepare the reaction mix for each assay in a separate microcentrifuge tube before transferring it to the reaction plate for thermal cycling and fluorescence analysis.

Assay Setup Recommendations

Follow Applied Biosystems recommendations for designing assay setup to ensure optimal performance of TaqMan PDARs for AD.

- Run controls for each assay for a high confidence level.
  - Four No Template Controls (NTCs)
  - Four Allele 1 Controls (AL1)
  - Four Allele 2 Controls (AL2)
- Run one assay per plate for high throughput.

The figure below shows an example of the placement of control and sample reactions.



Preparing the Reaction Mix

The reaction mix for TaqMan PDARs for AD assays contains TaqMan Universal PCR Master Mix, AD Assay Mix, and DNase-free water.

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

To prepare the reaction mix:

1.	Calculate the number of reactions to be performed for each assay. **Note:** Include at least 4 NTCs, 4 AL1 Controls, and 4 AL2 Controls on each plate for a high confidence level for allele calls.

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

2. Calculate the volume of components needed for each assay, using the table below.

Component	Volume (µL) per Reaction
TaqMan Universal PCR Master Mix (2×)	12.5
Allelic Discrimination Assay Mix (10×)	2.5
DNase-free water	5.0
Total	**20.0**

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

3. Gently swirl the bottle of 2× TaqMan Universal PCR Master Mix to resuspend.

4. Vortex and briefly centrifuge the 10× AD Assay Mix.

5. Pipette the required volumes of 2× TaqMan Universal PCR Master Mix and 10X AD Assay Mix into a sterile tube.

If the assay contains ...	Then use ...
1–75 samples and controls	1.5-mL tube
75–100 samples and controls	2.0-mL tube
100–500 samples and controls	5.0-mL tube

6. Invert the tube(s) to mix.

7. Briefly centrifuge the tube(s) to spin down the contents and to eliminate any air bubbles from the solution.

Preparing the Reaction Plate

Follow the assay setup recommendations on page 8 for preparing the reaction plate.

To prepare the reaction plate:

1. Pipette one control or sample into each well of a MicroAmp Optical 96-Well Reaction Plate.

   Note: You may run multiple allelic discrimination assays on one reaction plate.

Sample Type	Component	Volume (µL) per Well	Well Position[a]
No Template Control (NTC)	1× TE buffer	5	A1–A4
Allele 1 Control (AL1)	5× AL1 control	5	A5–A8
Allele 2 Control (AL2)	5× AL2 control	5	A9–A12
Unknown (UNKN)	Genomic DNA (2 to 20 ng/µL)	5	B1–H12

   a. Recommended placement for running one assay per plate.

   Note: Use a calibrated, positive-displacement pipettor to minimize contamination and error.

2. Invert the reaction mix tube(s) prepared on page 9 to remix.
3. Briefly centrifuge the tube(s) to spin down the contents and to eliminate air bubbles.
4. Pipette 20 µL of reaction mix into each well. Be sure that no cross-contamination occurs from well to well.
5. Cap the plate with MicroAmp® Optical Caps.
6. Centrifuge the plate to spin down the contents and to eliminate any air bubbles.

Selecting a Thermal Cycler

Because the data acquired during PCR amplification is not necessary for analysis, any of the following instruments can be used for PCR amplification:

- ABI PRISM 7700 Sequence Detection System

  Note: Use of the 7700 Sequence Detection system allows for real-time analysis of PCR, which is helpful for troubleshooting.

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

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

Performing PCR

To perform PCR:

1. Program the thermal cycling conditions.

   IMPORTANT! These conditions are optimized for use only with TaqMan PDARs for AD because of the modified TaqMan probes.

	Initial Steps		Each of 50 Cycles	
Stage	Hold	Hold	Melt	Anneal/Extend
Temp (°C)	50	95	92	60
Time	2 min	10 min	15 sec	90 sec

   Note: The 2-minute, 50 °C step is required for optimal AmpErase UNG enzyme activity. The 10-minute, 95 °C step is required to activate the AmpliTaq Gold DNA Polymerase.

2. Set the reaction volume to **25 µL**.
3. Load the reaction plate into the thermal cycler.
4. Begin thermal cycling.

Endpoint Plate Read

Endpoint Plate Read Overview

In this step, you use the 7700 SDS and SDS software v. 1.7 or later to:

- Create and set up a plate read file
- Run an endpoint plate read

Calibrating the 7700 System

Before using TaqMan PDARs for AD, the 7700 System should be calibrated for the VIC dye using the Sequence Detection Systems Spectral Calibration Kit. This kit provides the standards needed to calibrate the instrument for use with products containing TaqMan VIC dyes. For more information, refer to *ABI PRISM 7700 Sequence Detection System User Bulletin: Generating New Spectra Components*.

