User Bulletin

All PCR Instruments

May 26, 2000

SUBJECT: Primer Express Version 1.5 and TaqMan MGB Probes for Allelic Discrimination

Release of Primer Express Version 1.5

Version 1.5 of the Primer Express® Oligo Design software is now available from Applied Biosystems. Primer Express is a Macintosh®-based oligonucleotide design program created to support Applied Biosystems PCR and Sequence Detection Systems instruments. The new release features support for designing TaqMan® MGB probe assays and *turbo* TaqMan® probe assays, Y2K compliance, and increased program stability.

Introducing TaqMan MGB Probes

Applied Biosystems offers custom TaqMan MGB probes for allelic discrimination assays. The new TaqMan MGB probes contain the following features:

- A nonfluorescent quencher at the 3´ end

  Because the quencher does not fluoresce, the Sequence Detection Systems instruments can now measure the reporter dye contributions more precisely.

- A minor groove binder at the 3´ end

  The minor groove binder increases the melting temperature (T_m) of probes (Afonina *et al.*, 1997; Kutyavin *et al.*, 1997), allowing the use of shorter probes. Consequently, the TaqMan MGB probes exhibit greater differences in T_m values between matched and mismatched probes, which provides more accurate allelic discrimination.

In This Bulletin

This user bulletin contains information on the following topics:

Allelic Discrimination Using the 5´-Nuclease Assay

How Allelic Discrimination Assays Work

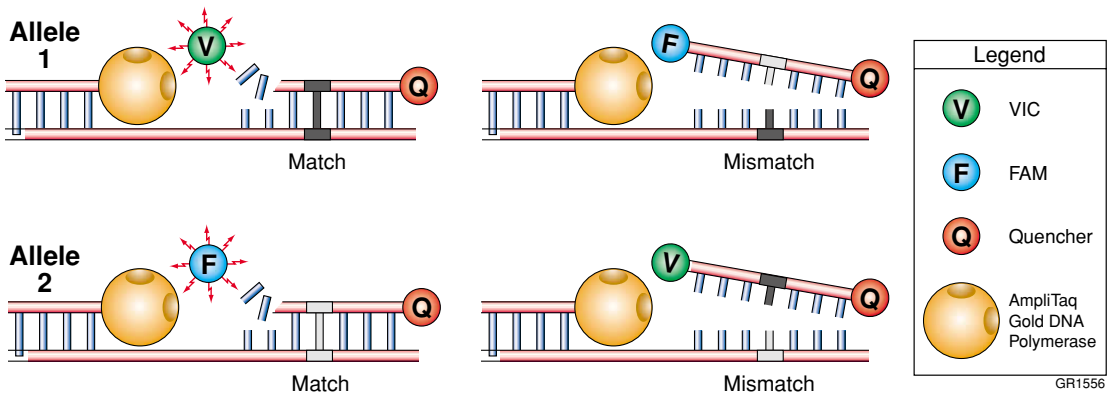
In allelic discrimination assays, the PCR assay includes a specific, fluorescent, dye-labeled probe for each allele. The probes contain different fluorescent reporter dyes (FAM and VIC™) to differentiate the amplification of each allele.

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 allele. Cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter dye. Thus, the fluorescence signal(s) generated by PCR amplification indicate(s) the alleles that are present in the sample.

Mismatches Between Probe and Allele Sequences

Mismatches between a probe and allele 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 figure below illustrates results from matches and mismatches between allele and probe sequences in allelic discrimination assays (Livak *et al.*, 1995; Livak *et al.*, 1999).



The table below summarizes the possible results of the example allelic discrimination assay shown above.

A substantial increase in…	Indicates…
VIC fluorescence only	homozygosity for Allele 1.
FAM fluorescence only	homozygosity for Allele 2.
both fluorescent signals	heterozygosity.

About TaqMan Probes

Applied Biosystems TaqMan Probes

Applied Biosystems offers three different types of probes for TaqMan assays:

- TaqMan probes
- TaqMan MGB probes
- *turbo* TaqMan probes

Probe Features

The following table describes the features of the three different types of probes:

TaqMan Probe	5´ Label	3´ Label	Features
TaqMan	6-FAM, VIC, or TET	TAMRA	None
TaqMan MGB	6-FAM, VIC, or TET	Nonfluorescent quencher	Minor groove binder
turbo TaqMan	6-FAM, VIC, or TET	TAMRA	5-propyne-2´-deoxyuridine in place of thymine[a]

a. See *ABI PRISM 7700 Sequence Detection Systems User Bulletin 6: turbo TaqMan Probes* (P/N 4311463) for more information about designing assays with *turbo* TaqMan probes.

Recommendations for Using TaqMan MGB Probes

Applied Biosystems recommends the general use of TaqMan MGB probes for allelic discrimination assays, especially when conventional TaqMan probes exceed 30 nucleotides. The procedures on the following pages explain how to design TaqMan MGB probes with Primer Express 1.5. For instructions on designing TaqMan or *turbo* TaqMan probes, see “Designing TaqMan and *turbo* TaqMan Assays for Allelic Discrimination” on page 17.

Applied Biosystems recommends using TaqMan MGB probes:

- To achieve estimated T_m values of 65–67 °C using probes shorter than 20 nucleotides
- To obtain greater differences in T_m values between matched and mismatched probes than with *turbo* TaqMan probes and conventional TaqMan probes
- To obtain more precise measurements of dye contributions

Designing Allelic Discrimination Assays Using Primer Express Version 1.5

About Primer Express Version 1.5

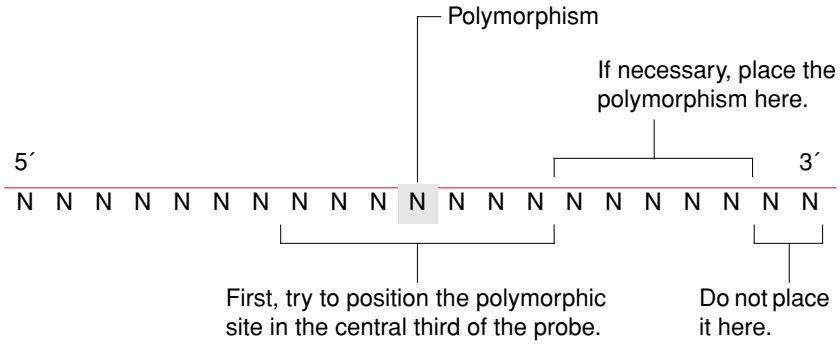
This section describes the guidelines for designing TaqMan MGB probes using Primer Express software version 1.5. To design conventional TaqMan or *turbo* TaqMan allelic discrimination assays, see “Designing TaqMan and *turbo* TaqMan Assays for Allelic Discrimination” on page 17. The new release of Primer Express features several improvements on the existing software. For a summary of the software system requirements and a explanation of how to obtain and install the update, see “Obtaining and Installing Primer Express Version 1.5” on page 24.

