# User Bulletin

## Applied Biosystems 7900HT Fast Real-Time PCR System

September 2, 2004

**SUBJECT: Performing Fast Gene Quantification**

### In This User Bulletin

This user bulletin covers:

- Procedures for performing fast gene quantification (page 3)
- Maintenance procedures (page 10)
- Troubleshooting (page 17)

### System Overview

Fast gene quantification involves running TaqMan® Gene Expression Assays on the Applied Biosystems 7900HT Fast Real-Time PCR System with Fast 96-Well Block Module in about 35 minutes.

### System Requirements

- 7900HT System with fast hardware and software:
  - Fast 96-Well Block Module (thermal cycler block)
  - Fast 96-Well Plate Adapter
  - Upgraded 48V-650W power supply
  - Sequence Detection Systems Software v2.2.1
- Fast reagents and plastics:
  - 7900HT System Fast 96-Well Spectral Calibration Kit (PN 4351653)
  - Optical 96-Well Fast Thermal Cycling Plate with Barcode (code 128) (PN 4346906)
  - TaqMan® RNase P Fast 96-Well Instrument Verification Plate (PN 4351979)
  - TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG (PN 4352042)

### Getting Started

Before you perform fast gene quantification, make sure that:

- You are familiar with the safety information in the *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684)
- SDS software v2.2.1 is installed.
- The Fast 96-Well Block and the Fast 96-Well Plate Adapter are installed (page 10).
- A background run has been performed in the last 6 months (page 10).
- A pure dye run has been performed in the last 6 months (page 12).
- Instrument performance has been verified (page 15).

Applied Biosystems

## Fast Gene Quantification Products

| Product | Applied Biosystems Part Number |
|---|---|
| Applied Biosystems 7900HT Fast Real-Time PCR System with Fast 96-Well Block Module | Contact your local Applied Biosystems sales office. |
| 7900HT System Fast Service Upgrade | 4351412 |
| 7900HT System Fast 96-Well Spectral Calibration Kit | 4351653 |
| Optical 96-Well Fast Thermal Cycling Plate with Barcode (code 128), 20 plates | 4346906 |
| Sequence Detection Systems Software v2.2.1 | 4352620 |
| TaqMan® Fast Reagents Starter Kit | 4352407 |
| TaqMan® RNase P Fast 96-Well Instrument Verification Plate | 4351979 |
| TaqMan® Fast Universal PCR Master Mix (2X), No AmpErase® UNG | 4352042 |
| TaqMan® Gene Expression Assays | 4331182 |

## Related Documents

| Document | Applied Biosystems Part Number |
|---|---|
| *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* | 4351684 |
| *Applied Biosystems 7900HT Fast Real-Time PCR System Quick Reference Card: Performing Fast Gene Quantification* | 4351892 |
| *Real-Time PCR Systems Chemistry Guide* | 4348358 |
| *TaqMan® Fast Universal PCR Master Mix (2X) Protocol* | 4351891 |
| *TaqMan® Gene Expression Assays Protocol* | 4333458 |

# Performing Fast Gene Quantification

## Procedural Overview



## Running Assays Using Fast Thermal Cycling Conditions

Run assays using Fast thermal cycling conditions.

- The performance of Fast thermal cycling and the TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG, has been verified for quantitative applications only and not for endpoint applications, such as allelic discrimination.
- The performance of Applied Biosystems TaqMan Gene Expression Assays and Custom TaqMan® Gene Expression Assays has been verified using the default Fast thermal cycling conditions and the TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG.
- It is expected that the vast majority of custom 5′ nuclease quantification assays designed with the Applied Biosystems Assay Design Guidelines will provide comparable performance when run using the default Fast thermal cycling conditions and the TaqMan Fast Universal PCR Maser Mix (2×), No AmpErase UNG (as compared to running the standard thermal cycling conditions and the TaqMan® 2× Universal PCR Master Mix). If you encounter poor performance, see “Troubleshooting” on page 17.
- When performing multiplex applications (when more than one target is amplified in a single tube), it may be necessary to perform some assay reoptimization. Before performing any multiplex applications, see the troubleshooting information on page 18 for further information.

## Starting the Software

1. Do one of the following:
   - Double-click (SDS 2.2.1) on the computer desktop.
   - Select Start > **All Programs** > **Applied Biosystems** > **SDS 2.2.1** > **SDS 2.2**.1
2. (Database Only) If you use an SDS Enterprise Database on your local area network, the software prompts you to log in. In the Login dialog box, enter your user name and password, then click **OK**.

   **Note:** See the *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684) for more information on assigning and modifying user accounts.

At startup, the software attempts to establish communication with the 7900HT instrument. If the connection is successful, the software displays Connected to 'PlateName' (Connected) in the status bar when a plate document is open.