SDS Software

Allelic discrimination assays using TaqMan PDARs for AD requires installation of SDS software v. 1.7 or later.

If you do not have the latest version, you can receive it by contacting Applied Biosystems technical support (see the "Preface" on page v).

Creating a New Plate Read File

To create a new plate read file:

1.	Launch the SDS software.
2.	Close the untitled window that opens.

To create a new plate read file: ***(continued)***

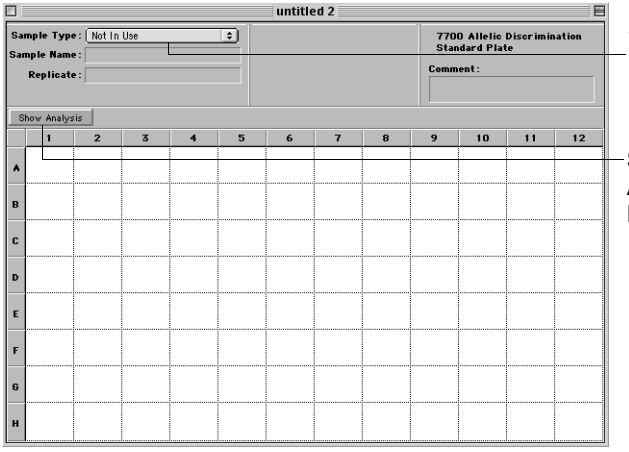
3. Create a new plate read file.

   a. From the File menu, select **New Plate**.

   b. In the **New Plate** dialog box, select the following options:

Plate Type	**Allelic Discrimination**
Plate Format	**Standard Plate**
Run	**Plate Read**

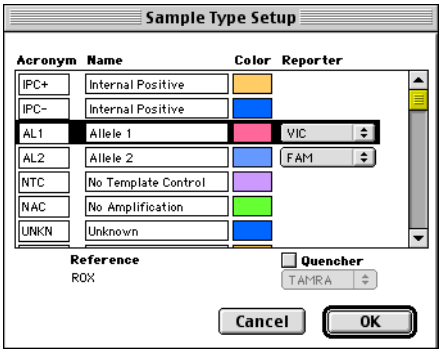
   c. Click **OK** to open the plate read window.



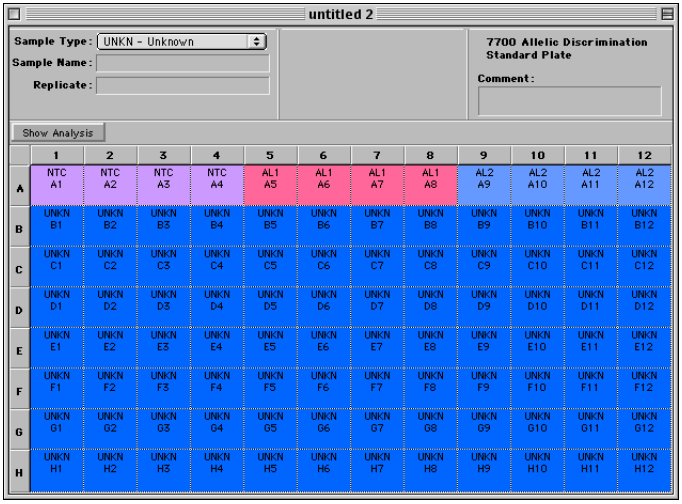
Setting Up a Plate Read File

In this procedure, the plate read file is configured with the appropriate sample types, reporter dyes, quenchers, and assay setup.

To set up a plate read file:

1.	From the **Sample Type** pop-up menu, select **Sample Type Setup** to open the Sample Type Setup dialog box. 
2.	Complete the Sample Type Setup dialog box. • For Allele 1 (AL1), select **VIC** for the reporter dye. • For Allele 2 (AL2), select **FAM** for the reporter dye. • Uncheck the box next to **Quencher**. • Click **OK**.
3.	Assign labels to the No Template Control wells (NTC). a. Select the wells representing NTC by clicking them. b. From the Sample Type pop-up menu, select **NTC - No Template Control**.
4.	Assign labels to the control wells with Allele 1 Control (AL1). a. Select the wells representing AL1 by clicking them. b. From the Sample Type pop-up menu, select **AL1-Allele 1**.

