TaqMan® Allelic Discrimination

Demonstration Kit

Protocol

Applied Biosystems

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Introduction

Overview

The TaqMan® Allelic Discrimination Demonstration Kit is a model assay to show the allelic discrimination capabilities of the Applied Biosystems Sequence Detection Systems. It has been optimized for use with TaqMan Universal PCR Master Mix (P/N 4304437).

Direct detection of polymerase chain reaction (PCR) product with no downstream processing is accomplished within minutes of PCR completion by measuring the increase in fluorescence of dye-labeled DNA probes. This method permits the analysis of thousands of samples per day with high sample-to-sample reproducibility.

5´ Nuclease Assay

The TaqMan Allelic Discrimination Demonstration Kit employs a probe technology that exploits the 5´-3´ nuclease activity of AmpliTaq Gold® DNA Polymerase to allow direct detection of the PCR product by the release of a fluorescent reporter as a result of PCR. This PCR system is optimized for yield. AmpErase® UNG is required for the prevention of PCR product carryover (Longo *et al.*, 1990). For more information on the 5´ nuclease assay, refer to Lawyer *et al.*, 1989, Holland *et al.*, 1991, and Lyamichev *et al.*, 1993.

Two TaqMan probes are used in this allelic discrimination assay, one probe for each allele in a two-allele system. Each probe consists of an oligonucleotide with a 5´-reporter dye and a 3´-quencher dye. TET (6-carboxy-4,7,2´,7´-tetrachlorofluorescein) is covalently linked to the 5´ end of the probe for the detection of Allele 1. FAM (6-carboxyfluorescein) is covalently linked to the 5´ end of the probe for the detection of Allele 2. Each of the reporters is quenched by TAMRA (6-carboxy-N,N,N´,N´-tetramethylrhodamine) attached via a linker arm located at the 3´ end of each probe.

When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence, primarily by Förster-type energy transfer (Förster, 1948; Lakowicz, 1983). During PCR, forward and reverse primers hybridize to a specific sequence of the target DNA. The TaqMan probe hybridizes to a target sequence within the PCR product. The AmpliTaq Gold enzyme cleaves the TaqMan probe with its 5´–3´ nuclease activity. The reporter dye and quencher dye are separated upon cleavage, resulting in increased fluorescence of the reporter (Figure 1 on page 2). The 3´ end of the TaqMan probe is blocked to prevent extension of the probe during PCR.

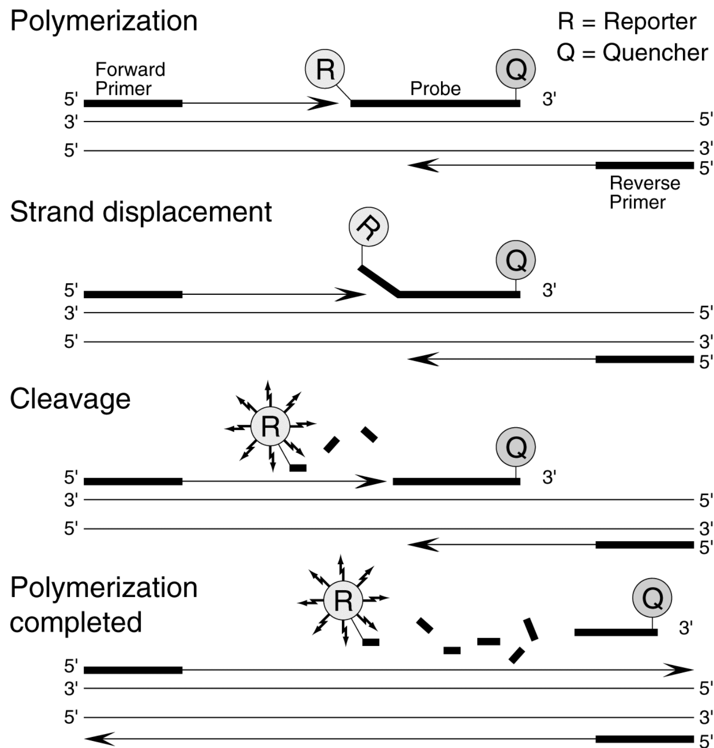

Figure 1 The fork-like-structure-dependent, polymerization-associated, 5´–3´ nuclease activity of AmpliTaq Gold DNA Polymerase during one extension phase of PCR (Lyamichev *et al.*, 1993)

This process occurs in every cycle and does not interfere with the exponential accumulation of product. The separation of the reporter dyes from the quencher dye results in increase in fluorescence for each of the FAM and TET reporters. The increase in fluorescence is measured, and is a direct consequence of target amplification during PCR.

Both primer and probe must hybridize to their targets for amplification and cleavage to occur. The fluorescence signals are generated only if the target sequences for the probes are amplified during PCR. Because of these requirements, non-specific amplification is not detected. For information on release of a fluorescent reporter during the PCR, refer to Lee *et al.*, 1993, and Livak *et al.*, 1995.

continued on next page

Sequence Detection

The Sequence Detection Systems from Applied Biosystems are used to measure the increase of reporter fluorescence following PCR. Reporter signals are normalized to the emission of a passive reference:

$$R_{n\,(AL1)} = \frac{\text{Emission Intensity of Allele 1 Reporter}}{\text{Emission Intensity of Passive Reference}}$$

$$R_{n\,(AL2)} = \frac{\text{Emission Intensity of Allele 2 Reporter}}{\text{Emission Intensity of Passive Reference}}$$

These parameters are used in the Allelic Discrimination analysis software described on pages 19–21.

Allelic Discrimination

The TaqMan Allelic Discrimination Demonstration Kit illustrates discrimination between the alleles of a two-allele system. It contains enough PCR reagents for up to 200 reactions of 50 µL each. During amplification, the Plasmid Allele 1 and Plasmid Allele 2 standards supplied with the kit generate reporter fluorescent signals such that allele calls may be made on unknown samples.

Allele 1 and Allele 2 probes supplied in the Probe and Primer Mix with the TaqMan Allelic Discrimination Demonstration Kit can be used with the specific Genomic Control DNA included in the kit. Custom probes must be designed for detection of any other templates. See Appendix A, "Guidelines for Custom Applications," on page 23.

System Performance Guarantee

Using the Genomic Control DNA and protocol for the TaqMan Allelic Discrimination Demonstration Kit, automated allele calls will be reported by the Sequence Detection System with a 99.7% confidence level.

Demonstrated Performance

The minimum and maximum detection range is from 10–100 ng of Genomic Control DNA, which is approximately 10^4–10^5 copies of a single copy gene.

Materials and Equipment

Kit Contents

The TaqMan Allelic Discrimination Demonstration Kit (P/N 4303263) has been designed to provide 200 reactions of 50 µL each. Experiments have been performed with the ABI PRISM® 7700 and ABI PRISM 7200 Sequence Detectors showing that a 25-µL final reaction volume will provide the same precision for TaqMan allelic discrimination assays. We do not recommend final reaction volumes lower than 25 µL.

The contents of the TaqMan Allelic Discrimination Demonstration Kit are listed in Table 1.