## Default Thermal Cycler Mode

The default thermal cycler mode is set according to whether the computer is connected directly to the 7900HT instrument (see below) or the computer is offline (see page 4).

### Computer is Connected Directly to the 7900HT Instrument

When the computer is connected directly to the 7900HT instrument, the SDS software detects the thermal cycler block type installed and sets the appropriate default thermal cycler mode for new plate documents:

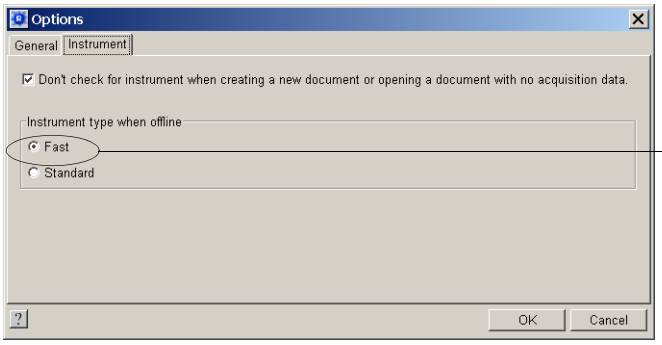
| Thermal Cycler Block Type Detected | Default Thermal Cycler Mode Selected | Available Thermal Cycler Modes |
|---|---|---|
| Fast block | Fast | • Fast<br>• Standard<br>• 9600 Emulation |
| Standard block | Standard | • Standard<br>• 9600 Emulation |

### Computer is Offline

If your computer is offline and is not connected directly to the 7900HT instrument, you can set the default thermal cycler mode for new plate documents.

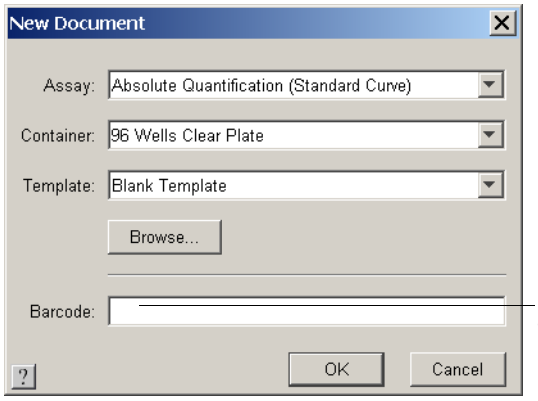
**IMPORTANT!** Plate documents created using the Fast thermal cycler mode can be run only when the Fast 96-Well Block is installed.

1. Select **Tools > Options**, then click the **Instrument** tab.
2. For Instrument type when offline, select **Fast**.



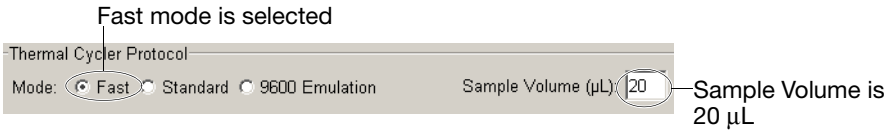

## Creating and Setting Up a New Plate Document

1. Create a plate document for fast gene quantification:
   a. From the SDS software, click (or select **File > New**).
   b. Complete the New Document dialog box with the following settings, then click **OK**:

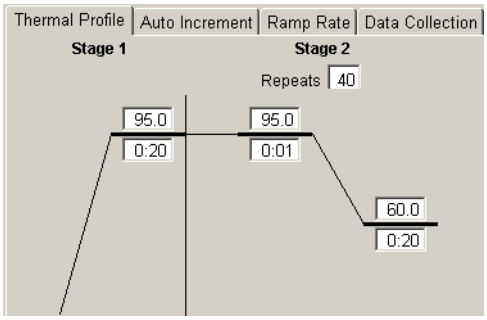



2. Select the **Instrument** tab, then verify the default thermal cycler protocol settings:

   a. Verify that the Fast mode is selected and the Sample Volume is 20 µL.

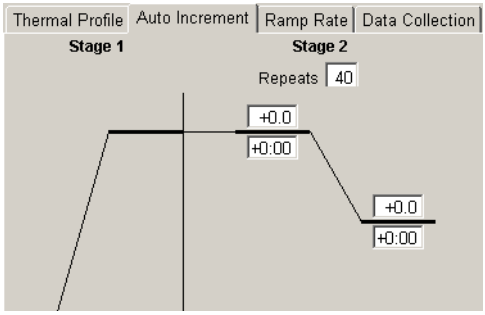



   b. Verify the default thermal profile settings:

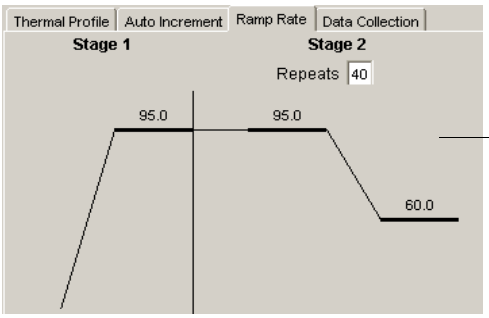



**Note:** If you choose to use AmpErase® UNG, you need to add an UNG activation step to the beginning of the thermal profile: 50 °C for 2 minutes.

   c. Select the **Auto Increment** tab, then verify the default settings:

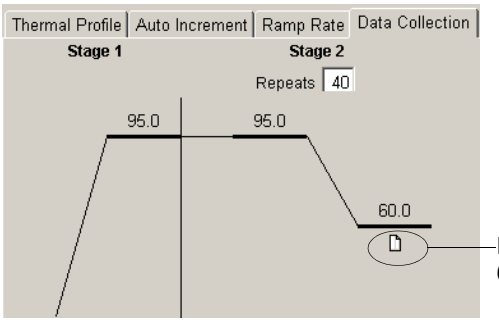


d. Select the **Ramp Rate** tab, then verify the default settings:




Ramp rates cannot be modified with the Fast mode selected

e. Select the **Data Collection** tab, then verify the default settings:




Data collection occurs only during the 60 °C extension step

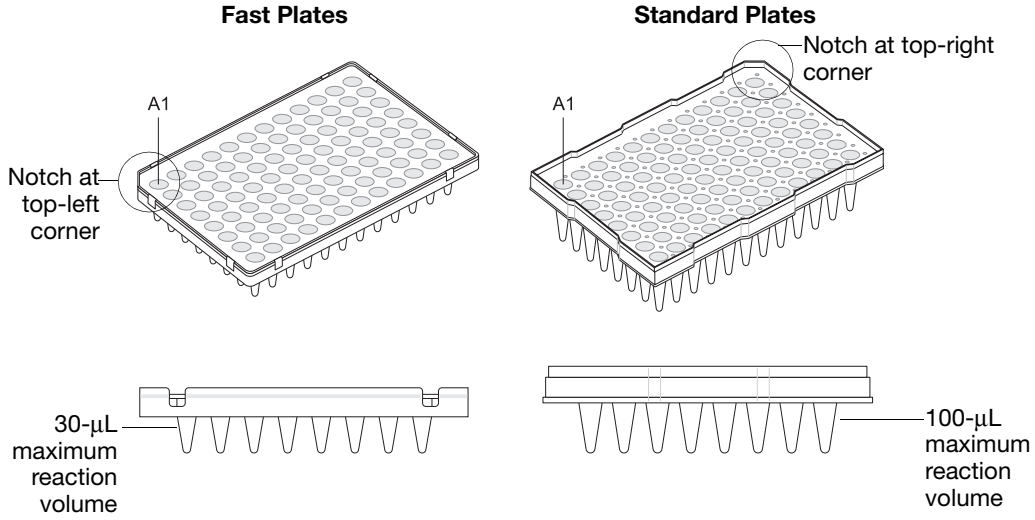
3. Complete the plate document setup as described in the *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684):
   a. Create detectors (the first time only).
   b. Copy detectors to the plate document.
   c. Apply detectors and tasks for samples.
   d. Add sample names.

   **IMPORTANT!** Set up the plate document so that it corresponds exactly to the arrangement of samples in the wells of the reaction plate.

4. Save the plate document:
   a. Click  (or select **File** > **Save As**).
   b. For Files of Type, select **SDS 7900HT Document (*.sds)**.
   c. Navigate to where you want to save the plate document file.
   d. In the File Name field, enter a name for the plate document.
   e. Click **Save**.

## Fast vs. Standard Plates

**IMPORTANT!** Make sure that you use the Optical 96-Well Fast Plate for fast gene quantification. Standard plates will not function properly and may be crushed when using the Fast 96-Well Block.




## Preparing Plates

For safety and biohazard guidelines, refer to the "Safety" section in the *TaqMan® Fast Universal PCR Master Mix (2×) Protocol* (PN 4351891). For all chemicals in **bold** type, read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

For more information on setting up the reactions, see the *TaqMan® Fast Universal PCR Master Mix (2×) Protocol* (PN 4351891).

**IMPORTANT!** The TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG, does provide a hot-start capability. However, to ensure optimal results, Applied Biosystems recommends running the reaction plate as soon as possible after completing the reaction setup. If you cannot run a reaction plate within 2 hours after completing the reaction setup, refrigerate or freeze the reaction plate until you can load and run it on the 7900HT instrument.