To set up a plate read file: ***(continued)***

5.	Assign labels to the control wells with Allele 2 Control (AL2). a. Select the wells representing AL2 by clicking them. b. From the Sample Type pop-up menu, select **AL2-Allele 2**.
6.	Assign labels to the sample wells with unknown genotype (UNKN). a. Select the wells representing UNKN samples by clicking them. b. From the Sample Type pop-up menu, select **UNKN-Unknown**. All wells in use should now be labeled with the sample type. 
7.	Save the changes to the plate read file.

Loading the Reaction Plate

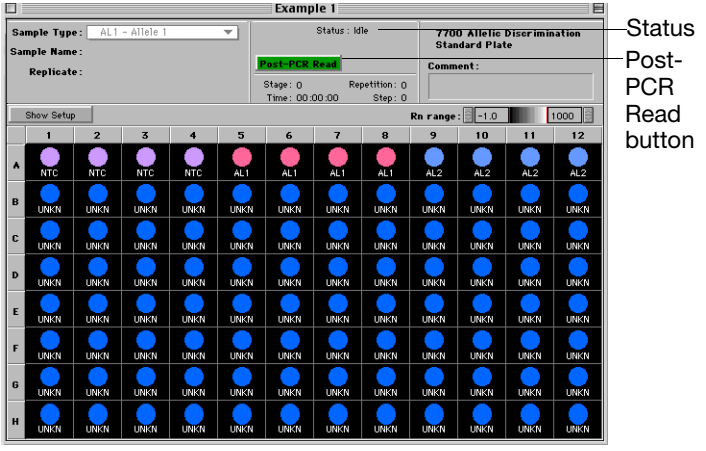
To load the reaction plate:

1.	Place the reaction plate into the sample compartment.
2.	Pull the heated cover forward, and secure it in place by tightening the knob.
3.	Turn the knob clockwise to lower and secure the heated cover over the sample plate. **IMPORTANT!** When securing the heated cover in place, be careful not to overtighten the knob. Damage to the instrument could occur.

Running a Plate Read

The 7700 System can detect and measure the fluorescence signals generated from the PCR amplification during an endpoint plate read.

To run a plate read:

1.	From the Setup view of a plate read file, click **Show Analysis**. 
2.	Click **Post-PCR Read**. The plate read runs, which should take about 10 seconds.
3.	After the plate read is complete, save the plate read file.

To run a plate read: ***(continued)***

4.	Remove the reaction plate from the instrument. **IMPORTANT!** To avoid PCR contamination with amplified product, do not remove the caps from the plate.
5.	Discard the reaction plate after analyzing the plate read, when you are confident that the plate read was successful.

Plate Read Analysis

Plate Read Analysis Overview

After the plate read is completed, SDS software calculates the fluorescence measurements made during the plate read and plots Rn values based on the signals from each well. Using the software, you can determine which alleles are present in each sample.

In this step you use SDS software v. 1.7 or later to:

- Analyze a plate read
- Call allele types manually

Setting Up the Plate Read Analysis

Note: You can open completed endpoint plate read files at a later time for analysis.

To set up the plate read analysis:

1.	Launch the SDS software and close the untitled window that opens.
2.	From the File menu, select **Open Plate.**

To set up the plate read analysis: ***(continued)***

3. **S**elect a Plate Read file to analyze, then click **Open**.

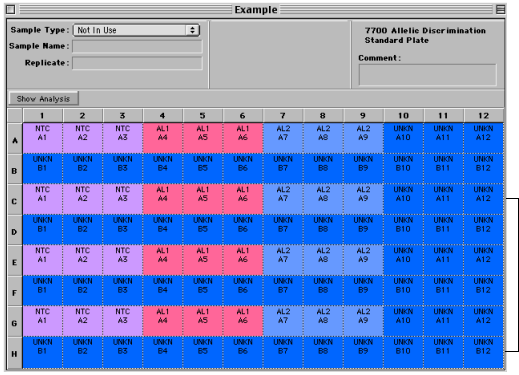
If the plate contains ...	Then ...
One assay	Go to step 5.
Multiple assays	Follow the actions below to label wells from all but one assay as **Not In Use**.

Note: You can only analyze one assay at a time.