Table 1. Kit Components

Component	Volume	Description
TaqMan Universal PCR Master Mix	5.75 mL	One bottle, sufficient for 200 reactions of 50 µL each
Probe and Primer Mix	3.45 mL	Two tubes, sufficient for 200 reactions of 50 µL each, containing the following: ♦ Forward primer: 5´-CAG TGG TGC CAG CTC AGC A-3´ ♦ Reverse primer: 5´-GGT GAG GCT GTG GCT GAA CA-3´ ♦ TaqMan Plasmid Allele 1 probe: 5´-TET-CCA GCA ACC AAT GAT GCC CGT T-TAMRA-3´ ♦ TaqMan Plasmid Allele 2 probe: 5´-FAM-CCA GCA AGC ACT GAT GCC TGT TC-TAMRA-3´
Plasmid Allele 1 standard	250 µL	One tube (10 fg/µL), sufficient for 100 reactions
Plasmid Allele 2 standard	250 µL	One tube (10 fg/µL), sufficient for 100 reactions
Genomic Control DNA (human)	1.0 mL	Two tubes (10 ng/µL), sufficient for 200 reactions

Storage and Stability

Store the TaqMan Allelic Discrimination Demonstration Kit or its components at 2–6 °C. If stored under the recommended conditions, the product will maintain performance through the control date printed on the label.

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Instruments Required

One of the following instrument systems in Table 2 is required.

Table 2. Instrument Platforms for Allelic Discrimination

Equipment Item	Source
♦ ABI PRISM® 7700 Sequence Detector ♦ ABI PRISM® 7200 Sequence Detector and GeneAmp® PCR System 9600 or GeneAmp® PCR System 9700 in 9600 Emulation Mode ♦ TaqMan® LS-50B PCR Detection System and GeneAmp PCR System 9600 or GeneAmp PCR System 9700 in 9600 Emulation Mode	Applied Biosystems (call your regional sales office for the instrument best suited your needs)

User-Supplied Materials

The following items in Table 3 may be required in addition to the reagents supplied in the TaqMan Allelic Discrimination Demonstration Kit.

Table 3. User-supplied Materials

Reagent/Equipment Item	Source
Deionized water or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)	Major laboratory suppliers (MLS)
TaqMan Universal PCR Master Mix	Applied Biosystems (P/N 4304437)
MicroAmp® Optical 96-Well Reaction Plate and Optical Caps	Applied Biosystems (P/N 403012)
96-Well Microplate (Portvair)	Applied Biosystems (P/N L225-1692)
Primer Express™ software	Applied Biosystems (P/N 402089)

Note The ABI PRISM 7700 and ABI PRISM 7200 Sequence Detectors use the MicroAmp Optical 96-Well Reaction Plate and MicroAmp Optical Caps. The LS-50B PCR Detection System uses the 96-Well Microplate (Portvair).

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Preventing Contamination

Overview

The DNA amplification capability of the PCR process makes special laboratory practices necessary. Small levels of DNA carryover from samples with high DNA concentrations, from the Genomic Control DNA, or from previous PCR amplifications can result in product even in the absence of added template DNA.

See the references in Appendix C on page 40 for more information on PCR and laboratory practices for preventing contamination.

Prevention of PCR Product Carryover

Treatment with uracil-N-glycosylase (UNG, EC 3.2.2–) can prevent the reamplification of carryover PCR products. This method involves substituting dUTP for dTTP in the Reagent Master Mix and adding AmpErase UNG to the mix prior to amplification (Kwok and Higuchi, 1989; Longo *et al.*, 1990). PCR products from previous amplifications are not reamplified. Misprimed, nonspecific PCR products created before thermal cycling are degraded, but native DNA template is not affected.

When dUTP replaces dTTP as a dNTP substrate in PCR, AmpErase UNG treatment can remove up to 100,000 copies of contaminating amplicon per 25–µL reaction.

The 2-minute hold cycle at 50 °C is necessary for optimum AmpErase UNG cleavage of the uracil-deoxyribose linkage. The 10-minute hold cycle at 95 °C necessary to activate AmpliTaq Gold DNA Polymerase also cleaves the phosphate ester backbone of the PCR products that contained uracil nucleotides and reduces the AmpErase UNG activity substantially. Because UNG is not completely deactivated during the 95 °C incubation, it is important to keep the reaction temperatures greater than 55 °C to prevent amplicon degradation.

Do not use AmpErase UNG in subsequent amplification of dU-containing PCR template, such as in nested-PCR protocols. The UNG will degrade the dU-containing PCR product, preventing further amplification.

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General PCR Practices

Although the protocol and reagents described above are capable of degrading or eliminating large numbers of carried-over PCR products, we encourage users to use the following precautions and those referenced in Appendix C on page 40 to minimize sample cross-contamination and PCR product carryover:

- 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 and dedicated equipment and supplies for sample preparation, for PCR setup, and for PCR amplification and analysis of PCR products.
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Use positive-displacement or air-displacement pipettors with filter-plugged tips. Change tips after each use.
- Keep reactions and components capped as much as possible.
- Clean lab benches and equipment periodically with 10% bleach solution.

Fluorescent Contaminants

Because sample protein and fluorescent contaminants may interfere with this assay and give false positive results, it may be necessary to include a No Amplification Control tube that contains the sample and no enzyme. If the absolute fluorescence of the No Amplification Control is greater than that of the No Template Control after PCR, fluorescent contaminants may be present in the sample.

Preparing Control Reactions and Control DNA Samples

Overview

This procedure involves PCR amplification of the target DNA followed by fluorescence analysis. When performing allelic discrimination using the Plasmid Allele 1 and Plasmid Allele 2 standards, the analysis requires that the controls and samples shown below in Figure 2 be run. For custom applications, see Appendix A on page 23.

Note The TaqMan LS-50B PCR Detection System uses a Buffer well, which must be placed in position A1. The ABI PRISM 7700 and 7200 Sequence Detectors do not use Buffer wells.

- Eight No Template Control wells (NTC)
- Eight Plasmid Allele 1 wells (AL1)
- Eight Plasmid Allele 2 wells (AL2)
- Seventy-two Genomic Control DNA wells (UNKN)

IMPORTANT Eight replicates of No Template Control, Plasmid Allele 1, and Plasmid Allele 2 must be run to make allele calls at a 99.7% confidence level using the automated allele calling routine. Manual allele calling with less than eight replicates is possible. Refer to Chapter 4 of the ABI PRISM 7200 *Sequence Detector User's Manual*.

NTC A1	NTC A2	NTC A3	NTC A4	NTC A5	NTC A6	NTC A7	NTC A8	AL1 A9	AL1 A10	AL1 A11	AL1 A12
AL1 B1	AL1 B2	AL1 B3	AL1 B4	AL2 B5	AL2 B6	AL2 B7	AL2 B8	AL2 B9	AL2 B10	AL2 B11	AL2 B12
UNKN C1	UNKN C2	UNKN C3	UNKN C4	UNKN C5	UNKN C6	UNKN C7	UNKN C8	UNKN C9	UNKN C10	UNKN C11	UNKN C12
UNKN D1	UNKN D2	UNKN D3	UNKN D4	UNKN D5	UNKN D6	UNKN D7	UNKN D8	UNKN D9	UNKN D10	UNKN D11	UNKN D12
UNKN E1	UNKN E2	UNKN E3	UNKN E4	UNKN E5	UNKN E6	UNKN E7	UNKN E8	UNKN E9	UNKN E10	UNKN E11	UNKN E12
UNKN F1	UNKN F2	UNKN F3	UNKN F4	UNKN F5	UNKN F6	UNKN F7	UNKN F8	UNKN F9	UNKN F10	UNKN F11	UNKN F12
UNKN G1	UNKN G2	UNKN G3	UNKN G4	UNKN G5	UNKN G6	UNKN G7	UNKN G8	UNKN G9	UNKN G10	UNKN G11	UNKN G12
UNKN H1	UNKN H2	UNKN H3	UNKN H4	UNKN H5	UNKN H6	UNKN H7	UNKN H8	UNKN H9	UNKN H10	UNKN H11	UNKN H12

