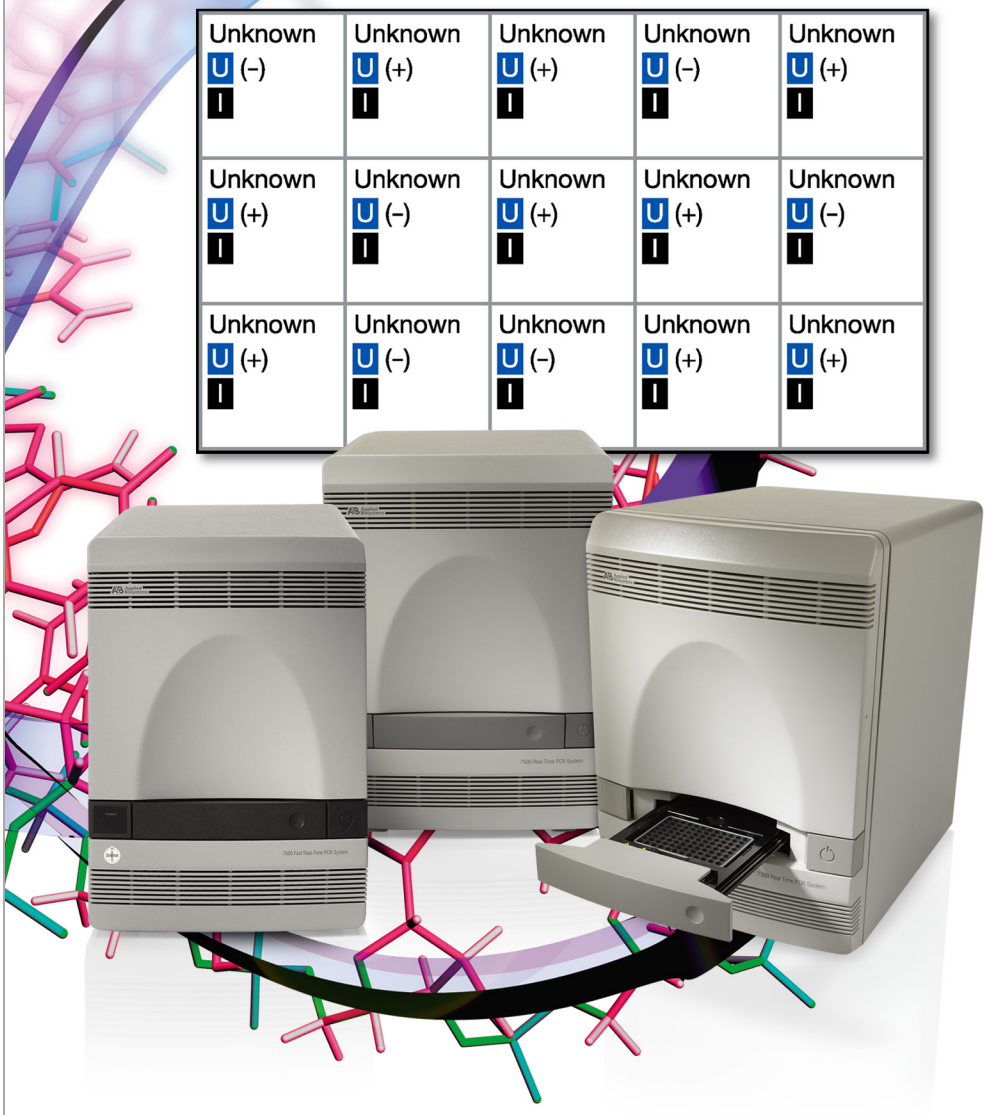
Applied Biosystems

Plus/Minus Assay Getting Started Guide



Introduction

Designing a Plus/Minus Experiment

Setting Up the Reaction Plate

Performing the Plus/Minus Pre-Read Run

Generating Amplification Data

Performing the Plus/Minus Post-Read Run

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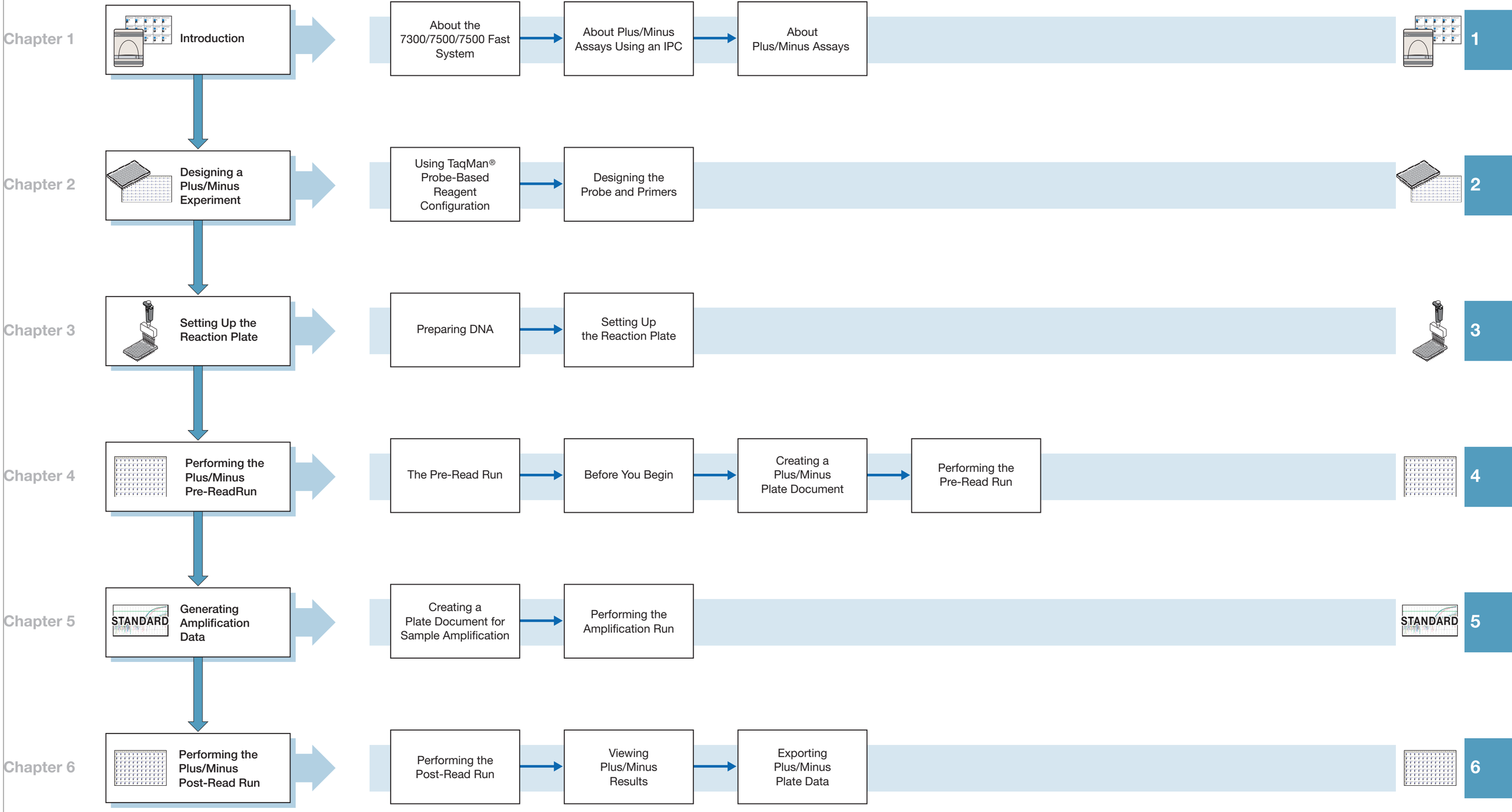
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Preface

How to Use This Guide

Purpose of This Guide

This manual is written for principal investigators and laboratory staff who run plus/minus assays using the Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System (7300/7500/7500 Fast system).

Assumptions

This guide assumes that you have:

- Familiarity with Microsoft® Windows® XP operating system.
- Knowledge of general techniques for handling DNA samples and preparing them for PCR.
- A general understanding of hard drives and data storage, file transfers, and copying and pasting.
- Networking experience if you want to integrate the 7300/7500/7500 Fast system into your existing laboratory data flow system.

Text Conventions

This guide uses the following conventions:

- **Bold** indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow bracket (>) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File > Open > Spot Set**.

User Attention Words

The following user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note – Provides information that may be of interest or help but is not critical to the use of the product.