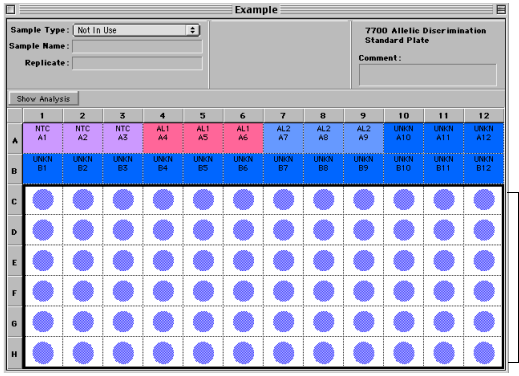
To set up the plate read analysis: ***(continued)***

4. Select the wells that will not be analyzed at this time.

   The example below shows a setup view with four assays on one plate.

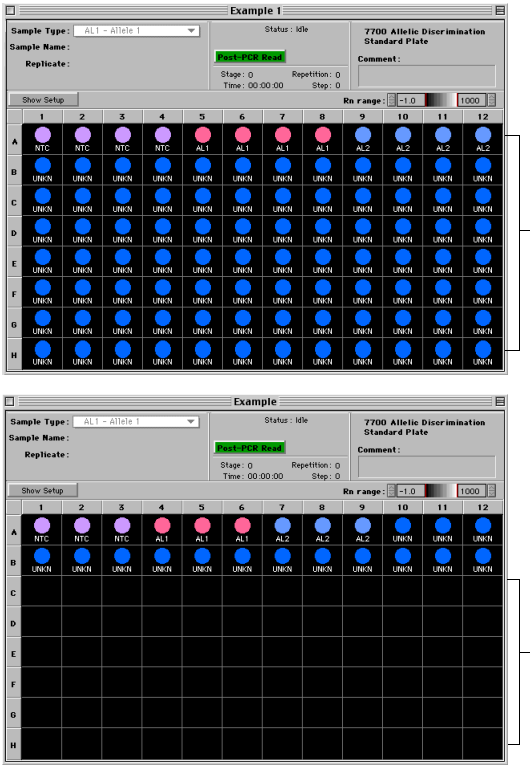


   a. From the Sample Type pop-up menu, select **Not In Use.**



To set up the plate read analysis: ***(continued)***

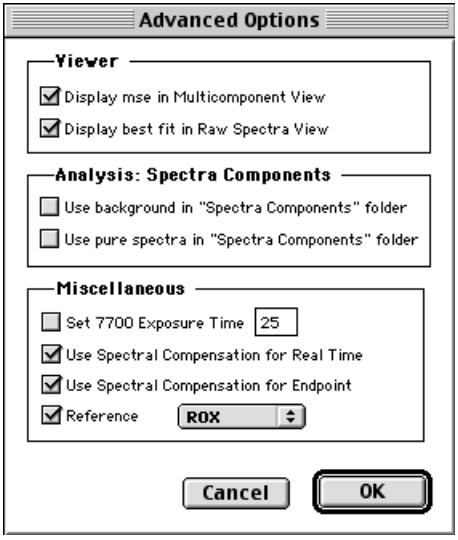
5. Click **Show Analysis** to open the Analysis view window.



Confirming the SDS Software Configuration

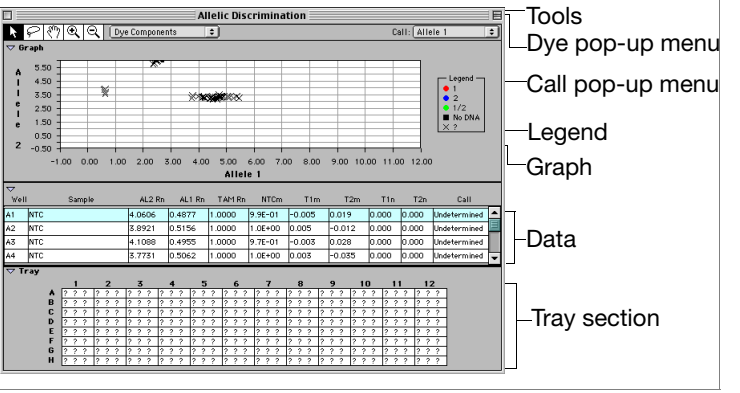
Perform this step to confirm that the software uses spectral compensation for endpoint plate reads. Once confirmed, this step is not required for subsequent plate read analyses.

To confirm the SDS software configuration:

1.	Select **Instrument** > **Diagnostics** > **Advanced Options**. 
2.	Select the **Use Spectral Compensation for Endpoint** and click **OK**.
3.	When the warning message opens, click **OK**.  **Note:** You do not need to quit and relaunch the SDS software at this time.

Analyzing a Plate Read

To analyze a plate read:

1.	In the Analysis menu, select **Analyze**. If the event log opens, close it.
2.	In the Analysis menu, select **Allelic Discrimination**. 