Figure 2 Plate diagram showing placement of control and sample reactions

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Prepare Controls and Unknowns

Prepare reactions in a MicroAmp Optical 96-Well Reaction Plate. The plate wells should contain the following:

Table 4. Plate Well Setup

Well	If preparing…	Then…
A1–A8	NTC	Combine the following and deliver 50 µL of the mixture to each of the 8 wells: ♦ 220 µL of 2X Master Mix ♦ 132 µL of Probe and Primer Mix ♦ 88 µL of TE[a] buffer
A9–A12 B1–B4	AL1	Combine the following and deliver 50 µL of the mixture to each of the 8 wells: ♦ 220 µL of 2X Master Mix ♦ 132 µL of Probe and Primer Mix ♦ 44 µL of Plasmid Allele 1 standard ♦ 44 µL of TE buffer
B5–B12	AL2	Combine the following and deliver 50 µL of the mixture to each of the 8 wells: ♦ 220 µL of 2X Master Mix ♦ 132 µL of Probe and Primer Mix ♦ 44 µL of Plasmid Allele 2 standard ♦ 44 µL of TE buffer
C1–H12	Unknowns (UNKN)	Combine the following and deliver 50 µL of the mixture to each of the 72 wells: ♦ 2000 µL of 2X Master Mix ♦ 1200 µL of Probe and Primer Mix ♦ 400 µL of Genomic Control DNA ♦ 400 µL of TE buffer

a. TE buffer = 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

IMPORTANT With the ABI PRISM 7700 and ABI PRISM 7200 Sequence Detectors, you must use MicroAmp Optical disposables. Do not use MicroAmp Optical Tubes with the ABI PRISM 7200.

PCR Amplification

Thermal Cycling Parameters

The thermal cycling parameters in Table 5 are used for the TaqMan Allelic Discrimination Demonstration Kit control reactions on the GeneAmp PCR Systems 9600 and 9700 and the ABI PRISM 7700 Sequence Detector. The GeneAmp PCR System 9600 or GeneAmp PCR System 9700 in 9600 Emulation Mode is used to perform PCR amplification when the TaqMan LS-50B PCR Detection System or ABI PRISM 7200 Sequence Detector is used for fluorescence analysis.

IMPORTANT All reaction volumes are 50 µL. The 2-minute, 50 °C step is required for optimal AmpErase UNG activity. The 10-minute, 95 °C step is required to activate AmpliTaq Gold DNA Polymerase.

Table 5. Thermal Cycling Conditions

Thermal Cycler	Times and Temperatures			
	Initial Steps		Each of 40 Cycles	
			Melt	Anneal/ Extend
GeneAmp PCR System 9600 or 9700	HOLD	HOLD	CYCLE	
	2 min 50 °C	10 min 95 °C	15 sec 95 °C	1 min 62 °C
ABI PRISM 7700 Sequence Detector	HOLD	HOLD	CYCLE	
	2 min 50 °C	10 min 95 °C	15 sec 95 °C	1 min 62 °C

continued on next page

Real Time Run on the ABI PRISM 7700

Use the following procedure to perform a Real Time run on the ABI PRISM 7700 Sequence Detector. Refer to the ABI PRISM 7700 *Sequence Detector User's Manual* for more information.

Step	Action
1	Create a Real Time plate document. Refer to the ABI PRISM 7700 *Sequence Detector User's Manual* for details.
2	Place the MicroAmp Optical 96-Well Reaction Plate in the ABI PRISM 7700 Sequence Detector.
3	Perform a Real Time run using the thermal cycling conditions shown in Table 5.
4	Save the Real Time plate results.
5	Close the Sequence Detection Systems software.
6	Leave the MicroAmp Optical 96-Well Reaction Plate in the ABI PRISM 7700 Sequence Detector.

Performing PCR on the GeneAmp 9600 and 9700

Use the following procedure to amplify samples in the GeneAmp PCR System 9600 or GeneAmp PCR System 9700 in 9600 Emulation Mode.

Step	Action
1	Place the MicroAmp Optical 96-Well Reaction Plate in the GeneAmp PCR System 9600 or GeneAmp PCR System 9700 in 9600 Emulation Mode.
2	Program the thermal cycler with the parameters shown in Table 5 on page 17.
3	Perform PCR amplification.
4	Store the PCR products at 2–6 °C until you are ready to analyze them in the ABI PRISM 7200 Sequence Detector or TaqMan LS-50B PCR Detection System.

Allelic Discrimination on the ABI PRISM 7700 and 7200

Overview

The TaqMan Allelic Discrimination Demonstration Kit is designed for Plate Read (end point) detection. Plate Read detection collects one fluorescence scan per tube after PCR is completed and can be used to perform the allelic discrimination assay on either the ABI PRISM 7700 or ABI PRISM 7200 Sequence Detector.

Analysis on the ABI PRISM 7700 or 7200

The ABI PRISM 7700 or ABI PRISM 7200 Sequence Detector performs the Plate Read and generates multicomponented columns for No DNA, Allele 1, and Allele 2. The data is then normalized for each allele and a genotype call is made for Allele 1 (homozygote 1), Allele 2 (homozygote 2), or Allele 1/2 (heterozygote). Samples run from the Genomic Control DNA included in this kit should all receive Allele 1/2 calls. Refer to your instrument user's manual for more information.

To perform allelic discrimination:

Step	Action
1	Launch the Sequence Detection Systems software.
2	**If the untitled plate that opens is not the correct Allelic Discrimination plate for your instrument:** a. Close the untitled plate. b. From the File menu, choose New Plate (⌘ N). c. In the New Plate dialog box, choose Allelic Discrimination from the Plate Type pop-up menu. (The Run pop-up menu will disappear.) d. Choose the correct instrument from the Instrument pop-up menu. **Note** The correct plate type and instrument can be set in Preferences… under the Edit menu.
3	Set up the plate as shown in Figure 2 on page 15. **Note** See your instrument user's manual for more information.
4	Click the Show Analysis button.
5	Click the Post PCR Read button. The software will perform the Plate Read.
6	From the File menu, choose Save as… to save the plate.
7	Click the Show Analysis button.

To perform allelic discrimination: *(continued)*

Step	Action
8	From the Analysis menu, choose Analyze (⌘ L). The computer analyzes the data.
9	From the Analysis menu, choose Allelic Discrimination (⌘ K). The Allelic Discrimination Viewer appears.
10	Examine data to confirm that allele calls have been made.

Allelic Discrimination on the LS-50B

LS-50B Settings

The excitation and emission settings for the TaqMan LS-50B PCR Detection System are summarized in Table 6.

Table 6. TaqMan LS-50B PCR Detection System Settings

Dye	Excitation λ (nm)	Excitation Slit (nm)	Emission λ (nm)	Emission Slit (nm)	Emission Filter (nm)
FAM	488	4	518	8	515
TET	488	4	538	8	515
TAMRA	488	4	582	8	515

Measure Fluorescence

To perform allelic discrimination, use the following procedure. Refer to the *LS-50B Luminescence Spectrometer User's Manual* for details.

The macro receives data from your output file and generates multicomponented data. The data is then normalized for each allele and a genotype call is made for Homo 1 (homozygote 1), Homo 2 (homozygote 2), or Hetero 1-2 (heterozygote). Samples run from the Genomic Control DNA included in this kit should all receive Hetero 1-2 calls.