1. Prepare the reaction mix for each sample (for four 20-µL reactions):

| Component | Volume (µL) for Four 20-µL Reactions[a] |
|---|---|
| TaqMan® Gene Expression Assay (20×) | 5.0 |
| cDNA template (10 to 100 ng of cDNA) + RNase-free water | 45.0[b] |
| **TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG** | 50.0 |
| **Total Volume** | 100.0 |

a. Volumes are calculated for five reactions to provide excess volume for the loss that occurs during reagent transfers.

b. If you choose to use UNG, decrease the volume of cDNA template and RNase-free water to 8.8 µL per 20-µL reaction and add 0.2 µL of UNG stock (1 U/µL).

2. Cap the tube(s), mix by gentle inversion, then centrifuge briefly.

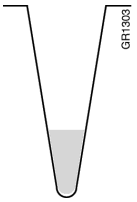
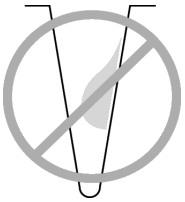
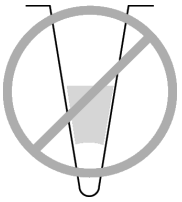
3. Transfer 20 µL of reaction mix to wells of an Optical 96-Well Fast Plate.

   **Note:** The arrangement of the reactions (samples and assays) on the plate must match the arrangement (sample names and detectors/markers) in the plate document used for the run.

4. Seal the reaction plate with an optical adhesive cover:
   - ABI PRISM™ Optical Adhesive Cover, quantity 100 (PN 4311971)
   - Optical Adhesive Cover, quantity 25 (PN 4360954)

5. Centrifuge the plate briefly.

6. Verify that each reaction is positioned in the bottom of the well.

| Correct Position | Incorrect Positions | |
|---|---|---|
|   The reaction is positioned correctly in the bottom of the well. |  The reaction lies on the side wall because the plate was not centrifuged. |  An air bubble lies at the bottom of the well because the plate was not centrifuged with sufficient force or for sufficient time. |

## Running Plates

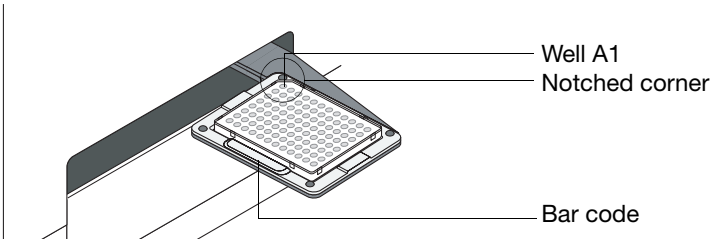
### Running One Plate

To run the plate, use the SDS software.

**IMPORTANT!** The TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG, does provide a hot-start capability. However, to ensure optimal results, Applied Biosystems recommends running the reaction plate as soon as possible after completing the reaction setup. If you cannot run a reaction plate within 2 hours after completing the reaction setup, refrigerate or freeze the reaction plate until you can load and run it on the 7900HT instrument.

1. If not already opened, open the plate document in the SDS software.
2. Select the **Instrument** tab of the plate document, then select the **Real-Time** tab.
3. Check if   ) (Connected) is displayed in the status bar. If the software is not connected to the instrument, click **Connect**.
4. If the instrument tray is inside the instrument, click **Open/Close** to rotate the instrument tray to the OUT position.
5. Verify that the Fast 96-Well Plate Adapter is installed in the instrument tray.
6. Place the prepared reaction plate into the instrument tray so that:
   - Well A1 and the notched corner are in the top left corner.

- The bar code is toward the front of the instrument.




7. In the Real-Time tab of the Instrument tab, click **Start**.

   Before beginning the run, the instrument pauses approximately 40 seconds to perform a laser check. If the instrument has just been powered up, it may then pause up to 15 minutes more to allow the heated cover to heat to the appropriate temperature.

   During the run, the SDS software collects and saves the run data and the Real-Time tab displays the instrument status and run progress.

8. When the run is complete and the Run Complete dialog box opens, click **OK** to close the dialog box, click **Open/Close**, then remove the plate from the instrument tray.

### Running Multiple Plates

**IMPORTANT!** Performance of the Optical 96-Well Fast Thermal Cycling Plates with the Zymark® Twister Microplate Handler (the Automation Accessory) has not been verified. Therefore, when running multiple Optical 96-Well Fast Plates on the 7900HT instrument, Applied Biosystems recommends that you load and unload the plates manually.

## Analyzing Results

For instructions on analyzing the results, see the *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684).

The general process for analyzing the results from gene expression assays:

1. View the amplification plots.
2. Set the baseline and threshold values.
3. Use the standard curve method or the relative quantification ($\Delta\Delta C_T$) method to analyze your data.