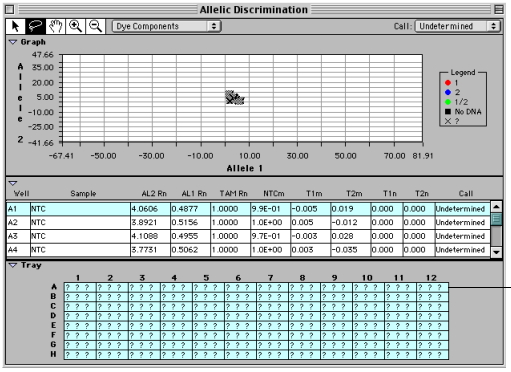
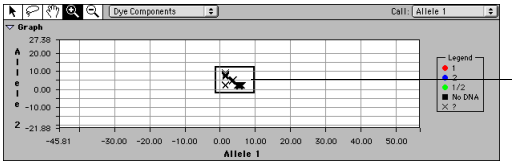
Calling Allele Types

Accurate allelic discrimination using TaqMan PDARs for AD requires making manual allele calls.

To call allele types:

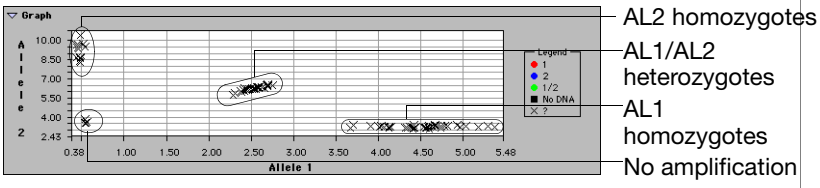
1.	Using the Dye pop-up menu at the top of the Allelic Discrimination window, change the view to **Dye Components**.
2.	Zoom out until all crossmarks are visible in the graph.
3.	Click the zoom out magnifying glass tool to select it. 

To call allele types: ***(continued)***

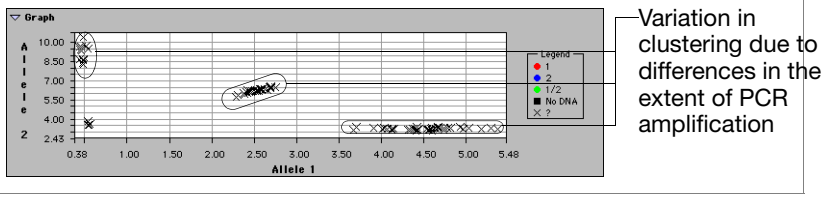
4.	Click the zoom out magnifying glass on the graph to zoom out. **Note:** Select the marks with the lasso tool to confirm that all appropriate wells have been selected. 
5.	If allele types were automatically called, designate the crossmarks as **Undetermined** by using the Call pop-up menu. **IMPORTANT!** Manual allele calling is required.
6.	Crop and zoom the crossmarks. a. Click the zoom in magnifying glass tool to select it.  b. Click and drag the zoom in magnifying glass on the graph to crop and magnify all marks. 

To call allele types: ***(continued)***

7.	Repeat step 6 until the marks are clearly clustered in distinct regions of the graph.



Note: The clustering of crossmarks can vary along the horizontal axis (AL1), vertical axis (AL2), or diagonal (AL1/AL2). This variation is due to differences in the extent of PCR amplification, and could be the result of differences in the initial DNA concentration.



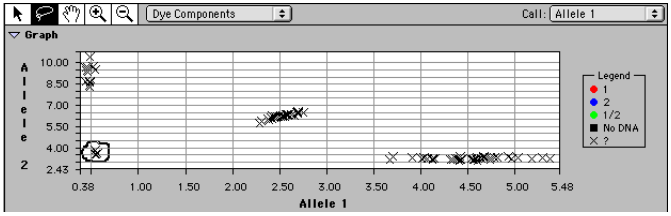
To call allele types: ***(continued)***

8. Manually call allele type.

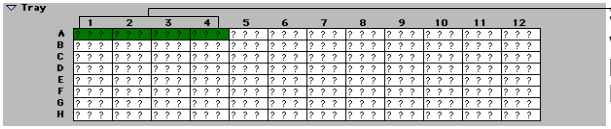
   a. Click the lasso tool to select it.

   b. Circle a cluster of marks with the lasso tool as shown below.

   The corresponding wells are highlighted in the Tray section of the Allelic Discrimination window.