Step	Action
1	Transfer the contents of each optical tube from the PCR amplification reactions into the corresponding well of a 96-Well Microplate (Portvair). Be sure to follow the allelic discrimination plate configuration shown in Figure 2 on page 15.
2	Under the Setup Instrument tab, configure the TaqMan LS-50B PCR Detection System as shown in Table 6.
3	Run the plate read. Name and store the output file.
4	Double-click on the Standard WPR Multicomponenting Macro.
5	When prompted for the spreadsheet name, type gtypewpr.xls.
6	Select the location and name of your output file. The macro analyzes the data and makes genotype calls.
7	Name and save your spreadsheet.

continued on next page

Troubleshooting on the TaqMan LS-50B PCR Detection System

Observation	Outcome	Probable Cause	Solution
Diffuse distribution of heterozygote normalized results in plotted data	Incorrect allele calls	Weak PCR amplifications	Repeat reactions, paying particular attention to pipetting technique and pipet calibration. Use fresh reagents and prepare a master mix. You can also try a larger reaction volume.
Distorted distribution (vertically or horizontally elongated) of heterozygote normalized results in plotted data	Incorrect allele calls	Poor reproducibility of NTC, AL1, or AL2 reactions	Allow spreadsheet to recalculate distributions and calls in the absence of the replicate value(s) farthest from the mean.
		Weak PCR amplifications for AL1 or AL2	Repeat reactions, paying particular attention to pipetting technique and pipet calibration. Use fresh reagents and prepare a Master Mix. You can also try a larger reaction volume.

Appendix A. Guidelines for Custom Applications

Nine-Step Program

We recommend the following steps for the development of custom 5´ nuclease assays for allelic discrimination applications:

Step	Action	See page
1	Install and use Primer Express software	–
2	Identify target sequence	23
3	Design TaqMan probe	23
4	Design primers	24
5	Order reagents	24
6	Quantitate probe and primers	24
7	Optimize primer concentrations	25
8	Optimize probe concentrations	28
9	Set up and run an Allelic Discrimination plate	33

Identify Target Sequence

A target is a nucleotide sequence, two primers, and a probe.

- For allelic discrimination, each allele associated with a target has a probe labeled with its own fluorescent reporter dye.
- The shortest amplicons work the best. Consistent results are obtained for amplicon ranges from 50–150 bp.
- Primers are common and have complete homology for both alleles.

Design TaqMan Probe

Use the following guidelines:

- Keep the G-C content in the 20–80% range (if possible).
- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- Do not put a G on the 5´ end.
- Using Primer Express™ software, the melting temperature (T_m) should be 65–67 °C.
- Select the strand that gives the probe with more Cs than Gs.
- Position the polymorphic site approximately in the middle third of the sequence.
- Adjust the probe lengths so that both probes have the same T_m.

Design Primers

Use the following guidelines:

- ♦ Keep the G-C content in 30–80% range.
- ♦ Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- ♦ Using Primer Express software, the T_m should be 58–60 °C.
- ♦ The five nucleotides at the 3´ end should have no more than two G and/or C bases.
- ♦ Place the forward and reverse primers as close as possible to the probe without overlapping the probe.

Order Reagents

Refer to “User-Supplied Materials” on page 5 for a list of required reagents and equipment.

Quantitate Probes and Primers

Use a spectrophotometric method to determine the concentrations of the probes and primers received:

- ♦ Measure the absorbance at 260 nm of a 1:100 dilution of each oligonucleotide in TE buffer.
- ♦ Calculate the oligonucleotide concentration (C) in µM using the method shown in the table below.

Chromophore	Extinction Coefficient	Number	Extinction Coefficient Contribution
A	15,200	1	15,200
C	7,050	6	42,300
G	12,010	5	60,050
T	8,400	6	50,400
FAM	20,958	1	20,958
TAMRA	31,980	1	31,980
TET	16,255	0	–
Total	–	–	220,888

Absorbance (260 nm) = sum of extinction coefficient contributions × cuvette pathlength × oligonucleotide concentration/100

$0.13 = 220{,}888\ M^{-1}cm^{-1} \times 0.3\ cm \times C/100$

$C = 196\ \mu M$

Optimize Primer Concentrations

The purpose of this procedure is to determine the minimum primer concentrations that give the maximum R_n. The ABI PRISM 7700 Sequence Detector can provide additional data for optimization using the minimum threshold cycle (C_T). See Appendix B on page 39 for more information regarding C_T.

- ♦ Use the TaqMan Universal PCR Master Mix.
- ♦ Use the thermal cycler conditions in the table below.

Thermal Cycler	Times and Temperatures			
	Initial Steps		Each of 40 Cycles	
			Melt	Anneal/Extend
GeneAmp PCR System 9600 or 9700 in 9600 Emulation Mode	HOLD	HOLD	CYCLE	
	2 min 50 °C	10 min 95 °C	15 sec 95 °C	1 min 62 °C
ABI PRISM 7700 Sequence Detector	HOLD	HOLD	CYCLE	
	2 min 50 °C	10 min 95 °C	15 sec 95 °C	1 min 62 °C

IMPORTANT The two-minute, 50 °C step is required for optimal AmpErase UNG activity. The 10-minute, 95 °C step is required to activate AmpliTaq Gold DNA Polymerase.

- ♦ Use one of the allelic discrimination probes with its target at a concentration of 100 nM.
- ♦ Run at least four replicates of each of the nine conditions defined by the 3 × 3 matrix below, as well as four No Template Control (NTC) and four No Amplification Control (NAC) replicates. The NTC and NAC replicates should be run at 900 nM forward and reverse primer concentrations.

	Forward Primer (nM)		
Reverse Primer (nM)	**50**	**300**	**900**
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

Wells	Universal PCR Master Mix (µL)	10 µM FAM Probe (µL)	FAM Template Target (µL)	20 µM Forward Primer (µL)	20 µM Reverse Primer (µL)	Deionized Water	Total Volume/ Well (µL)
A1–A4	25	0.5	5.0	0.125	0.125	19.25	50
A5–A8	25	0.5	5.0	0.125	0.75	18.625	50
A9–A12	25	0.5	5.0	0.125	2.25	17.125	50
B1–B4	25	0.5	5.0	0.75	0.125	18.625	50
B5–B8	25	0.5	5.0	0.75	0.75	18.0	50
B9–B12	25	0.5	5.0	0.75	2.25	16.5	50
C1–C4	25	0.5	5.0	2.25	0.125	17.125	50
C5–C8	25	0.5	5.0	2.25	0.75	16.5	50
C9–C12	25	0.5	5.0	2.25	2.25	15.0	50
D1–D4 (NTC)	25	0.5	0	2.25	2.25	20.0	50
D5–D8 (NAC)[a]	25	0.5	5.0	2.25	2.25	14	50[a]