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

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

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

Safety

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

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

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

To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose
4. To have a copy of a document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.
5. After you enter the required information, click **View/Deliver Selected Documents Now**.

Refer to the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Getting Started Guide* (PN 4378657) and the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Site Preparation Guide* (PN 4378654) for important safety information.

How to Obtain More Information

Related Documentation

For more information about using the 7300/7500/7500 Fast system, refer to the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Online Help* or the documents shown below.

Document Title	Online Help P/N	P/N
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Allelic Discrimination Getting Started Guide	4347822	4378653
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantification Getting Started Guide	4347824	4378655
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4347825	4378656
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Site Preparation Guide	4347823	4378654
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide	4347828	4378657
Real-Time PCR Systems Chemistry Guide	4348358	4378658
Applied Biosystems 7500 FAST Real-Time PCR System, QRC	4362285	4378659
Applied Biosystems Real-Time System Computer Set Up Guide, QRC	4365367	4378660

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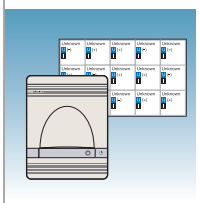
techpubs@appliedbiosystems.com

How to Obtain Support

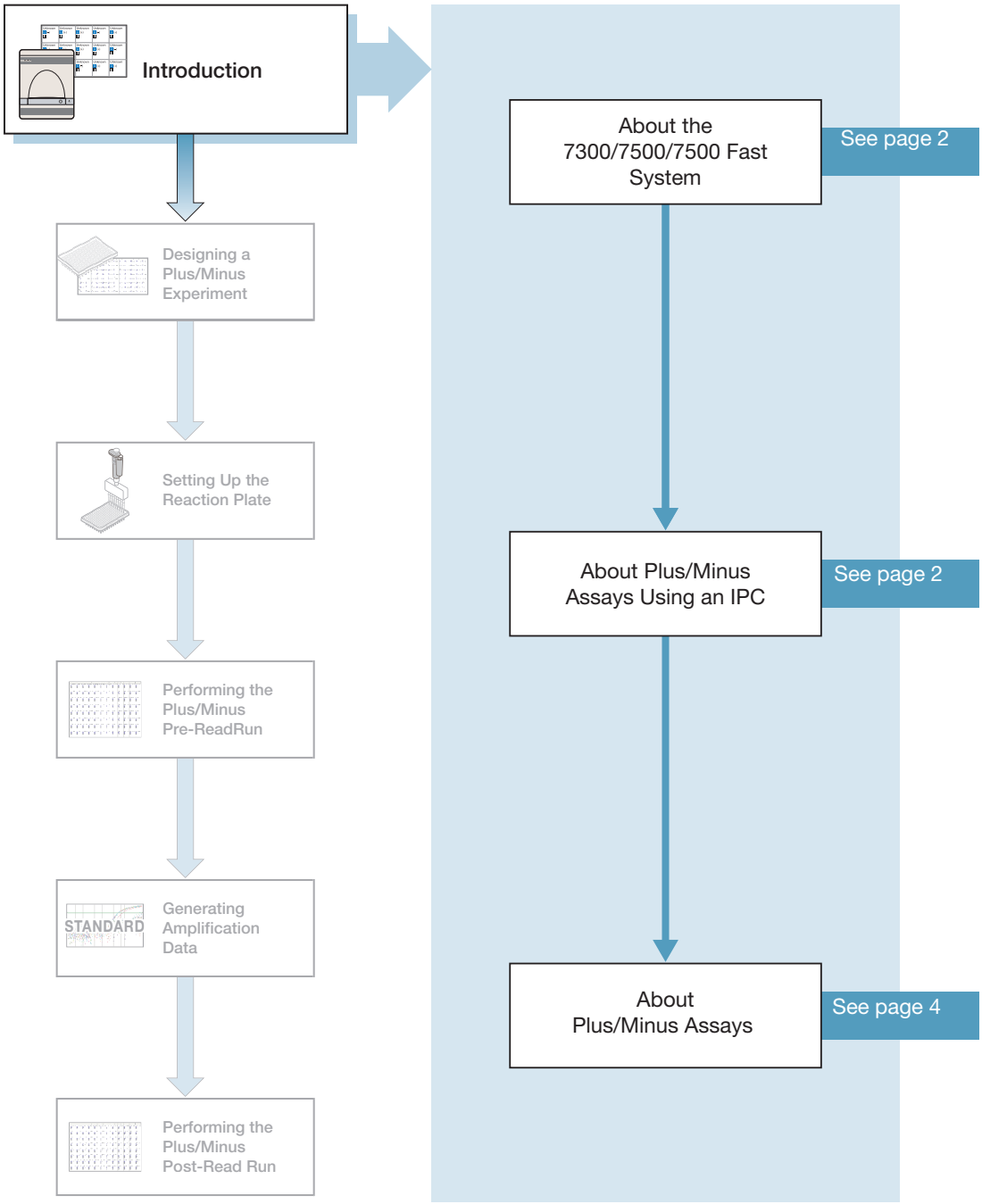
For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