Note The following procedures are valid only for version 1.5 of the Primer Express software. Primer Express is optimized for use with Applied Biosystems assays, reagents, and thermal cycling parameters. Melting temperatures may differ substantially if another T_m calculator is used to design the TaqMan MGB probes or *turbo* TaqMan probes.

TaqMan MGB Probe Design Guidelines

IMPORTANT When designing probes, it is important to consider probes from both strands.

Follow the guidelines in the table below for designing TaqMan MGB probes:

Priority	Guideline
1	Avoid probes with a guanine residue at the 5´ end of the probe. A guanine residue adjacent to the reporter dye will quench the reporter fluorescence, even after cleavage.
2	Select probes with a Primer Express software–estimated T_m of 65–67 °C.
3	Make TaqMan MGB probes as short as possible without being shorter than 13 nucleotides.
4	Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more should be avoided.
5	Position the polymorphic site in the central third of the probe. **Note** The polymorphic site can be shifted toward the 3´ end to meet the above guidelines, however, the site must be located more than two nucleotides upstream from the 3´ terminus. The following figure illustrates the placement of a polymorphism in an example probe (N = Nucleotide). 

Designing TaqMan MGB Assays for Allelic Discrimination

Loading the Sequence

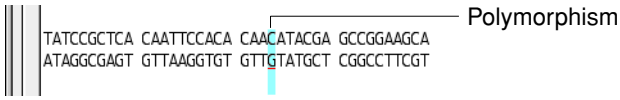
IMPORTANT Because of the asymmetric placement of the minor groove binder at the 3´ end, complementary TaqMan MGB probes do not necessarily have the same T_m.

To load the sequence:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Launch the Primer Express software.</td></tr>
<tr><td>2</td><td>Import a DNA sequence for designing probes and primers.
<table>
<tr><th>To select a probe from…</th><th>Then…</th></tr>
<tr><td>a DNA file</td><td>a. From the File menu, scroll to New, and select TaqMan® MGB Probe & Primer Design.
A TaqMan® MGB Probe document appears.
b. Click Import DNA File.
c. Locate and select a DNA file in the browser.
d. Click Open.
The software loads the sequence and displays it in the Sequence tab.</td></tr>
<tr><td>an existing primer/probe document (located in the Document Archive)</td><td>a. From the File menu, select Open.
The Document Archive dialog box appears.
b. Double-click the document to load, or select the sequence and click Open.
The software loads the sequence and displays it in the Sequence tab.</td></tr>
<tr><td>a text document or GenBank sequence</td><td>a. Select the sequence from the text document or the navigator window.
b. From the Edit menu, select Copy.
c. From the File menu, scroll to the New submenu, and select TaqMan® MGB Probe & Primer Design.
A TaqMan® MGB Probe document appears.
d. From the Edit menu, select Paste.
The software pastes the nucleotide sequence into the Sequence tab.
Note Edit the GenBank sequence before saving it as a Primer Express document.</td></tr>
</table></td></tr>
<tr><td>3</td><td>Select the following checkboxes for primer selection:
♦ Double Stranded
♦ Limit 3´ G+C
 

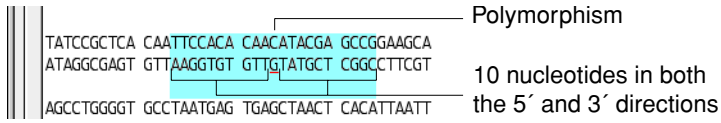
Select these checkboxes
The sense and antisense sequences appear on the Sequence tab.</td></tr>
</table>

To load the sequence: *(continued)*

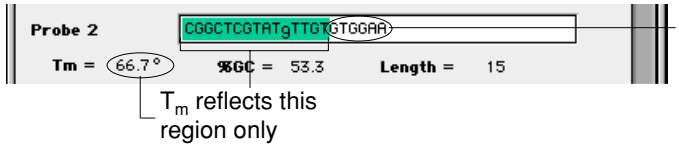
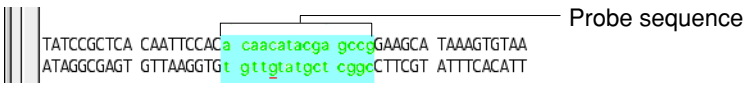
Step	Action
4	Label the polymorphism within the sequence using the **Line** tool: a. From the **Tools** palette, click the **Line** tool.  b. Select the polymorphic sequence.  The software automatically underlines the polymorphism.
5	Following steps 1-4, import the sequence for the other allele into a separate **TaqMan® MGB Probe** document.

Designing the Allele 1 Probe

To design the probe for Allele 1:

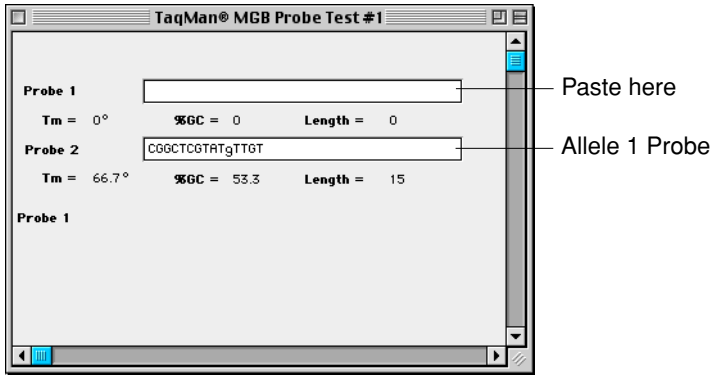
Step	Action
1	From the **TaqMan® MGB Probe** document for Allele 1, click the **Sequence** tab. The **Sequence** tab comes to the front of the dialog box.
2	Select a region containing potential probe sequences. a. Highlight the polymorphism and approximately 10 nucleotides in both the 5´ and 3´ directions.  b. From the **Edit** menu, select **Copy**. c. From the **File** menu, scroll to **New**, and select **TaqMan® MGB Probe Test Document**. A **TaqMan® MGB Probe Test** document appears. d. Click the **Probe 1** text box. e. From the **Edit** menu, select **Paste**. Primer Express copies the sequence into the TaqMan® MGB Probe Test document and calculates the T_m using a specialized algorithm for TaqMan MGB probes.
3	Test potential probe sequences in the complementary strand. a. Return to the **Sequence** tab in the **TaqMan® MGB Probe** document for Allele 1. The polymorphic sequence and surrounding nucleotides should still be selected. b. From the **Edit** menu, select **Copy Complement**. c. Return to the **TaqMan® MGB Probe Test** document, and click the **Probe 2** text box. d. From the **Edit** menu, select **Paste**. Primer Express copies the complementary sequence into the test document and calculates the T_m of the oligonucleotide.