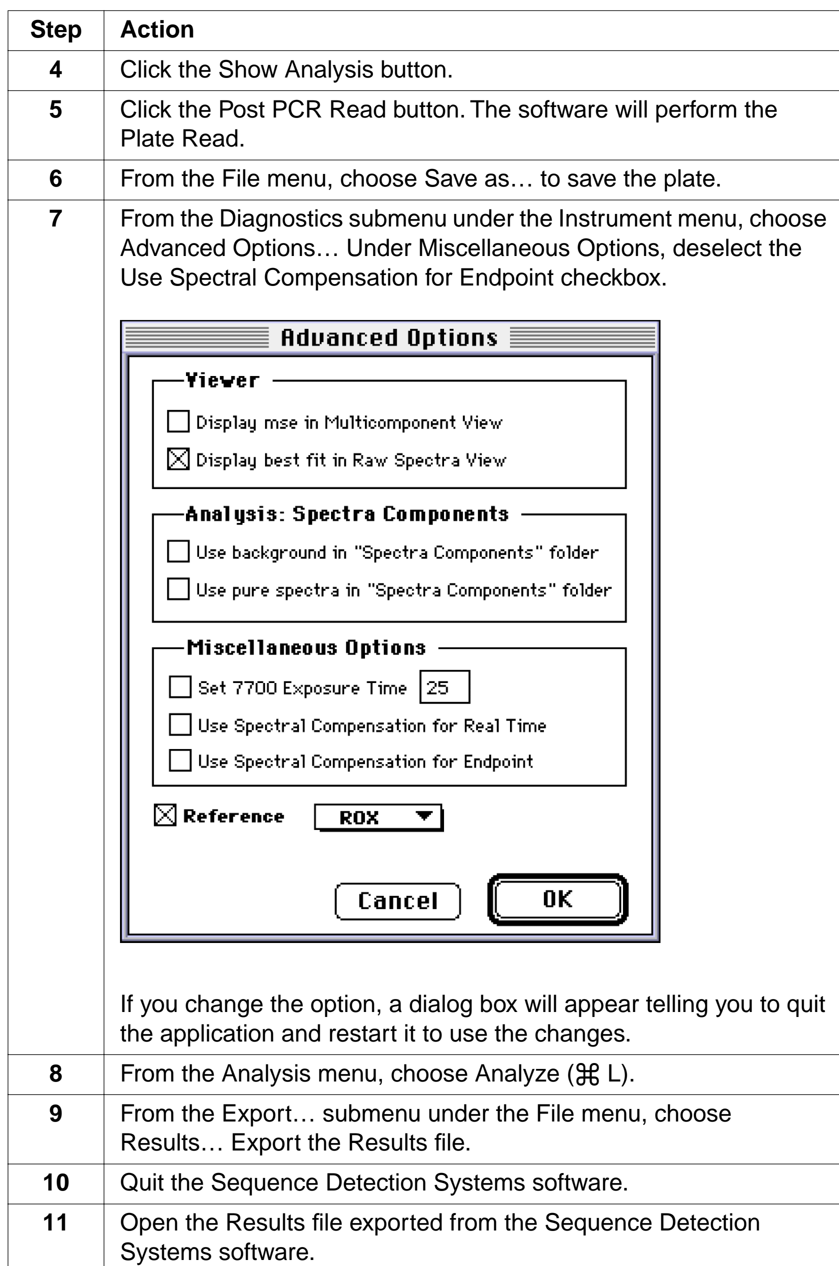
a. Add 1 µL of 0.5% sodium dodecyl sulfate (SDS) to each of the four NAC wells to inhibit any enzyme activity in those wells.

To optimize primer concentrations:

Step	Action
1	Launch the Sequence Detection Systems software.
2	**If the untitled plate that opens is not a Single Reporter Plate Read document for your instrument:**
	a. Close the untitled plate. b. From the File menu, choose New Plate (⌘ N). c. In the New Plate dialog box, choose Single Reporter from the Plate Type pop-up menu and Plate Read from the Run pop-up menu. d. Choose the correct instrument from the Instrument pop-up menu.
3	Select wells as follows: ♦ A1–C12, unknowns (UNKN) ♦ D1–D4, No Template Controls (NTC) ♦ D5–D8, No Amplification Controls (NAC)

To optimize primer concentrations: *(continued)*

Step	Action
4	Click the Show Analysis button.
5	Click the Post PCR Read button. The software will perform the Plate Read.
6	From the File menu, choose Save as… to save the plate.
7	From the Diagnostics submenu under the Instrument menu, choose Advanced Options… Under Miscellaneous Options, deselect the Use Spectral Compensation for Endpoint checkbox.



Step	Action
	If you change the option, a dialog box will appear telling you to quit the application and restart it to use the changes.
8	From the Analysis menu, choose Analyze (⌘ L).
9	From the Export… submenu under the File menu, choose Results… Export the Results file.
10	Quit the Sequence Detection Systems software.
11	Open the Results file exported from the Sequence Detection Systems software.

To optimize primer concentrations: *(continued)*

Step	Action
12	Tabulate the results for R_n (and C_T if using the ABI PRISM 7700 instrument). Choose the minimum forward and reverse primer concentrations that yield the maximum R_n (and minimum C_T).
13	Use these primer concentrations in your allelic discrimination assay.
14	If wells D1–D4 are different from wells D5–D8, check for sources of contamination. If a run with fresh reagents still shows significant differences between these wells, remove possible interactions between primers and probes by redesigning one of the primers.

Optimize Probe Concentrations

The purpose of this procedure is to determine the probe concentrations that give the most reliable autocalls.

- ♦ The initial fluorescence signals from the two probes are matched approximately.
- ♦ Fluorescence is measured directly. No thermal cycling is required.
- ♦ The procedure is instrument-dependent, reflecting the optical differences between the ABI PRISM 7200 Sequence Detector (page 28) and the ABI PRISM 7700 Sequence Detector (page 31).

ABI PRISM 7200 Sequence Detector

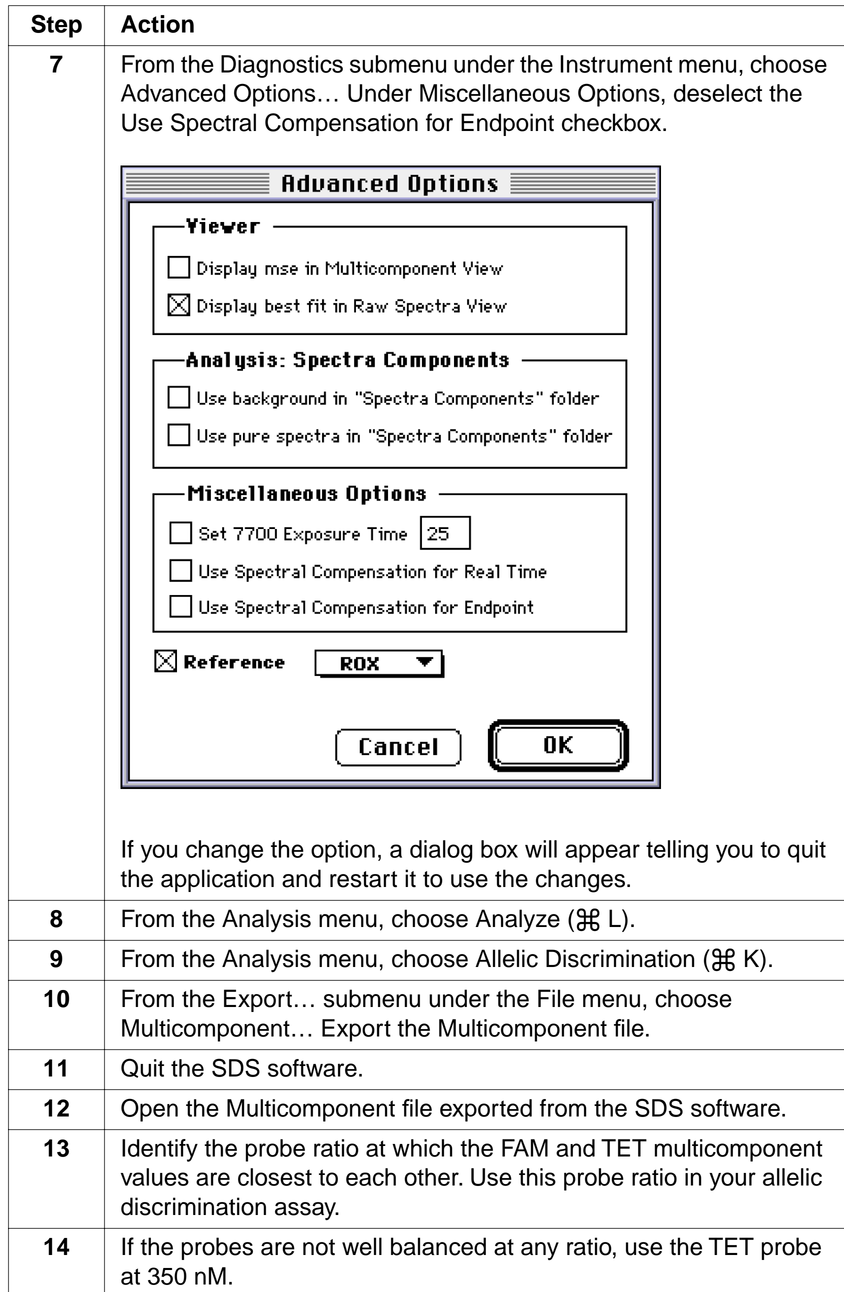
Prepare the plate shown in the table below. Use 50 µL of the indicated solution in each well.