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



Introduction



Notes

About the 7300/7500/7500 Fast System

Description

The Applied Biosystems Real Time PCR System (7300/7500/7500 Fast system) uses fluorescent-based PCR chemistries to provide:

- Quantitative detection of nucleic acid sequence using real-time analysis.
- Qualitative detection of nucleic acid sequence using end-point and dissociation-curve analysis.

Plus/Minus Assay

The 7300/7500/7500 Fast system allows you to perform several assay types with plates in the 96-well format. This guide describes the plus/minus assay, which determines whether or not a specific target sequence is present in a sample.

Note: For information about the other assay types, refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4378658) and the Online Help for the 7300/7500/7500 Fast system.

Note: Plus/Minus Assays may be run on a 7500 Fast system using standard reagents; Plus/Minus Assays are not supported using Fast reagents and protocols.

About Plus/Minus Assays Using an IPC

Definition

A plus/minus assay is an end point assay that determines if a specific target sequence is present (plus) or not present (minus) in a sample. In an end-point assay, data are collected at the end of the PCR process.

What Is An IPC?

An IPC is an internal positive control (see TaqMan® Exogenous Internal Positive Control Reagents kit, PN 4308323) that is used in plus/minus assays to monitor the PCR process and to ensure that a negative result is not due to failed PCR. The IPC consists of a template, a primer set, and a dye-labeled (VIC®) probe added to each well of a reaction plate (the IPC is part of the reaction mix, see "Preparing the PCR Reaction Mix" on page 14).

Plus/Minus assays with an IPC use fluorogenic 5′ nuclease chemistry (also known as "TaqMan probe-based chemistry"). During amplification, the sample target and the IPC target generate reporter fluorescence signals, so that positive or negative calls may be made on unknown samples.

Note: The SYBR® Green I dye chemistry is not supported for plus/minus assays using an IPC.

Notes

Terms Used in Plus/Minus Analysis

Term	Definition
Internal positive control (IPC)	A second TaqMan® probe and primer set added to the reaction plate to monitor the PCR process and to ensure that a negative result is not due to failed PCR in the sample
No amplification control (NAC)	Wells that contain no target template and blocked IPC (the IPC target template has been blocked by a blocking agent)
No template control (NTC)	A sample that contains no target template
Nucleic acid target	Nucleotide sequence that you want to identify as present or absent
Unknown sample (U)	The sample for which you want to determine the presence or absence of a specific target

Notes

About Plus/Minus Assays

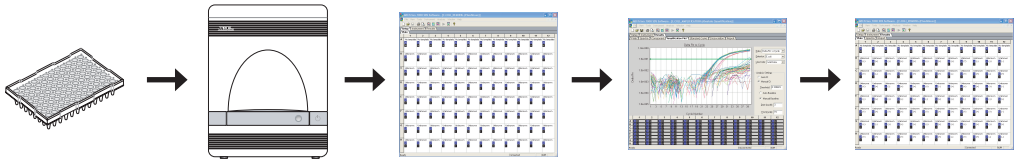
Plus/Minus Experiment Workflow

This document uses the term "plus/minus assay" to refer to the entire process of analyzing samples of extracted DNA from data collected at the end of the PCR process.

Design the experiment and isolate the DNA, then conduct a plus/minus assay by performing:

- **A pre-read run** on a plus/minus plate document to determine the baseline fluorescence associated with primers and probes before amplification.
- **An amplification run** using an AQ plate document to generate real-time PCR data, which can be used to analyze and troubleshoot the PCR data for the plus/minus assay, if needed.
- **A post-read run** using the original plus/minus plate document, which automatically subtracts the baseline fluorescence determined during the pre-read run to calculate the result.