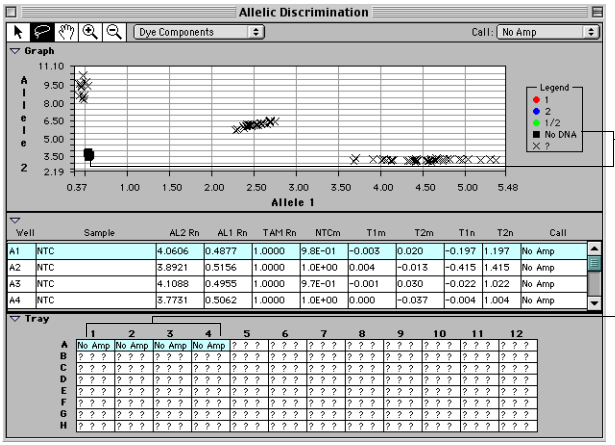
   

   Selected wells are high-lighted

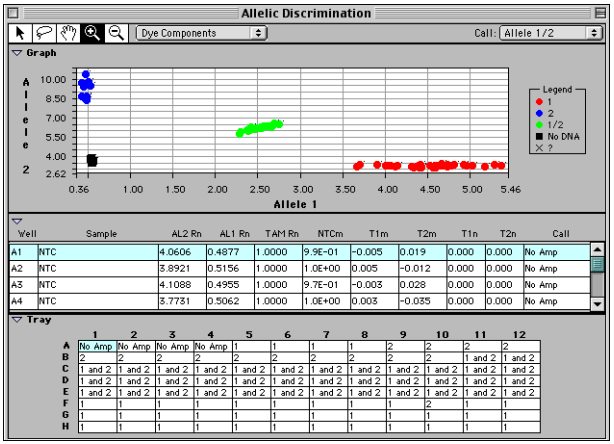
To call allele types: ***(continued)***

9. Using the Call pop-up menu, designate the appropriate allele type for the selected cluster, based on the location on the graph.

- The symbols on the graph are updated to match the Legend.
- The wells in the Tray section of the Allelic Discrimination window are updated with the call.



To call allele types: ***(continued)***

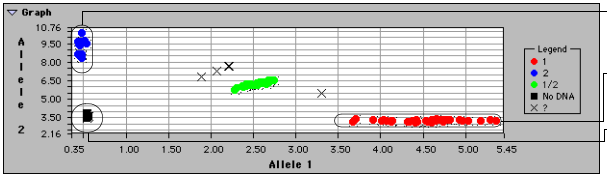
10.	Select clusters and call allele types (step 8 and step 9) until all calls are made. 
11.	Print the Allelic Discrimination window and export the results.
12.	Analyze any additional assays on the plate. a. Close the Allelic Discrimination window to activate the plate read window. b. Click Show Setup to return to the Setup view. c. Use the Sample Type pop-up menu to label assay wells NTC, AL1, AL2, or UNKN and wells from other assays as **Not In Use**. d. Click **Show Analysis**. e. Repeat "Analyzing a Plate Read" on page 24 and "Calling Allele Types" on page 24. **Note:** You can analyze only one assay at a time.

Analyzing Allele Types

Review the results to confirm allele types.

To analyze allele types:

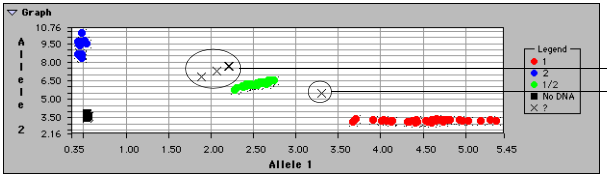
1. Verify the calls for NTC, AL1, and AL2 Controls.



2. Designate samples that did not cluster tightly as **Undetermined**.

   Samples that did not cluster tightly may:

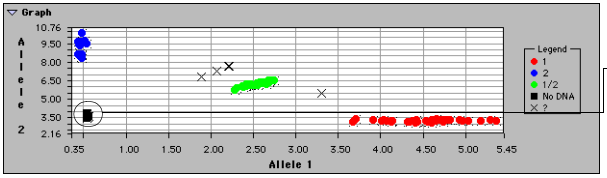
   - Contain rare sequence variations
   - Contain sequence duplications



3. Check whether UNKN samples clustered with NTCs.

   Samples that clustered with NTCs may:

   - Contain no DNA
   - Contain PCR inhibitors
   - Be homozygous for a sequence deletion