Wells	Universal PCR Master Mix (µL)	1 µM FAM Probe (µL)	1 µM TET Probe (µL)	Deionized Water (µL)	Total Volume/ Well (µL)	Final FAM Probe Conc. (nM)	Final TET Probe Conc. (nM)
A1–A4	25	2.5	2.5	20	50	50	50
A5–A8	25	2.5	5.0	17.5	50	50	100
A9–A12	25	2.5	7.5	15	50	50	150
B1–B4	25	2.5	10	12.5	50	50	200
B5–B8	25	2.5	12.5	10	50	50	250
B9–B12	25	2.5	15	7.5	50	50	300
C1–C4	25	2.5	17.5	5	50	50	350

To optimize probe concentrations on the 7200:

Step	Action
1	Launch the Sequence Detection Systems software.
2	**If the untitled plate that opens is not the correct Allelic Discrimination plate for your instrument:** a. Close the untitled plate. b. From the File menu, choose New Plate (⌘ N). c. In the New Plate dialog box, choose Allelic Discrimination from the Plate Type pop-up menu. (The Run pop-up menu will disappear.) d. Choose the correct instrument from the Instrument pop-up menu. **Note** The correct plate type and instrument can be set in Preferences… under the Edit menu.
3	Select wells A1–C12 as unknowns (UNKN).
4	Click the Show Analysis button.
5	Click the Post PCR Read button. The software will perform the Plate Read.
6	From the File menu, choose Save as… to save the plate.

To optimize probe concentrations on the 7200: *(continued)*



Step	Action
7	From the Diagnostics submenu under the Instrument menu, choose Advanced Options… Under Miscellaneous Options, deselect the Use Spectral Compensation for Endpoint checkbox. If you change the option, a dialog box will appear telling you to quit the application and restart it to use the changes.
8	From the Analysis menu, choose Analyze (⌘ L).
9	From the Analysis menu, choose Allelic Discrimination (⌘ K).
10	From the Export… submenu under the File menu, choose Multicomponent… Export the Multicomponent file.
11	Quit the SDS software.
12	Open the Multicomponent file exported from the SDS software.
13	Identify the probe ratio at which the FAM and TET multicomponent values are closest to each other. Use this probe ratio in your allelic discrimination assay.
14	If the probes are not well balanced at any ratio, use the TET probe at 350 nM.

ABI PRISM 7700 Sequence Detector

Prepare the plate shown in the table below. Use 50 µL of the indicated solution in each well.

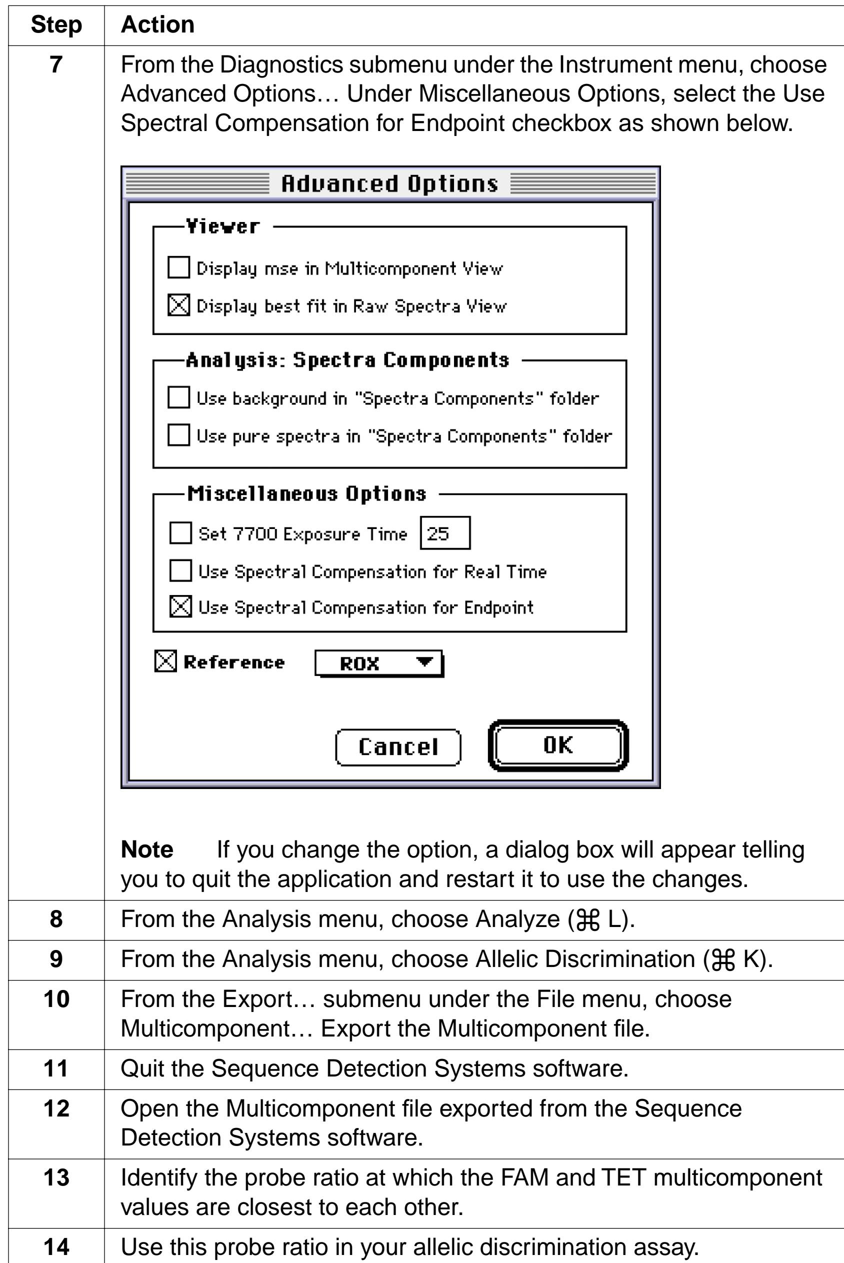
Wells	Universal PCR Master Mix (µL)	1 µM FAM Probe (µL)	1 µM TET Probe (µL)	Deionized Water (µL)	Total Volume (µL)	Final FAM Probe Conc. (nM)	Final TET Probe Conc. (nM)
A1–A4	125	12.5	50	62.5	250	50	200
A5–A8	125	25	50	50	250	100	200
A9–A12	125	25	25	75	250	100	100
B1–B4	125	50	25	50	250	200	100
B5–B8	125	50	12.5	62.5	250	200	50

To optimize probe concentrations on the 7700:

Step	Action
1	Launch the Sequence Detection Systems software.
2	**If the untitled plate that opens is not the correct Allelic Discrimination plate for your instrument:** a. Close the untitled plate. b. From the File menu, choose New Plate (⌘ N). c. In the New Plate dialog box, choose Allelic Discrimination from the Plate Type pop-up menu. (The Run pop-up menu will disappear.) d. Choose the correct instrument from the Instrument pop-up menu. **Note** The correct plate type and instrument can be set in Preferences… under the Edit menu.
3	Select wells A1–B8 as unknowns (UNKN).
4	Click the Show Analysis button.
5	Click the Post PCR Read button. The software will perform the Plate Read.
6	From the File menu, choose Save as… to save the plate.