# Maintaining the 7900HT Instrument

## Overview

This section provides information on:

- Instrument procedures (page 10)
- Performing a background run (page 10)
- Performing a pure dye run (page 12)
- Verifying instrument performance (page 15)

## Instrument Procedures

For the following instrument procedures, perform them as described in the *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684), with any changes as noted below:

- Installing the Fast 96-Well Block and Fast 96-Well Plate Adapter

  **IMPORTANT!** When you install the Fast 96-Well Block, make sure that you also install the Fast 96-Well Plate Adapter.

- Decontaminating the thermal cycler block

  **IMPORTANT!** For the Fast 96-Well Block, use 100 µL of 10% bleach solution to decontaminate the block and then rinse with three treatments of 100 µL deionized water.

**WARNING** **CHEMICAL HAZARD. Sodium hypochlorite (bleach)** is a liquid disinfectant that can be corrosive to the skin and can cause skin depigmentation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

## Performing a Background Run

Applied Biosystems recommends performing a background run every 6 months.

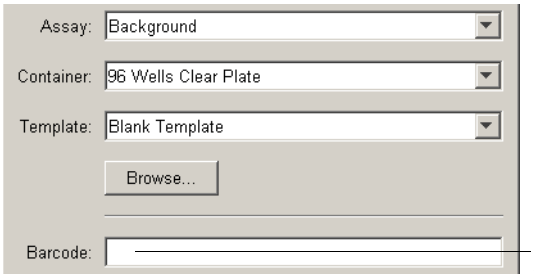
### Materials Required

- Background plate included in the 7900HT System Fast 96-Well Spectral Calibration Kit (PN 4351653)
- Tabletop centrifuge with 96-well plate holders

### Procedure

1. Prepare the background plate:
   a. Remove the background plate from the freezer and allow it to thaw to room temperature.
   b. Centrifuge the background plate briefly.
2. Create a background plate document:
   a. From the SDS software, click  (or select **File > New**).

b. Complete the New Document dialog box, then click **OK**:

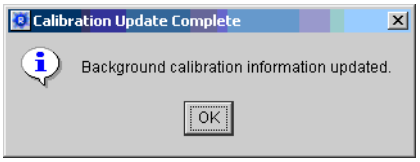



**IMPORTANT!** Do not modify the plate document. The method for a background run (one hold at 60 °C for 2 minutes) is coded into the SDS software. Because the plate contains only buffer, sample and detector labels are not required.

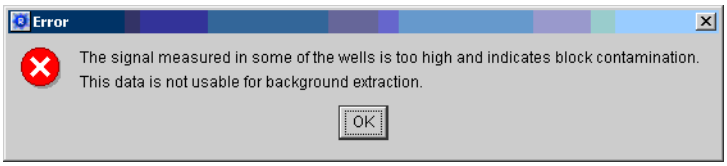
3. Save the background plate document:
   a. Click (or select **File** > **Save**).
   b. For Files of Type, select **SDS 7900HT Document (*.sds)**.
   c. In the File name field, type:
      **Background_96Fast_*<date in MMDDYY format>*.sds**
      For example, for a background plate run on May 31, 2004, type:
      Background_96Fast_053104.sds
   d. Click **Save**.
4. Run the background plate:
   a. In the plate document in the SDS software, select the **Instrument** tab, then select the **Real-Time** tab.
   b. Check if Connected to 'PlateName' (Connected) is displayed in the status bar. If the software is not connected to the instrument, click **Connect**.
   c. If the instrument tray is inside the instrument, click **Open/Close**.
   d. Verify that the Fast 96-Well Plate Adapter is installed in the instrument tray.
   e. Place the background plate into the instrument tray so that Well A1 and the notched corner are in the top left corner and the bar code is toward the front of the instrument.
   f. In the Real-Time tab of the Instrument tab, click **Start**.
      **Note:** Before starting the run, the instrument pauses approximately 40 seconds to perform a laser check and then may pause up to 15 minutes to allow the heated cover to heat to the appropriate temperature.
   g. After the background run is complete, click **OK** to close the Run Complete dialog box, click **Open/Close**, then remove the plate from the instrument tray.
5. Extract the background:
   a. Select all wells in the background plate document.

b. Select **Analysis** > **Extract Background**. The software extracts the background signal and displays the success of the extraction in a dialog box. If the software displays:

– Calibration Update Complete box – The run is successful because the raw spectra conform to acceptable limits. Proceed to step 5c.




– Error box – The run is unsuccessful because one or more raw spectra exceed 2500 fluorescence spectral units (FSU). Troubleshoot and decontaminate the sample block (see "Instrument Procedures" on page 10).




c. Click  (or select **File** > **Save**), then select **File > Close**.

## Performing a Pure Dye Run

Applied Biosystems recommends performing a pure dye run every 6 months, depending on instrument use.