To design the probe for Allele 1: *(continued)*

Step	Action
4	For easier identification, label the polymorphism within each probe sequence. a. Select the polymorphism within the sequence in the **Probe 1** text box.  b. Press the key corresponding to the letter of the polymorphic base. Primer Express replaces the uppercase letter of the base with a lowercase letter.  c. Repeat steps a and b for the sequence in the **Probe 2** text box.
5	Highlight potential probe sequences until you identify a probe that meets the guidelines listed in “TaqMan MGB Probe Design Guidelines” on page 4. **Note** Look at potential probes from the complementary sequence. **IMPORTANT** Primer Express calculates the T_m for only the *highlighted* nucleotide sequence and excludes residues outside the selected region from the calculation. 
6	From the **Edit** menu, select **Trim**. The software eliminates all but the selected nucleotide sequence (in blue) from the **TaqMan® MGB Probe Test** document.
7	Copy and paste the final sequence for the Allele 1 probe into a text document for ordering.
8	Double-click the unused Allele 1 probe sequence and press the **delete** key. The software clears the unused probe sequence from the **TaqMan® MGB Probe Test** document.
9	Label the selected Allele 1 probe. a. From the **TaqMan® MGB Probe** document for Allele 1, click the **Sequence** tab. b. Click the **Probe** tool.  c. Highlight the final probe sequence.  The software labels the probe in green lowercase letters.

Designing the Allele 2 Probe

To design the probe for Allele 2:

Step	Action
1	In the **TaqMan® MGB Probe** document for Allele 2, click the **Sequence** tab. The **Sequence** tab appears.
2	Select a region containing potential probe sequences. a. Highlight the polymorphic sequence and approximately 10 nucleotides in both the 5´ and 3´ directions. b. Copy the sequence for the Allele 2 probe. **If the Allele 1 probe is on the…** / **Then go to the Edit menu and select…** sense strand, / **Copy**. antisense (complementary) strand, / **Copy Complement.** **IMPORTANT** Both probe sequences in the allelic discrimination assay must come from same strand, otherwise the two probes will hybridize to each other. c. Return to the **TaqMan® MGB Probe Test** document, and click the empty **Probe** text box. d. From the **Edit** menu, select **Paste**.  Primer Express copies the appropriate sequence into the test document and calculates the T_m of the oligonucleotide.
3	For easier identification, label the polymorphism within the Allele 2 probe sequence. a. Select the polymorphism within the Allele 2 sequence. b. Press the key corresponding to the letter of the polymorphic base. Primer Express replaces the uppercase letter of the base with a lowercase letter.
4	Highlight potential probe sequences until you identify a probe that meets the guidelines listed in “TaqMan MGB Probe Design Guidelines” on page 4.
5	With the desired probe sequence highlighted, select **Trim** from the **Edit** menu. The software eliminates all but the selected nucleotide sequence (in blue) from the probe test document.
6	Copy and paste the final sequence for the Allele 2 probe into a text document for ordering.

Primer Design Guidelines

After selecting probes for the assay, choose primers based on the guidelines below. Consequently, amplicons are usually 75–150 bp. By limiting the parameters for amplicon design (such as amplicon size), it is possible to run all reactions with a single reaction buffer (such as TaqMan® Universal PCR Master Mix (P/N 4304437)) and a single thermal cycling protocol.

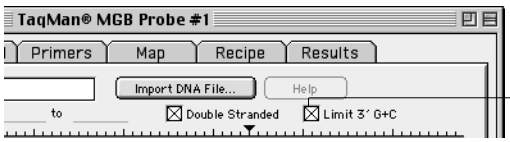
Guidelines for Designing Primers

- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more should be avoided.
- Set a Primer Express software–estimated T_m for the primers of 58–60 °C.
- Keep the guanine + cytosine content within 20–80%.
- Make sure the last five nucleotides at the 3´ end contain no more than two guanine + cytosine residues.
- Place the forward and reverse primers as close as possible to the probe without overlapping it.

Designing Primers

IMPORTANT Design primers after designing the probe with the probe tool. The probe should appear in green.

To design primers for the allelic discrimination assay:

Step	Action
1	Click the **Sequence** tab from the **TaqMan® MGB Probe** document for Allele 1. The **Sequence** tab comes to the front of the dialog box.
2	Ensure that the **Limit G+C** checkbox from the **Sequence** tab is checked. 
3	Select **Find Primers/Probes Now** from the **Options** menu. **If the software…** / **Then…** finds acceptable primers: a. Click the **Primers** tab. b. Select a primer sequence from the list that satisfies all requirements listed above. cannot find acceptable primers: design the forward and reverse primers manually according to the guidelines listed above.
4	Select a set of primers from the list that will produce the shortest amplicon while satisfying the guidelines above.
5	Copy and paste the final primer sequences into a text document for ordering.
6	Select **Save** from the **File** menu, and assign a name to the file to save the results. **IMPORTANT** It is not necessary to design primers for the Allele 2 probe.

Performing Allelic Discrimination Using TaqMan MGB Probes

Assay Setup Recommendations

Note See the *Pre-Developed TaqMan Assay Reagents Allelic Discrimination Protocol* (P/N 4312214) for more information about how to set up and analyze allelic discrimination assays using TaqMan probes with a minor groove binder and a nonfluorescent quencher.

Follow Applied Biosystems recommendations for setting up allelic discrimination assays to ensure accurate allelic discrimination analysis:

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

Note Applied Biosystems recommends using eight replicates of each control assay. You can use fewer replicates if you do not intend to use the autocalling feature.

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

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

Reaction Recommendations

Applied Biosystems recommends the following guidelines for preparing reactions using custom TaqMan MGB probes:

- Keep probes protected from light, in the freezer, until you are ready to use them. Excessive exposure to light will damage the fluorescent probes.
- Dilute probes with TE buffer (10 mM TrisHCl, pH 8.0, 1 mM EDTA)
- Set up 25-µL reactions:
  - Up to 5 µL of genomic DNA (2 to 20 ng/µL) as the template
  - 200 nM of each probe
  - 900 nM of forward and reverse primers
  - 12.5 µL TaqMan Universal PCR Master Mix (P/N 4304437)

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

Note Because the results from allelic discrimination assays are not based on quantification, a reaction volume of 25 µL generally provides adequate performance for SDS autocalling.

Selecting a Thermal Cycler

Because the data acquired during PCR amplification are not necessary for analysis, use any of the following instruments for PCR amplifications:

- ABI PRISM 7700 Sequence Detection System

IMPORTANT ABI PRISM 7700 instruments must be running SDS software version 1.7 or later to analyze data from allelic discrimination assays using custom TaqMan MGB probes.

Note Using 7700 instruments 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 thermal cycling conditions if you choose to use other thermal cyclers.

Performing PCR

To perform the PCR:

Step	Action
1	Program the thermal cycling conditions. **IMPORTANT** Sufficient probe binding requires an Anneal/Extend temperature of 60 °C. **IMPORTANT** These conditions are optimized for use only with allelic discrimination assays using TaqMan MGB probes because of the probe modifications.
2	Set the reaction volume to **25** µL.
3	Load the reaction plate into the thermal cycler.
4	Begin thermal cycling.