The following figure illustrates the complete process.



Reaction Plate | 7300/7500/7500 Fast System | Pre-Read Run | Amplification Run | Post-Read Run

Required User-Supplied Materials

Item	Source
DNA isolation and purification chemistry systems: • ABI PRISM™ 6100 Nucleic Acid PrepStation • BloodPrep™ Chemistry (genomic DNA from fresh or frozen blood or cells) • NucPrep™ Chemistry (DNA from animal and plant tissue) • PrepMan™ Ultra Sample Preparation Reagent Kit	 • Applied Biosystems (PN 6100-01) • Applied Biosystems (PN 4346860) • Applied Biosystems (PN 4340274) • Applied Biosystems (PN 4322547)
Labeled primers and probes source: • Primer Express® Software (custom-designed primers and probes)	 • PN 4330710 (1-user license) PN 4330709 (10-user license) PN 4330708 (50-user license)
MicroAmp® Optical 96-Well Reaction Plates	Applied Biosystems (PN 4306757)
Optical Adhesive Covers	Applied Biosystems (PN 4311971)
Reagent tubes with caps, 10-mL	Applied Biosystems (PN 4305932)

Notes

Item	Source
TaqMan® Exogenous Internal Positive Control Reagents (VIC® Probe)	Applied Biosystems (PN 4308323)
TaqMan® Universal PCR Master Mix	Applied Biosystems (PN 4304437)
Centrifuge with adapter for 96-well plates	Major Laboratory Supplier (MLS)
Gloves	MLS
Microcentrifuge	MLS
Microcentrifuge tubes, sterile 1.5-mL	MLS
Nuclease-free water	MLS
Pipette tips, with filter plugs	MLS
Pipettors, positive-displacement	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Vortexer	MLS

Example Plus/Minus Experiment

To better illustrate how to design, perform, and analyze plus/minus experiments, this document provides an example experiment. The example experiment represents a typical plus/minus experiment that you can use as a quick-start procedure to familiarize yourself with the plus/minus workflow. Details about the plus/minus workflow are described in the subsequent chapters of this guide. Example Experiment boxes appear in subsequent chapters to illustrate workflow details. Refer to Appendix C, "Example Plus/Minus Experiment," for more information.