To optimize probe concentrations on the 7700: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>7</td><td>From the Diagnostics submenu under the Instrument menu, choose Advanced Options… Under Miscellaneous Options, select the Use Spectral Compensation for Endpoint checkbox as shown below.



Note If you change the option, a dialog box will appear telling you to quit the application and restart it to use the changes.</td></tr>
<tr><td>8</td><td>From the Analysis menu, choose Analyze (⌘ L).</td></tr>
<tr><td>9</td><td>From the Analysis menu, choose Allelic Discrimination (⌘ K).</td></tr>
<tr><td>10</td><td>From the Export… submenu under the File menu, choose Multicomponent… Export the Multicomponent file.</td></tr>
<tr><td>11</td><td>Quit the Sequence Detection Systems software.</td></tr>
<tr><td>12</td><td>Open the Multicomponent file exported from the Sequence Detection Systems software.</td></tr>
<tr><td>13</td><td>Identify the probe ratio at which the FAM and TET multicomponent values are closest to each other.</td></tr>
<tr><td>14</td><td>Use this probe ratio in your allelic discrimination assay.</td></tr>
</table>

continued on next page

Set Up and Run an Allelic Discrimination Plate

This procedure involves PCR amplification of the target DNA followed by fluorescence analysis. When performing allelic discrimination using your Allele 1 and Allele 2 standards, the analysis requires that the controls and samples be run (Figure 3).

- ♦ Eight No Template Control wells (NTC)
- ♦ Eight Allele 1 standard wells (AL1)
- ♦ Eight Allele 2 standard wells (AL2)
- ♦ Seventy-two Genomic Control DNA wells (UNKN)

IMPORTANT Eight replicates of No Template Control, Allele 1 standard, and Allele 2 standard must be run to make allele calls at a 99.7% confidence level using the automated allele calling routine. Manual allele calling with less than eight replicates is possible. Refer to Chapter 4 of the ABI PRISM 7200 *Sequence Detector User's Manual*.

NTC A1	NTC A2	NTC A3	NTC A4	NTC A5	NTC A6	NTC A7	NTC A8	AL1 A9	AL1 A10	AL1 A11	AL1 A12
AL1 B1	AL1 B2	AL1 B3	AL1 B4	AL2 B5	AL2 B6	AL2 B7	AL2 B8	AL2 B9	AL2 B10	AL2 B11	AL2 B12
UNKN C1	UNKN C2	UNKN C3	UNKN C4	UNKN C5	UNKN C6	UNKN C7	UNKN C8	UNKN C9	UNKN C10	UNKN C11	UNKN C12
UNKN D1	UNKN D2	UNKN D3	UNKN D4	UNKN D5	UNKN D6	UNKN D7	UNKN D8	UNKN D9	UNKN D10	UNKN D11	UNKN D12
UNKN E1	UNKN E2	UNKN E3	UNKN E4	UNKN E5	UNKN E6	UNKN E7	UNKN E8	UNKN E9	UNKN E10	UNKN E11	UNKN E12
UNKN F1	UNKN F2	UNKN F3	UNKN F4	UNKN F5	UNKN F6	UNKN F7	UNKN F8	UNKN F9	UNKN F10	UNKN F11	UNKN F12
UNKN G1	UNKN G2	UNKN G3	UNKN G4	UNKN G5	UNKN G6	UNKN G7	UNKN G8	UNKN G9	UNKN G10	UNKN G11	UNKN G12
UNKN H1	UNKN H2	UNKN H3	UNKN H4	UNKN H5	UNKN H6	UNKN H7	UNKN H8	UNKN H9	UNKN H10	UNKN H11	UNKN H12

Figure 3 Plate diagram showing placement of control and sample reactions

Prepare Controls and Unknowns

<table>
<tr><th>Step</th><th colspan="2">Action</th></tr>
<tr><td>1</td><td colspan="2">Prepare 575 µL of a solution that contains your optimized primers and probes in concentrations 10X the optimal values you determined.</td></tr>
<tr><td>2</td><td colspan="2">Combine the following:
♦ TaqMan Universal PCR Master Mix for 115 reactions (2.875 mL) in 1.725 mL of water.
♦ 575 µL of 10X Primer and Probe Solution</td></tr>
<tr><td>3</td><td colspan="2">Deliver 45 µL of this mixture to each of the 96 wells in the plate.</td></tr>
<tr><td rowspan="5">4</td><td>If preparing…</td><td>Then add…</td></tr>
<tr><td>NTC</td><td>5 µL of TE buffer to wells A1–A8</td></tr>
<tr><td>AL1</td><td>5 µL of Allele 1 (TET) standard to wells A9–A12 and B1–B4</td></tr>
<tr><td>AL2</td><td>5 µL of Allele 2 (FAM) standard to wells B5–B12</td></tr>
<tr><td>UNKN</td><td>5 µL of each unknown sample to wells C1–B12</td></tr>
<tr><td>5</td><td colspan="2">Close the plate with MicroAmp Optical Caps.</td></tr>
<tr><td>6</td><td colspan="2">Centrifuge the plate to collect the liquid at the bottom of the tubes and remove the air bubbles.</td></tr>
</table>

Thermal Cycling

Use the thermal cycler conditions in “Optimize Primer Concentrations” on page 25.

Run Allelic Discrimination Plate

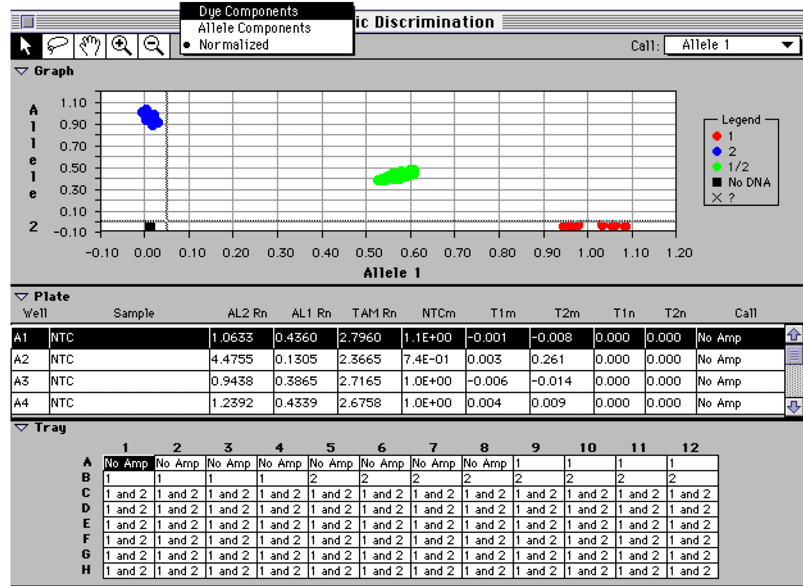
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Launch the Sequence Detection Systems software.</td></tr>
<tr><td rowspan="2">2</td><td>If the untitled plate that opens is not the correct Allelic Discrimination plate for your instrument:</td></tr>
<tr><td>a. Close the untitled plate.
b. From the File menu, choose New Plate (⌘ N).
c. In the New Plate dialog box, choose Allelic Discrimination from the Plate Type pop-up menu. (The Run pop-up menu will disappear.)
d. Choose the correct instrument from the Instrument pop-up menu.