	AmpErase UNG Activation	**AmpliTaq Gold Enzyme Activation**	**PCR**	
	HOLD	HOLD	CYCLE (35 cycles)	
			Denature	Anneal/ Extend
Temp.	50 °C	95 °C	92 °C	60 °C
Time	2 min	10 min	15 sec	1 min

Note See your instrument user's manual for help with programming your thermal cycler.

SDS Software Requirement

ABI PRISM 7700 instruments must be running SDS software version 1.7 or later to analyze data from allelic discrimination assays using custom TaqMan MGB probes. The SDS software version 1.7 upgrade is available from the Applied Biosystems Internet Web site (http://www.pebio.com).

Note For more information on version 1.7 of the SDS software, see the *Sequence Detection System Software Version 1.7 Update User Bulletin* (P/N 4317584).

Preparing a New Plate Read File

To prepare a new plate read file:

Step	Action
1	Launch SDS software.
2	Close the untitled window that appears.
3	Create a new plate read file with the following settings: **Plate Type** — Allelic Discrimination **Plate Format** — Standard Plate **Run** — Plate Read
4	Using the **Sample Type Setup** dialog box, set up sample types: a. Using the **Reporter** pop-up menu, select the reporter dye attached to the Allele 1 (AL1) probe. b. Using the **Reporter** pop-up menu, select the reporter dye attached to the Allele 2 (AL2) probe. c. Uncheck the box next to **Quencher**. TaqMan MGB probes contain a nonfluorescent quencher. d. Click **OK**. The dialog box closes, and the plate read window becomes active.
5	Label the wells with the sample types. ♦ Label wells serving as No Template Controls as **NTC**. ♦ Label wells serving as Allele 1 controls as **AL1**. ♦ Label wells serving as Allele 2 controls as **AL2**. ♦ Label wells containing Unknown samples as **UNKN**.
6	Save the changes to the plate read file.

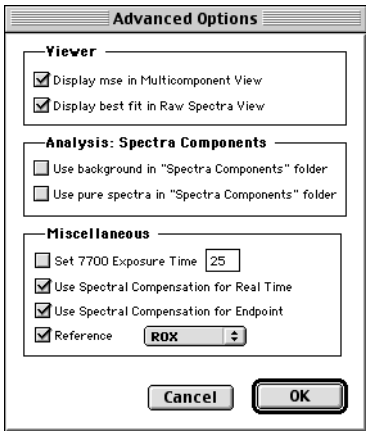
Running a Plate Read

To run a plate read:

Step	Action
1	Load the reaction plate. a. Place the reaction plate into the sample compartment. b. Pull the heated cover forward. c. Turn the knob clockwise to lower and secure the heated cover over the plate.
2	From the Setup view of a plate read file, click the **Show Analysis** button.
3	Click the **Post-PCR Read** button. The instrument will conduct a plate read, which should take about 10 seconds.
4	After the plate read is complete, save the plate read file.
5	Remove the reaction plate from the instrument. **IMPORTANT** To avoid PCR contamination with amplified product, do not remove the caps from the plate.
6	Discard the reaction plate after analyzing the plate read, when you are confident that the plate read was successful.

Setting Up the Plate Read Analysis

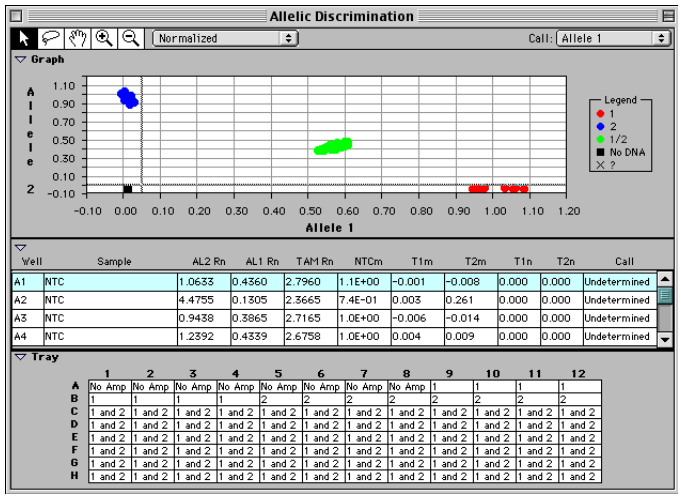
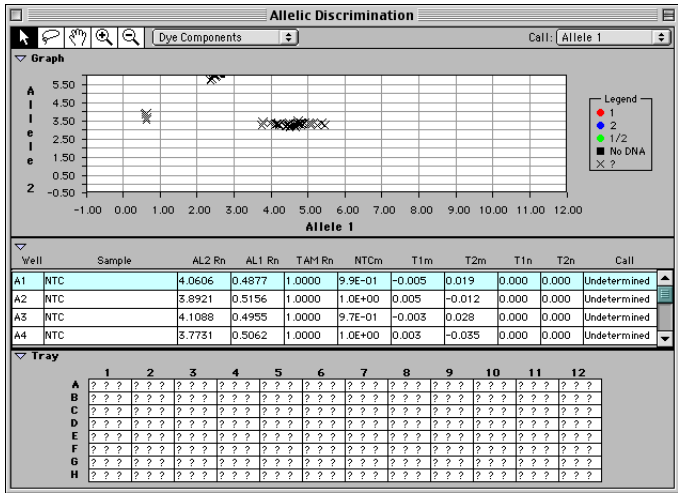
To set up the plate read analysis:

Step	Action
1	Launch SDS software.
2	Close the untitled window that appears.
3	Open the plate read file. a. From the **File** menu, select **Open Plate**. b. Choose a plate read file to analyze. c. Click **Open**. The file appears in the Setup view.
4	Click the **Show Analysis** button. The Analysis view appears.
5	From the **Instrument** menu, scroll to **Diagnostics**, and choose **Advanced Options**. The **Advanced Options** dialog box appears. 
6	Confirm that the **Use Spectral Compensation for Endpoint** check box is checked.
7	Click **OK**. The software displays a warning message requesting you to quit and relaunch the application. 
8	Click **OK**. **Note** It is not necessary to quit and relaunch the SDS software at this time.