**IMPORTANT!** Before performing a pure dye run, perform a background run (see page 10).

### Materials Required

- Pure dye plates included in the 7900HT System Fast 96-Well Spectral Calibration Kit (PN 4351653):
  - Pure Dye Plate 1 (with FAM™ dye, JOE™ dye, NED™ dye, and ROX™ dye)
  - Pure Dye Plate 2 (with SYBR® Green dye, TAMRA™ dye, TET™ dye, and VIC® dye)
- Tabletop centrifuge with 96-well plate holder

### Procedure

1. Remove one of the pure dye plates from the freezer, allow it to thaw to room temperature, then centrifuge the plate briefly.
2. Create a pure dye plate document:
   a. From the SDS software, click  (or select **File** > **New**).

b. Complete the New Document dialog box, then click **OK**:

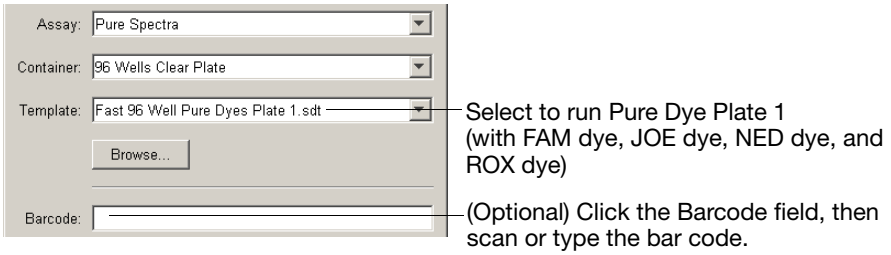



**Figure 1 New Document dialog box for Pure Dye Plate 1**

**IMPORTANT!** Do not modify the pure dye plate document. The method for a pure dye run (one hold at 60 °C for 2 minutes) is coded into the SDS software.

3. Save the pure dye plate document:
   a. Click (or select **File** > **Save**).
   b. For Files of type, select **SDS 7900HT Document (*.sds)**.
   c. In the File name field, type:
      **FastPureDye_Plate<*plate #*>_<*date in MMDDYY format*>.sds**
      For example, for a fast pure dye plate 1 run on May 31, 2004, type: FastPureDye_Plate1_053104.sds
   d. Click **Save**.
4. Run the pure dye plate:
   a. In the plate document in the SDS software, select the **Instrument** tab, then select the **Real-Time** tab.
   b. Check if Connected to 'PlateName' (Connected) is displayed in the status bar. If the software is not connected to the instrument, click **Connect**.
   c. If the instrument tray is inside the instrument, click **Open/Close**.
   d. Verify that the Fast 96-Well Plate Adapter is installed in the instrument tray.
   e. Place the pure dye plate into the instrument tray so that Well A1 and the notched corner are in the top left corner and the bar code is toward the front of the instrument.
   f. In the Real-Time tab of the Instrument tab, click **Start**.
      **Note:** Before starting the run, the instrument pauses approximately 40 seconds to perform a laser check and then may pause up to 15 minutes to allow the heated cover to heat to the appropriate temperature.
   g. After the pure dye run is complete, click **OK** to close the Run Complete dialog box, click **Open/Close**, then remove the plate from the instrument tray.
5. Extract the pure dye data:
   a. Select **Analysis** > **Extract Pure Dye Wizard**.
   b. Follow the Extract Pure Dye Wizard instructions to extract the pure dye spectra.

c. As each screen is displayed, inspect the spectra for shifts in peak location (Figure 2). Omit any outlying peaks by deselecting the check box of the associated well.

**Note:** Dye spectra are generally acceptable if they peak at the same location as their group but diverge slightly at other wavelengths.

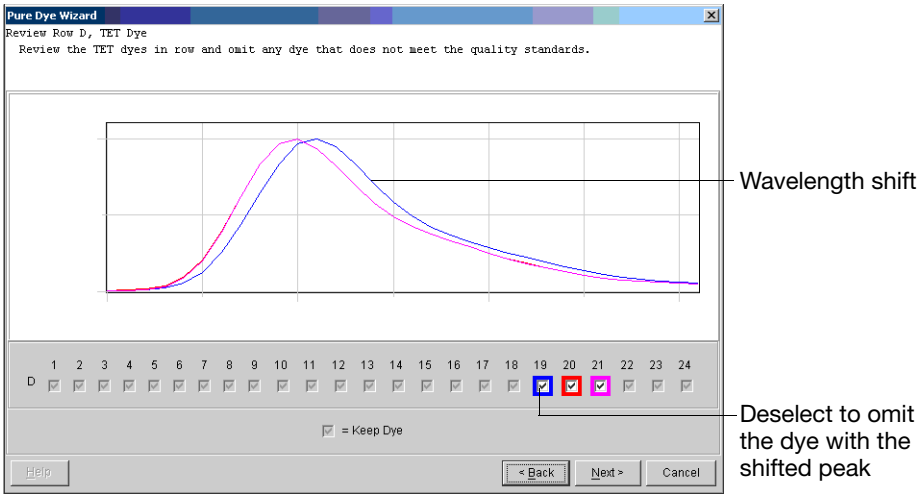



**Figure 2 Example of spectra from a pure dye plate**

d. Click **Next**.

e. Repeat steps b through d for all remaining wells.