4. Retest any samples that did not cluster tightly or clustered with NTCs to confirm results.

Troubleshooting

Observation	Possible Cause	Suggested Action
Wells in the Tray section of the graph remained uncalled after all crossmarks were typed.	Some samples are plotted beyond the range of the graph.	1. Zoom out with the "–" magnifying glass tool until all crossmarks are visible. 2. Confirm that all marks are visible in the graph by selecting all crossmarks in the graph with the lasso tool. The selected marks are highlighted in the Tray section of the Allelic Discrimination window. 3. Designate all crossmarks as undetermined, and proceed with "Calling Allele Types" on page 24.
NTCs generated fluorescence signals.	PCR contamination may have occurred.	Test your buffers for the presence of nucleic acid.
Distinct clusters were not observed.	Reporter dyes were not appropriately assigned. Quencher was selected.	1. Confirm the options in the **Sample Type Setup** dialog box (see "Setting Up a Plate Read File" on page 15). 2. Reanalyze the plate read.
A sample did not cluster with one specific allele type.	The sample may: • Contain more or less DNA than other samples • Contain a rare allelic variation or sequence duplication	1. Check the DNA concentrations of the samples. 2. Retest the sample to confirm. 3. Test the sample using a different TaqMan PDARs for AD assay.
UNKN samples did not generate fluorescence signals.	The sample may: • Contain no DNA • Contain PCR inhibitors • Be homozygous for a rare allelic variation	1. Check the DNA concentrations of the samples. 2. Retest the sample to confirm. 3. Test the sample using a different TaqMan PDARs for AD assay.

SDS Software Features for TaqMan PDARs for AD

Menu Items

The table below describes the commands used to perform allelic discrimination assays using TaqMan PDARs for AD.

Table 1 SDS Software Commands for AD Assays

Menu	Command	Function
File 	New Plate	Create a new plate read file.
	Open Plate	Open an existing plate read file.
	Close	Close the plate read file.
	Save	Save the plate read file.
	Save As	Save the plate read file with another name.
	Export	Export data and results (tab delimited).
	MultiComponent	Export post-read multicomponent fluorescence data.
	Results	Export allele calls and Rn, m, and n values.
	Experimental Report	Export run and sample information.
	Page Setup	Set up orientation, scale, and printer for printing.
	Print	Print the Allelic Discrimination window or the Experiment Report.
	Quit	Quit the SDS software.

Table 1 SDS Software Commands for AD Assays *(continued)*

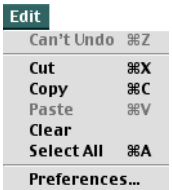
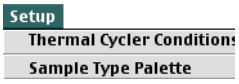
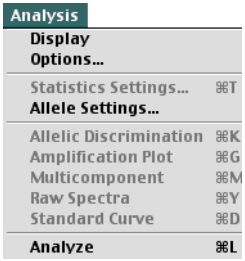
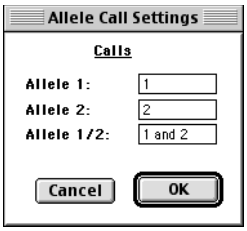
Menu	Command	Function
Edit 	Cut	Cut selected wells.
	Copy	Copy selected wells from the Setup view only.
	Paste	Paste cut or copied wells from the Setup view only.
	Clear	Clear wells.
	Select All	Select all wells.
	Preferences	Set up New Document Defaults and Document Settings.
Setup 	Sample Type Palette	Show sample types and dye information. Label wells with sample types after sample types are set.

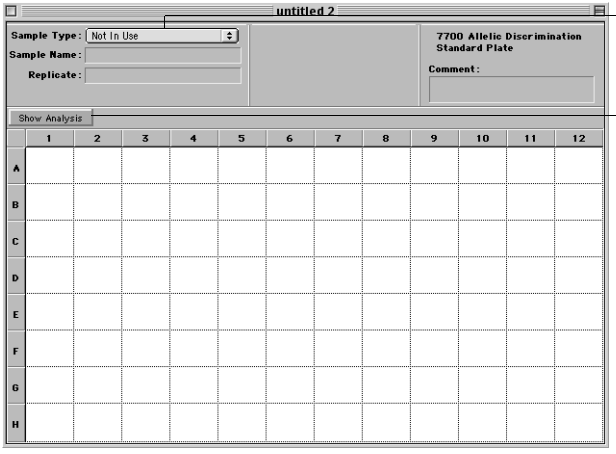
Table 1 SDS Software Commands for AD Assays *(continued)*

Menu	Command	Function
Analysis 	Allele Settings...	Set how calls appear in the **Tray** section of the **Allelic Discrimination** window. Default settings are shown below. 
	Allelic Discrimination	Open the Allelic Discrimination window after analyzing a plate read.
	Analyze	Analyze a plate read from the Analysis view.
Window 	Toggle Setup/Analysis	Switch between the Setup view and the Analysis view. Equivalent to Show Setup and Show Analysis buttons.
	Experiment Report	View the Experiment Report, which contains run and sample information.