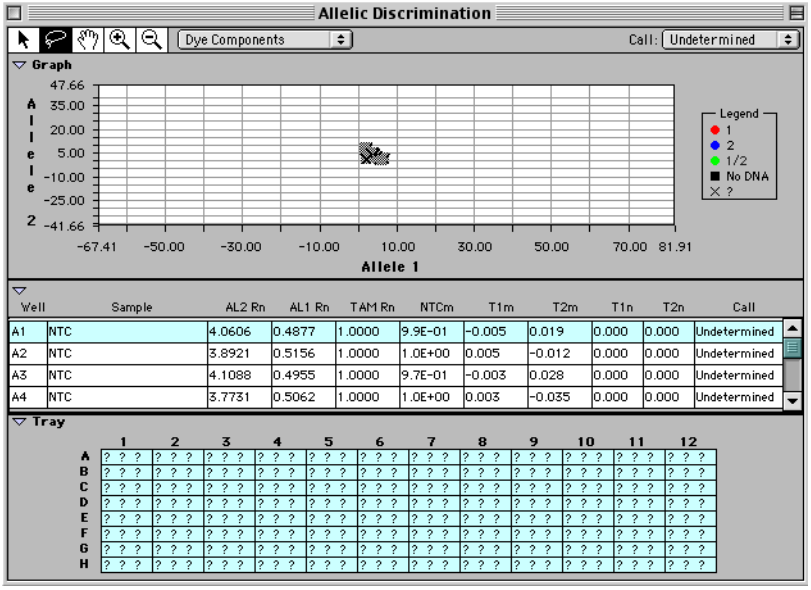
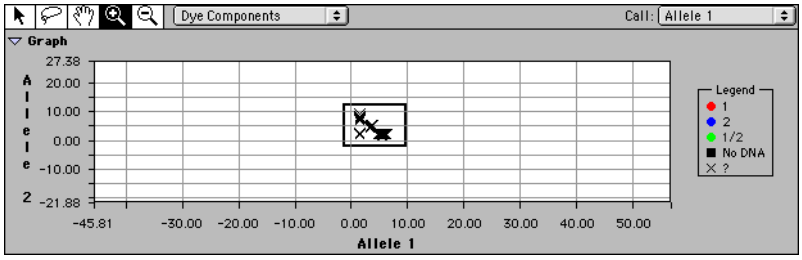
Analyzing a Plate Read

To analyze a plate read:

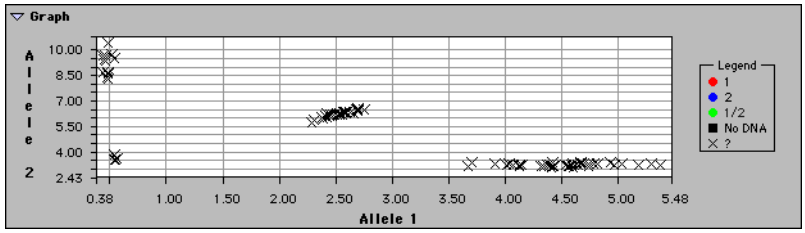
Step	Action
1	From the **Analysis** menu, select **Analyze**. An event log may appear. If the event log appears, close it.
2	From the **Analysis** menu, select **Allelic Discrimination.** The **Allelic Discrimination** window appears.

If the Allelic Discrimination window…	Then…
shows autocalls 	the results require no further modification.
does not show autocalls 	select **Dye Components** from the Dye pop-up menu and continue to the next step.

To analyze a plate read: *(continued)*

Step	Action
3	Zoom out until all crossmarks are visible in the graph. a. Select the "–" magnifying glass tool by clicking it.  b. Click the "–" magnifying glass on the graph to zoom out. **Note** Select the marks with the lasso tool to confirm that all appropriate wells are selected. 
4	Crop and zoom the crossmarks. a. Select the "+" magnifying glass tool by clicking it.  b. Click and drag the "+" magnifying glass on the graph to crop and magnify all marks. 

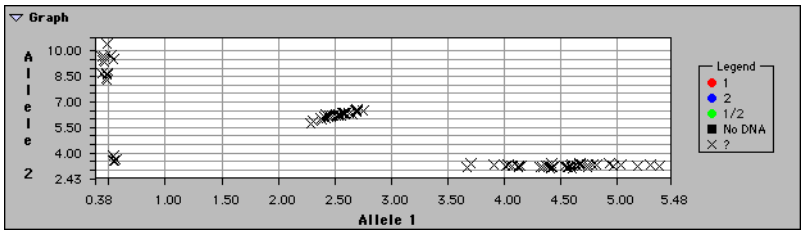
To analyze a plate read: *(continued)*

Step	Action
5	Repeat step 4 until the marks are clearly clustered in distinct regions of the graph. 
6	Manually call the allele types. a. Select the lasso tool by clicking it. b. Circle a cluster of marks with the lasso tool. The software highlights the corresponding wells in the **Tray** section of the **Allelic Discrimination** window.
7	Using the **Call** pop-up menu, designate the appropriate allele type for the selected cluster, based on the location of the graph. ♦ The software updates the symbols on the graph to match the **Legend**. ♦ The software updates the wells in the **Tray** section of the **Allelic Discrimination** window with the call.
8	Repeat step 6 and step 7 until you make all calls.

Cluster Variations

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, which could result from differences in initial DNA concentration.

The example below shows variation in clustering due to differences in the extent of PCR amplification.



Troubleshooting Information

See the *Pre-Developed TaqMan Assay Reagents Allelic Discrimination Protocol* (P/N 4312214) for more information about troubleshooting allelic discrimination assays using TaqMan probes with a minor groove binder and a nonfluorescent quencher.

Designing TaqMan and *turbo* TaqMan Assays for Allelic Discrimination

Overview

Primer Express version 1.5 also includes features for designing TaqMan and *turbo* TaqMan probes for allelic discrimination assays. This section describes how to design TaqMan probes and includes supplemental procedures for designing *turbo* TaqMan probes in instances where TaqMan probes exceed 30 nucleotides.

Note See *ABI Prism 7700 Sequence Detection Systems User Bulletin 6: turbo TaqMan Probes* (P/N 4311463) for more information for using *turbo* TaqMan probes.

TaqMan Probe Design Guidelines

The location of the polymorphism dictates the placement of the probe. Because mismatches near the end of probes tend not to be as disruptive to hybridization, Applied Biosystems generally recommends designing probes so that the polymorphic site is near the center of the probe.

Guidelines for Designing TaqMan Probes

- Use the VIC and FAM reporter dyes to label the allelic discrimination probes.
- Avoid runs of an identical nucleotide. This is especially true for guanosine, where runs of four or more should be avoided.
- The Primer Express® software-estimated T_m for the probes should be between 65–67 °C.
- The 5´ end of a probe cannot be a guanosine residue. A guanosine residue adjacent to the reporter dye will quench the reporter fluorescence somewhat, even after cleavage.
- Position the polymorphic site approximately in the middle third of the sequence.