The software extracts the pure spectra and stores the data as a component of the pure dye file.

6. Click (or select **File** > **Save**), then select **File > Close**.

7. Run the second pure dye plate (Pure Dye Plate 2):

   a. Prepare the pure dye plate (step 1).

   b. Create a pure dye plate document (step 2).

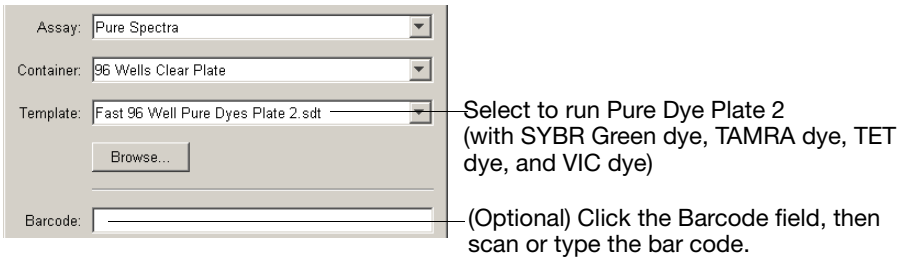



**Figure 3 New Document dialog box for Pure Dye Plate 2**

   c. Save the pure dye plate document (step 3).

   d. Run the pure dye plate (step 4).

   e. Extract the pure dye data (step 5).

## Verifying Instrument Performance

Applied Biosystems recommends running a TaqMan RNase P Fast 96-Well Instrument Verification Plate:

- When changing sample block formats for the first time
- As needed to verify the function of the 7900HT instrument

Figure 4 shows the arrangement of standards and samples on the RNase P plate. The RNase P plate consists of five sets of template standards (1250; 2500; 5000; 10,000; and 20,000 copies), two sets of "unknown" populations (5000 and 10,000 copies), and one set of no template controls (NTCs).

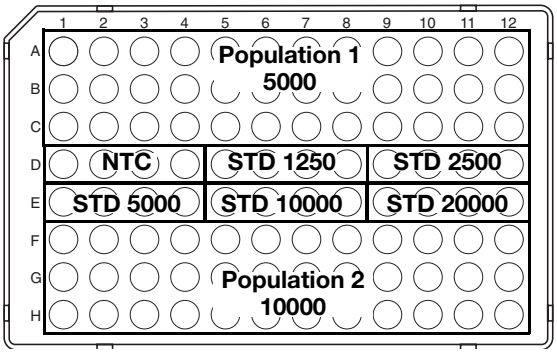


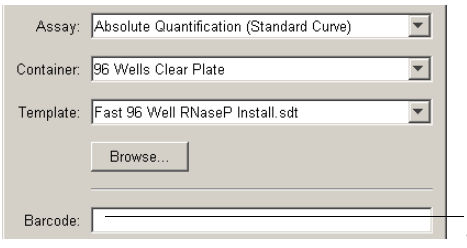

**Figure 4 Configuration of the TaqMan RNase P Fast 96-Well Instrument Verification Plate**

### Materials Required

- TaqMan RNase P Fast 96-Well Instrument Verification Plate (PN 4351979)
- Tabletop centrifuge with 96-well plate holder

### Procedure

1. Remove the RNase P plate from the freezer, allow it to thaw to room temperature, then centrifuge the RNase P plate briefly.
2. Create the plate document:
   a. From the SDS software, click (or select **File** > **New**).
   b. Complete the New Document dialog box, then click **OK**:




(Optional) Click the Barcode field, then scan or type the bar code.

**Note:** Do not modify the plate document. The plate document template contains all information needed for the run.

3. Save the plate document:
   a. Click (or select **File** > **Save**).
   b. For Files of Type, select **SDS 7900HT Document (*.sds)**.
   c. In the File name field, type a name for the plate document.
   d. Click **Save**.
4. Run the RNase P plate:
   a. In the plate document in the SDS software, select the **Instrument** tab, then select the **Real-Time** tab.
   b. Check if Connected to 'PlateName' (Connected) is displayed in the status bar. If the software is not connected to the instrument, click **Connect**.
   c. If the instrument tray is inside the instrument, click **Open/Close**.
   d. Verify that the Fast 96-Well Plate Adapter is installed in the instrument tray.
   e. Place the RNase P plate into the instrument tray so that Well A1 and the notched corner are in the top left corner and the bar code is toward the front of the instrument.
   f. In the Real-Time tab of the Instrument tab, click **Start**.