New Plate Read Setup View

From this view you can:

- Set up sample types and reporter dyes
- Assign sample types for each well



Old Plate Read Setup View

From this view you can:

- Confirm Sample Type Setup
- Designate wells as Not In Use, if necessary

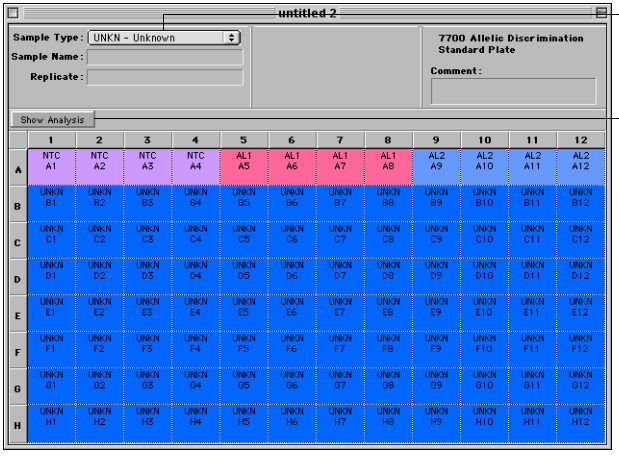
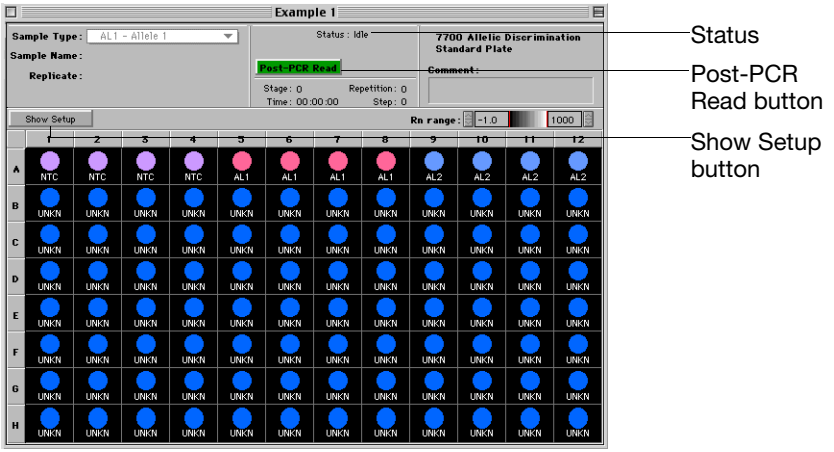


Plate Read Analysis View

From this view you can:

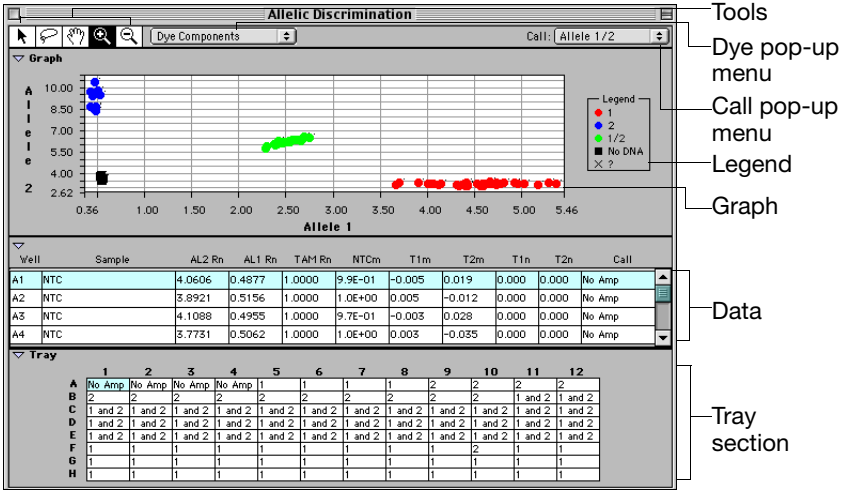
- Perform an endpoint plate read
- Analyze the plate read file



Allelic Discrimination Window

This window appears after analyzing the endpoint plate read and selecting Allelic Discrimination from the Analysis menu. From this window you can:

- Zoom in and zoom out of the graph to view crossmarks from each reaction
- Call allele types
- Print the window



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