Loading the Sequence

To import a DNA file for generating a list of potential primers and probes:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Launch the Primer Express software.</td></tr>
<tr><td>2</td><td>Import a DNA sequence for designing probes and primers.
<table>
<tr><th>To design a probe and primers from…</th><th>Then…</th></tr>
<tr><td>a DNA file</td><td>a. From the File menu, scroll to the New submenu, and select TaqMan® Probe & Primer Design.
A TaqMan® Probe document appears.
b. Click Import DNA File.
c. Locate and select a DNA file in the browser.
d. Click Open.
The software loads the sequence and displays it in the Sequence tab.</td></tr>
<tr><td>an existing TaqMan® Probe document (located in the Document Archive)</td><td>a. From the File menu, select Open.
The Document Archive dialog box appears.
b. Double-click the document to load, or select the sequence and click Open.
The software loads the sequence and displays it in the Sequence tab.</td></tr>
<tr><td>a text document or GenBank sequence</td><td>a. Select the sequence from the text document or the navigator window.
b. From the Edit menu, select Copy.
c. From the File menu, scroll to the New submenu, and select TaqMan® Probe & Primer Design.
A TaqMan® Probe document appears.
d. From the Edit menu, select Paste.
The software pastes the nucleotide sequence into the Sequence tab.
Note Edit the GenBank sequence before saving it as a Primer Express document.</td></tr>
</table></td></tr>
<tr><td>3</td><td>Click the following checkboxes for primer selection:
♦ Double Stranded
♦ Limit 3´ G+C
 

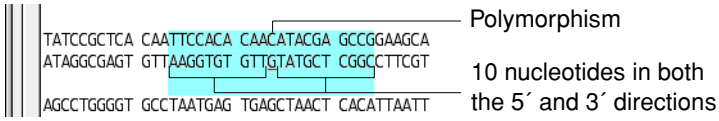
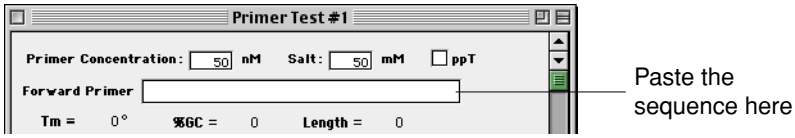
Select these checkboxes
The sense and antisense sequences appear on the Sequence tab.</td></tr>
<tr><td>4</td><td>Label the polymorphism within the sequence using the Line tool:
a. From the Tools palette, click the Line tool.
b. Select the polymorphic sequence.
The software automatically underlines the polymorphism.</td></tr>
</table>

To import a DNA file for generating a list of potential primers and probes: *(continued)*

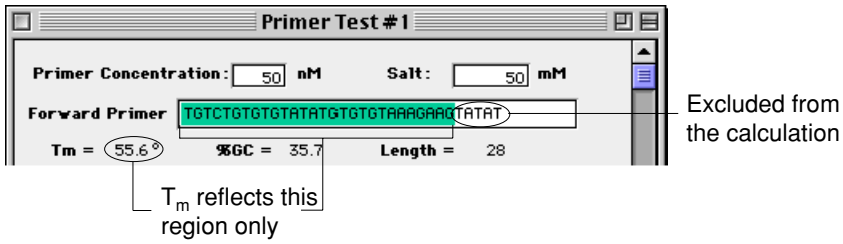
Step	Action
5	Following steps 1-4, import the sequence for the other allele into a separate **TaqMan® Probe** document.

Designing a TaqMan Probe for Allele 1

To design a probe for Allele 1:

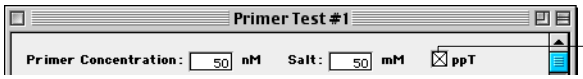
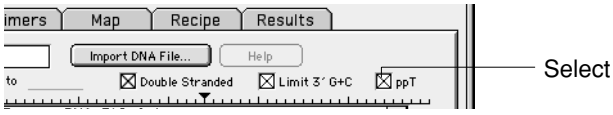
Step	Action
1	From the **TaqMan® Probe** document for Allele 1, click the **Sequence** tab. The **Sequence** tab comes to the front of the dialog box.
2	Select a region containing potential probe sequences. a. Highlight the polymorphism and approximately 10 nucleotides in both the 5´ and 3´ directions.  b. From the **Edit** menu, select **Copy**. c. From the **File** menu, scroll to **New**, and select **Primer Test Document**. A **Primer Test** document appears. d. Click the **Forward Primer** text box. e. From the **Edit** menu, select **Paste**. Primer Express copies the probe sequence into the **Primer Test** document and calculates the T_m using the nearest-neighbor algorithm (Rychlik *et al.*, 1990). 
3	Test potential probe sequences in the complementary strand. a. Return to the **Sequence** tab in the **TaqMan® Probe** document for Allele 1. The polymorphic sequence and surrounding nucleotides should still be selected. b. From the **Edit** menu, select **Copy Complement**. c. Return to the **Primer Test** document, and click the **Reverse Primer** text box. d. From the **Edit** menu, select **Paste**. Primer Express copies the complementary sequence into the test document and calculates the T_m of the oligonucleotide.
4	For easier identification, label the polymorphism within each probe sequence. a. Select the polymorphism within the sequence in the **Probe 1** text box. b. Press the key corresponding to the letter of the polymorphic base. Primer Express replaces the uppercase letter of the base with a lowercase letter. c. Repeat steps a and b for the sequence in the **Probe 2** text box.

To design a probe for Allele 1: *(continued)*

Step	Action
5	Highlight potential probe sequences until you identify a probe that meets the guidelines listed in "TaqMan Probe Design Guidelines" on page 17. **Note** Remember to look at potential probes from the complementary sequence. **IMPORTANT** Add/remove nucleotides evenly to/from both ends of the probe so that the polymorphic site remains within the center. **IMPORTANT** Primer Express calculates the T_m for only the *highlighted* nucleotide sequence and excludes residues outside the selected region from the calculation. 
6	From the **Edit** menu, select **Trim**. The software eliminates all but the selected nucleotide sequence (in blue) from the probe test document.
7	Count the number of nucleotides in the probe sequence. **If the probe is…** / **Then…** > 30 nucleotides: design a *turbo* TaqMan probe as explained in "Designing a turbo TaqMan Probe" on page 21. ≤ 30 nucleotides: a. From the sequence selected in step 8, identify the nucleotide strand (sense or antisense) with more cytosine than guanine residues. (This strand is the probe sequence of interest.) **IMPORTANT** The 5´ end of the probe must not be a guanine residue. b. Copy and paste the final sequence for the Allele 1 probe into a text document for ordering. c. Go on to the next step.
8	Select the probe sequence in the **Sequence** tab. a. From the **Windows** menu, select the **TaqMan® Probe** document. b. From the Primer Express **Tools** palette, click the probe tool. c. Select the edited probe sequence on the **Sequence** tab. Primer Express highlights the probe sequence in green. d. Go to "Designing the Allele 2 Probe" on page 22.