      **Note:** Before starting the run, the instrument pauses approximately 40 seconds to perform a laser check and then may pause up to 15 minutes to allow the heated cover to heat to the appropriate temperature.
5. After the run is complete, perform analysis:
   a. Analyze the run data.
   b. Set the baseline and threshold values for the analyzed data.
6. Verify that the 7900HT instrument meets the installation specifications:

   The 7900HT System is designed to distinguish between 5,000 and 10,000 genome equivalents with a 99.7% confidence level. The following equation verifies the 7900HT instrument installation specifications:

   $$[(\text{CopyPop}_2) - 3(\sigma_{\text{CopyPop2}})] > [(\text{CopyPop}_1) + 3(\sigma_{\text{CopyPop1}})]$$

   where:
   - $\text{CopyPop}_2$ – Average copy number of population 2 (10,000 copies)
   - $\sigma_{\text{CopyPop2}}$ – Standard deviation of population 2 (10,000 copies)
   - $\text{CopyPop}_1$ – Average copy number of population 1 (5000 copies)
   - $\sigma_{\text{CopyPop1}}$ – Standard deviation of population 1 (5000 copies)

   **Note:** The values above can be obtained from the experimental report window.

   **Note:** You can omit up to six wells from each replicate group to meet installation specifications.

# Troubleshooting

## General

| Observation | Possible Cause | Action |
|---|---|---|
| High $C_T$ values/poor precision or failed PCR reactions | Target is difficult to amplify | • Increase the annealing/extension time in the thermal cycler protocol.<br>• Increase the annealing/extension temperature to 62 °C. |
| | Insufficient cDNA template is present | Use 10 to 100 ng of cDNA template per 20-µL reaction. |
| | Quality of cDNA template is poor | 1. Quantify the amount of cDNA template.<br>2. Test the cDNA template for the presence of PCR inhibitors. |
| | Sample degradation | Prepare fresh cDNA, then repeat the experiment. |
| | The TaqMan 2× Universal PCR Master Mix was used instead of the TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG | Prepare the reactions as described on page 7. |
| | Primer-dimer formation | To ensure optimal results, run the reaction plate as soon as possible after completing the reaction setup. If you cannot run a reaction plate within 2 hours after completing the reaction setup, refrigerate or freeze the reaction plate until you can load and run it on the 7900HT instrument. |
| Low $\Delta R_n$ or $R_n$ values | Extension time is too short | Use the default thermal profile settings (see page 5). |
| | Primer-dimer formation | To ensure optimal results, run the reaction plate as soon as possible after completing the reaction setup. If you cannot run a reaction plate within 2 hours after completing the reaction setup, refrigerate or freeze the reaction plate until you can load and run it on the 7900HT instrument. |
| Run takes more than 40 minutes | Thermal cycler mode is set to Standard or 9600 Emulation | Make sure that the thermal cycler mode is set to Fast (see page 5). |
| Rn vs. Cycle plot is not displayed | ROX dye was not selected as the passive reference when the plate document was set up | Select ROX dye as the passive reference when you set up the plate document. |

| Observation | Possible Cause | Action |
|---|---|---|
| Extremely high $\Delta R_n$ or $R_n$ values | ROX dye was not selected as the passive reference when the plate document was set up | Select ROX dye as the passive reference when you set up the plate document. |
| | Evaporation | Make sure that the reaction plate is sealed completely, especially around the edges. |
| High variability across the reaction plate | ROX dye was not selected as the passive reference when the plate document was set up | Select ROX dye as the passive reference when you set up the plate document. |
| | Evaporation | Make sure that the reaction plate is sealed completely, especially around the edges. |
| High variability across replicates | Reaction mix was not mixed well | Mix the reaction mix gently by inversion, then centrifuge briefly before aliquoting to the reaction plate. |

## Multiplex Applications

**IMPORTANT!** Due to the challenging nature of multiplex applications and the complexity that can be encountered, it is impossible to guarantee assay performance. However, the recommendations listed below should be helpful when running multiplex applications using Fast thermal cycling conditions and the TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG.

Perform the recommendations in the order listed.

When running multiplex applications:

1. Increase the annealing/extension temperature to 62 °C.
2. If you do not obtain the expected performance by increasing the annealing/extension temperature to 62 °C, increase the annealing/extension time in the thermal cycling protocol to 30 seconds.
3. If you do not obtain acceptable performance by increasing both the annealing/extension temperature and time, assay reoptimization may be required. Refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4348358) for more information.

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