Note The correct plate type and instrument can be set in Preferences… under the Edit menu.</td></tr>
<tr><td>3</td><td>Define the plate wells as shown in “Set Up and Run an Allelic Discrimination Plate” on page 33.</td></tr>
<tr><td>4</td><td>Click the Show Analysis button.</td></tr>
<tr><td>5</td><td>Click the Post PCR Read button. The software will perform the Plate Read.</td></tr>
<tr><td>6</td><td>From the File menu, choose Save as… to save the plate.</td></tr>
<tr><td>7</td><td>From the Analysis menu, choose Analyze (⌘ L). The computer analyzes the data.

IMPORTANT Spectral Compensation for Endpoint must be on for the ABI PRISM 7700 Sequence Detector and off for the ABI PRISM 7200 Sequence Detector.</td></tr>
<tr><td>8</td><td>From the Analysis menu, choose Allelic Discrimination (⌘ K). The Allelic Discrimination window appears.</td></tr>
<tr><td>9</td><td>Check the Allelic Discrimination window and confirm that the No Amp (NTC), 1 (Allele 1 standard), 2 (Allele 2 standard), and 1 and 2 (heterozygote) calls have been made. If these calls are correct, the custom application is running under optimal conditions.</td></tr>
</table>

Troubleshooting Custom Allelic Discrimination Applications

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>If the Allelic Discrimination window does not show autocalls, select Dye Components from the pop-up menu to the right of the toolbar as shown below.</td></tr>
<tr><td colspan="2">



</td></tr>
<tr><td>2</td><td>Bring all samples on-scale. Confirm that the standards and samples in the plot under Graph are distributed into up to four populations.</td></tr>
<tr><td>3</td><td>If the populations are well distributed, use the lasso tool and the Call pop-up menu in the upper right corner of the Allelic Discrimination window (see below) to call them manually as No Amp, Allele 1, Allele 2, and Allele 1/2 (heterozygote).

Note Manual calling allows this application to be run without NTCs or standards, with up to 96 individual samples per plate.</td></tr>
</table>

Troubleshooting Custom Allelic Discrimination Applications *(continued)*

Step	Action

Allelic Discrimination

Dye Components Call: Allele 1

▽ Graph

Allele 2 (y axis): 14.00, 10.00, 6.00, 2.00, -2.00, -6.32

Allele 1 (x axis): -2.71, -1.00, 0.00, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00

Legend: 1; 2; 1/2; No DNA; ?

▽ Plate

Well	Sample	AL2 Rn	AL1 Rn	TAM Rn	NTCm	T1m	T2m	T1n	T2n	Call
A1	NTC	1.0633	0.4360	2.7960	1.1E+00	-0.001	-0.008	0.000	0.000	No Amp
A2	NTC	4.4755	0.1305	2.3665	7.4E-01	0.003	0.261	0.000	0.000	No Amp
A3	NTC	0.9438	0.3865	2.7165	1.0E+00	-0.006	-0.014	0.000	0.000	No Amp
A4	NTC	1.2392	0.4339	2.6758	1.0E+00	0.004	0.009	0.000	0.000	No Amp

▽ Tray

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	1	1	1	1
B	1	1	1	1	2	2	2	2	2	2	2	2
C	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2
D	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2
E	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2
F	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2
G	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2
H	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2

Step	Action
4	If the populations do not show good discrimination, choose Raw Spectra (⌘Y) from the Analysis menu. Check whether the signal for at least one replicate of each of the two allele standards is within the dynamic range of the detector. a. If the signal exceeds the dynamic range, rerun the plate with a proportional reduction in the concentrations of both probes. b. If the signal is within the dynamic range, proceed to step 5.
5	From the Diagnostics submenu under the Instrument menu, choose Advanced Options… Under Viewer, select the Display best fit in Raw Spectra View checkbox. a. If the Raw Spectra View shows a poor fit for the Pure Dye files, rerun the Pure Dye standards. b. If the Raw Spectra View shows a good fit for the raw data, proceed to the next step. **Note** Updating your Pure Dye files every 90 days is a good practice for running allelic discrimination assays. To do this, use the Sequence Detection Systems Spectral Calibration Kit (P/N 4305822). Refer to ABI PRISM 7700 *User Bulletin #4: Generating New Spectra Components* (P/N 4306234) for instructions on creating new Pure Dye standards.

Troubleshooting Custom Allelic Discrimination Applications *(continued)*

Step	Action
6	Rerun the reaction using an extension temperature of 64 °C to improve the separation between populations.
7	If the separation between populations still does not allow them to be called manually, reinspect the probe sequences and samples to confirm that they have been labeled and run correctly.

Appendix B. Real Time Detection on the ABI PRISM 7700

Threshold Cycle

Real Time detection on the ABI PRISM 7700 Sequence Detector monitors fluorescence and calculates R_n during each PCR cycle.The threshold cycle or C_T value is the cycle at which a statistically significant increase in ΔR_n, the difference between reporter fluorescence in the sample and that in the No Template Control, is first detected (Figure 4).

On the graph of R_n versus cycle number, the threshold cycle occurs when the Sequence Detection Application begins to detect the increase in signal associated with an exponential growth of PCR product.

For example, in a series of similar reactions where primer concentrations are varied, the optimum conditions are those that give the lowest C_T value.

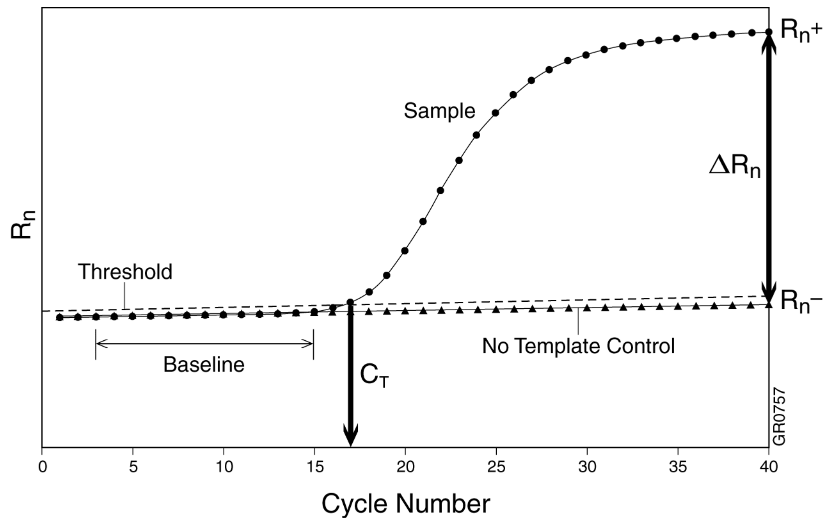


Figure 4 Amplification plot, R_n versus cycle number

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