Designing a *turbo* TaqMan Probe

To design a *turbo* TaqMan probe:

Step	Action
1	From the **Windows** menu, select the **Primer Test** document.
2	Click the **ppT** checkbox. **Note** The **ppT** checkbox adjusts the algorithm T_m calculation for the effect of thymine substitutions in *turbo* TaqMan probes.  Primer Express recalculates the adjusted T_m of the probe sequence based on thymine substitutions.
3	Compare the T_m of the probes to the desired T_m of 65–67 °C. **If the probe T_m is…** / **Then…** within 65–67 °C: go to the next step. greater than 67 °C or less than 65 °C: a. While observing the guidelines on page 17, highlight more or fewer nucleotides until you achieve the optimal T_m of 65–67 °C. **IMPORTANT** The 5´ end of the probe must not be a guanine residue. **IMPORTANT** Add/remove nucleotides evenly to/from both ends of the probe so that the polymorphic site remains within the center of the probe. b. From the **Edit** menu, select **Trim**.
4	Copy and paste the final sequence for the Allele 1 probe into a text document for ordering.
5	From the **Windows** menu, select the **TaqMan® Probe** document for Allele 1.
6	Click the **ppT** checkbox. **Note** The **ppT** checkbox adjusts the T_m calculation algorithm for the effect of thymine substitutions in *turbo* TaqMan probes. 
7	Select the probe sequence in the **Sequence** tab. a. From the Primer Express **Tools** palette, click the **Probe** tool. b. Select the edited probe sequence. Primer Express highlights the probe sequence in green.

Designing the Allele 2 Probe

To design the probe for Allele 2:

Step	Action
1	In the **TaqMan® Probe** document for Allele 2, click the **Sequence** tab. The **Sequence** tab appears.
2	Select a region containing potential probe sequences. a. Highlight the polymorphic sequence and approximately 10 nucleotides in both the 5´ and 3´ directions. b. Copy the sequence for the Allele 2 probe. **If the Allele 1 probe is on the…** / **Then go to the Edit menu and select…** sense strand, / **Copy**. antisense (complementary) strand, / **Copy Complement.** **IMPORTANT** Both probe sequences in the allelic discrimination assay must come from same strand, otherwise the two probes will hybridize to each other. c. Return to the **Primer Test** document, and click the **Primer** text box that does not contain the Allele 1 probe you selected. d. From the **Edit** menu, select **Paste**. Primer Express copies the appropriate sequence into the **Primer Test** document and calculates the T_m of the oligonucleotide.
3	For easier identification, label the polymorphism within the Allele 2 probe sequence. a. Select the polymorphism within the Allele 2 sequence in the **Primer** text box. b. Press the key corresponding to the letter of the polymorphic base. Primer Express replaces the uppercase letter of the base with a lowercase letter.
4	Highlight potential probe sequences until you identify a probe that meets the guidelines listed in “TaqMan Probe Design Guidelines” on page 17.
5	With the desired probe sequence highlighted, select **Trim** from the **Edit** menu. The software eliminates all but the selected nucleotide sequence (in blue) from the **Primer Test** document.
6	Copy and paste the final sequence for the Allele 2 probe into a text document for ordering.

Designing Primers

After selecting probes for the assay, choose primers based on the guidelines below. Consequently, amplicons are usually 75–150 bp. By limiting the parameters for amplicon design (such as amplicon size), it is possible to run all reactions with a single reaction buffer (TaqMan® Universal PCR Master Mix (P/N 4304437)) and a single thermal cycling protocol.

Primer Design Guidelines

- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more should be avoided.
- Set a Primer Express software–estimated T_m for the primers of 58–60 °C.
- Keep the guanine + cytosine content within 20–80%.
- Make sure the last five nucleotides at the 3´ end contain no more than two guanine + cytosine residues.
- Place the forward and reverse primers as close as possible to the probe without overlapping it.

Procedure

IMPORTANT The probe has been designed with Probe tool and is shown in green.

To select primers for the allelic discrimination assay:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Ensure that the Limit 3´ G+C checkbox from the Sequence tab is selected.</td></tr>
<tr><td>2</td><td>Select Find Primers/Probes Now from the Options menu.

IMPORTANT If you designed turbo TaqMan probes, both ppT checkboxes found in the TaqMan® Probe document and the Primer Test document must be checked before activating the Find Primers/Probes Now function. The software will not find primers and probes for the correct T_m if one or both of the checkboxes remain inactive.

<table>
<tr><th>If the software…</th><th>Then…</th></tr>
<tr><td>finds acceptable primers</td><td>a. Click the Primers tab.
b. Select a primer sequence from the list that satisfies all requirements listed above.</td></tr>
<tr><td>cannot find acceptable primers</td><td>design the forward and reverse primers manually according to the guidelines listed above.</td></tr>
</table>
</td></tr>
<tr><td>3</td><td>Select a set of primers from the list that will produce the shortest amplicon while satisfying the guidelines above.</td></tr>
<tr><td>4</td><td>Copy and paste the final primer sequences into a text document for ordering.</td></tr>
<tr><td>5</td><td>Select Save from the File menu, and assign a name to the file to save the results.</td></tr>
</table>

Obtaining and Installing Primer Express Version 1.5

System Requirements

Your Macintosh computer must meet the minimum following hardware and software requirements to install version 1.5 of the Primer Express software:

Macintosh Model	7200	4400	G3	G4
Macintosh OS Version	7.6.1	7.6.1/8.0	8.5.1/8.6	9.0
Memory Configuration	32 MB	32 MB	64 MB	64 MB
Virtual Memory Configuration	OFF	OFF	OFF	OFF
Storage Requirements	40 MB Free Disk Space			
Additional Hardware	CD-ROM Drive			

IMPORTANT Because of problems acknowledged by Apple regarding the Macintosh OS version 8.1, Applied Biosystems does not support Mac OS 8.1 for any Macintosh computer.

Obtaining Primer Express Version 1.5

The Primer Express software version 1.5 is available from the Applied Biosystems Internet Web site (http://www.pebio.com/ab/about/pcr/sds/software.html). Before downloading the upgrade, make sure that your computer meets or exceeds the minimum requirements specified above.

Note If necessary, the Primer Express software version 1.5 update can be downloaded from the Applied Biosystems Web site to a Windows®-compatible computer, then transferred to a Macintosh computer for use.

To obtain the update package:

Platform	Procedure
Macintosh	Click the **Primer Express 1.5** hyperlink from the Web site. The Macintosh downloads the compressed Primer Express 1.5 **.hqx** file. **Note** If unable to find the compressed Primer Express 1.5 **.hqx** file once downloaded, you can locate it using **Find (⌘–F)** option from the **File** menu.
Windows	a. Click the **Primer Express 1.5** hyperlink from the Web site. The internet browser prompts you with download options. b. Follow the instructions for downloading the software. The computer downloads the compressed Installer file from the Web site. c. Transfer the compressed Primer Express 1.5 **.hqx** file to the desktop of the Macintosh computer to which you wish to install the software. **Note** If unable to find the compressed Primer Express 1.5 **.hqx** file once downloaded, you can locate it using the **Find** utility from the **Start** menu.

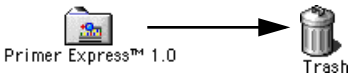
Extracting the Compressed Installer

IMPORTANT You must have Aladdin Stuffit Expander™ to use the Primer Express 1.5 Update from the Applied Biosystems Web site. The latest version of Stuffit Expander is free and available on the Aladdin Systems, Inc. Web site (http://www.aladdinsys.com).

Once downloaded, the Primer Express 1.5 Installer file must be decompressed for use. To extract the compressed Installer program, drag and drop the compressed .hqx file onto the Aladdin Stuffit Expander software icon. Stuffit Expander should extract the compressed file entitled “Install Primer Express™ 1.5.” If the installation fails, download the latest version of Stuffit Expander and re-attempt the extraction.

Installing the Software

After downloading and extracting the Primer Express version 1.5 update, follow the instructions below to install the software onto your computer.

Step	Action
1	Save the Primer Express Archive file from the existing **Primer Express™ 1.0** software folder. a. Locate and open the existing **Primer Express™ 1.0** software folder. b. Drag the Primer Express Archive file onto the desktop.  c. Close the **Primer Express™ 1.0** software folder.
2	Drag the **Primer Express™ 1.0** folder into the **Trash**. 
3	Load the **Primer Express Version 1.5** CD onto your CD-ROM drive or download the installer file from the internet (see “Obtaining Primer Express Version 1.5” on page 24). The icon for the Primer Express CD appears on your computer desktop and a window displaying the Primer Express Version 1.5 Installer opens.
4	Double-click the Installer icon and follow the instructions given by the software. During the installation, the Primer Express 1.5 Installer completely replaces the program files for the existing version of the Primer Express software. The update is completely automated and does not require you to make any further modifications to the existing files or to reboot your computer.
5	When the installation is complete, click **Quit** to exit the installer.
6	Drag the Primer Express Archive file (from step 1) into the new **Primer Express™ 1.5** software folder.

Using Primer Express Version 1.5 for the First Time

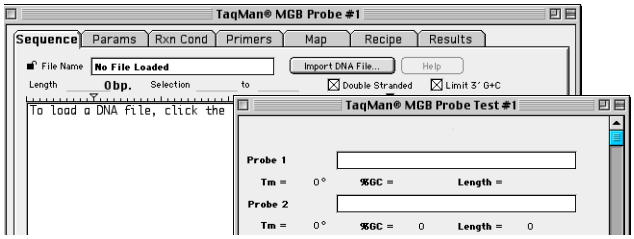
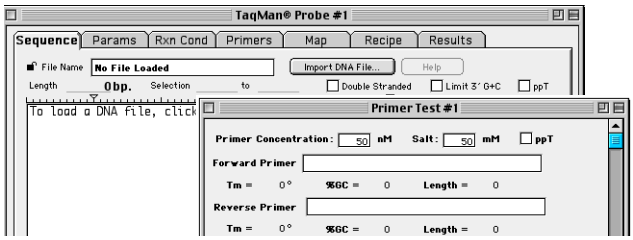
IMPORTANT After the installation you will be required to enter your personalized registration code to unlock the updated version of Primer Express. The registration code is located on the diskette sleeve in which the original CD-ROM arrived. If you do not have your Registration Code, please contact your local Applied Biosystems Sales and Service Office for assistance.

To run Primer Express Version 1.5 for the first time:

Step	Action
1	Double-click the **Primer Express 1.5** icon. The software prompts you for a registration code.
2	Enter your registration code and click **OK**. Primer Express prompts you to identify a Primer Express Archive file.
3	Select the Primer Express archive file you saved in the installation procedure.
4	From the **Edit** menu, select **Preferences**. Reconfigure the Primer Express software preferences as needed.

Summary of New Features and Modifications

Primer Express Version 1.5 features the following improvements and modifications:

Feature	Description
Support for designing TaqMan MGB probe assays	Primer Express Version 1.5 features a set of probe and primer functions specifically for designing and testing TaqMan MGB probe assays.  The function of these dialog boxes is demonstrated in “Designing TaqMan MGB Assays for Allelic Discrimination” on page 5.
Support for designing *turbo* TaqMan probe assays	The existing TaqMan probe and primer design documents were redesigned to incorporate a function for designing and testing *turbo* TaqMan probe assays.  The function of these dialog boxes is demonstrated in “Designing TaqMan and turbo TaqMan Assays for Allelic Discrimination” on page 17.
Y2K compliance	Primer Express version 1.5 is Year 2000 compliant.
Increased program stability	The program code has been revised to provide additional stability.

Outstanding Issues

Primer Express Version 1.5 contains the following outstanding issues:

- The Save As option allows you to save files with the same name.
- The application allows you to save data from the Results Page in the Results Archive with the same name.
- While in the Open dialog box, the application allows you to save results with an incorrect date.
- The application quits unexpectedly when the Close option is used with the Batch Processing document.

Purchasing and Contact Information

Over the Web or Via E-Mail

To receive more information about any of the products mentioned in this bulletin:

To reach us…	Contact the oligonucleotide manufacturing department at…
over the internet	one of the Web addresses in the following table. **If you have…** / **Go to…** an existing account → http://oligos.pebio.com never used the Applied Biosystems oligonucleotide department Web site → http://oligos.pebio.com/demo The Applied Biosystems Web site login appears. a. Click the **Username** text field and type **guestuser**. b. Click the **PIN** text field and type **3455224.** c. Click **Login**.
by e-mail	OligosUS@pebio.com

Ordering TaqMan Probes and Primers

The following table contains a list of the available primer and probe configurations:

Description	Quantities (pmol)	Part Number
TaqMan MGB probe ♦ 5´-fluorescent label: 6-FAM, VIC, or TET ♦ 3´-nonfluorescent quencher ♦ Minor groove binder	15,000–25,000	4316033
	50,000–100,000	4316032
turbo TaqMan probe ♦ 5´-fluorescent label: 6-FAM, VIC, or TET ♦ 3´-label: TAMRA ♦ Thymine residues replaced with 5-propyne-2´-deoxyuridine	5000–6000	4310390
	15,000–25,000	4310392
	50,000–100,000	4310394
TaqMan probe ♦ 5´-fluorescent label: 6-FAM, VIC, or TET ♦ 3´-label: TAMRA	5000–6000	450025
	15,000–25,000	450024
	50,000–100,000	450003
Sequence detection primer	4000 (minimum)	4304970
	40,000 (minimum)	4304971
	130,000 (minimum)	4304972

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.

Kuimelis, R.G., Livak, K.J., Mullah, B., and Andrus, A. 1997. Structural analogues of TaqMan probes for real-time quantitative PCR. *Nucleic Acids Symp. Ser.* 37:255–256.

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.

Livak, K.J., Flood, S.J.A., Marmaro, J., and Mullah, K.B., inventors; Applied Biosystems (Foster City, CA), assignee. 2 Mar. 1999. Hybridization assay using self-quenching fluorescence probe. United States patent 5,876,930.

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

Rychlik, W., Spencer, W.J., and Rhoads, R.E. 1990. Optimization of the annealing temperature for DNA amplification *in vitro* [published erratum appears in Nucleic Acids Res 1991 Feb 11;19(3):698]. *Nucleic Acids Res.* 18:6409–6412.

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