Notes

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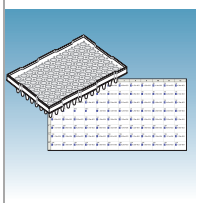
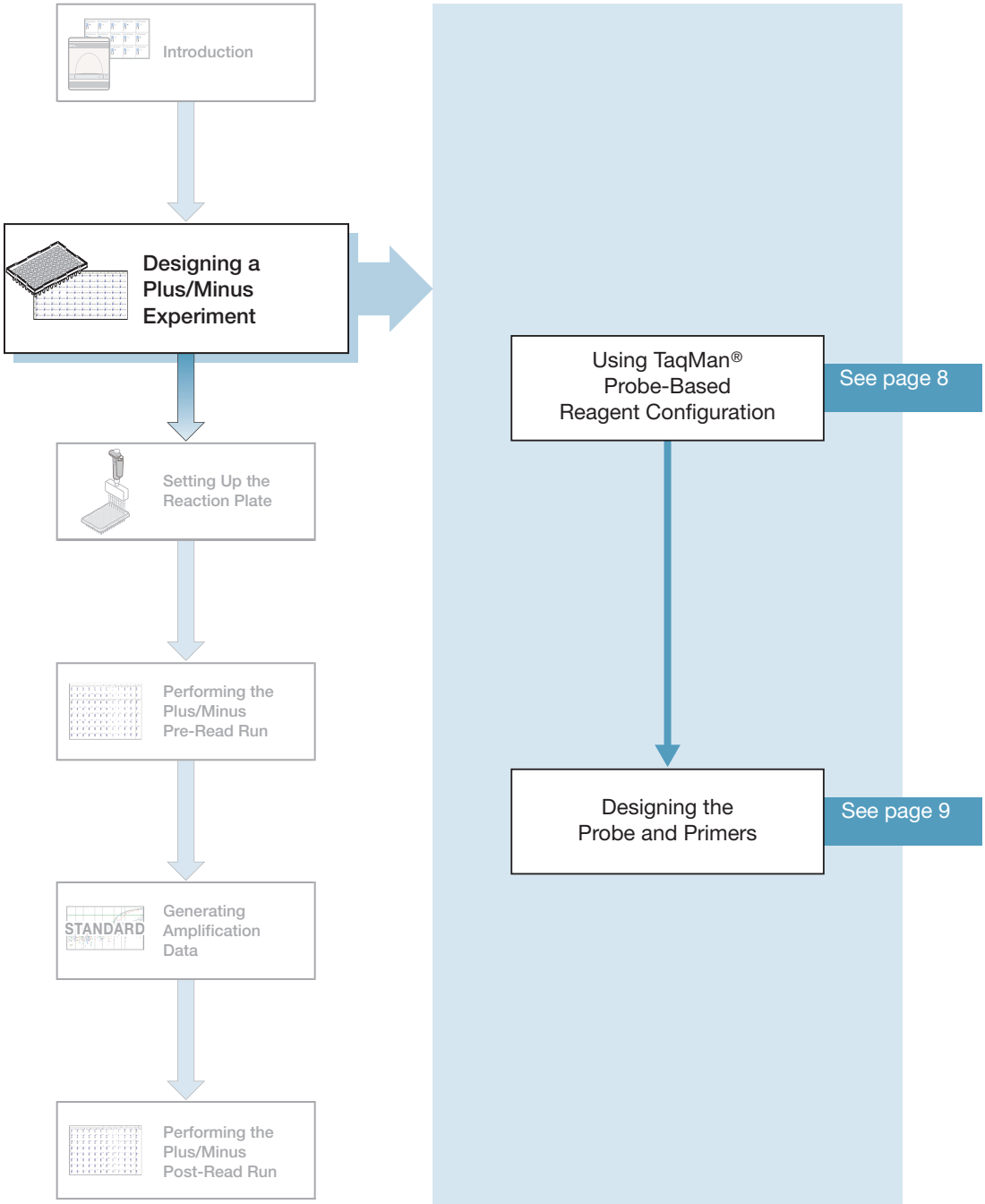


Designing a Plus/Minus Experiment

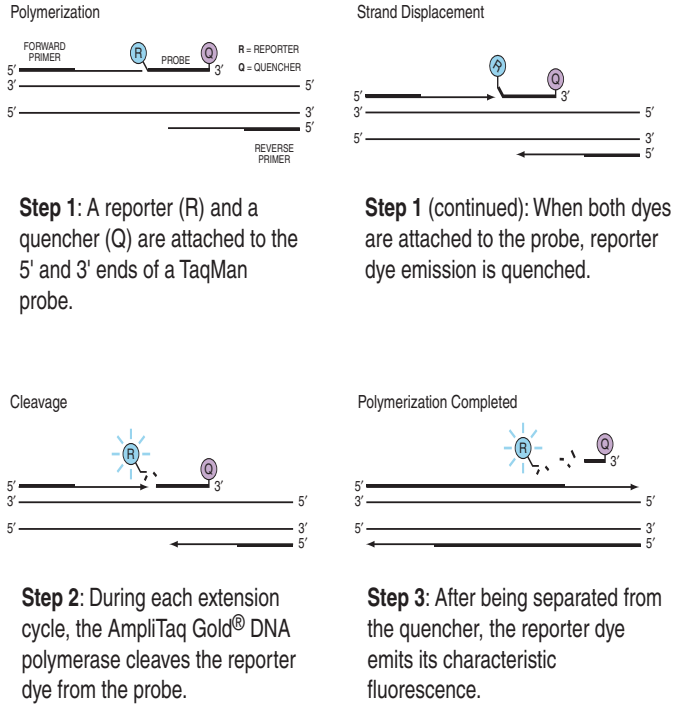


Notes

Using TaqMan® Probe-Based Reagent Configuration

About the Chemistry

Plus/minus assays with an IPC use the fluorogenic 5′ nuclease chemistry (also known as TaqMan® probe-based chemistry).

Chemistry	Process
TaqMan® reagents or kits **Description** TaqMan probe-based chemistry uses a fluorogenic probe to detect a specific PCR product as it accumulates during PCR cycles.	 **Step 1**: A reporter (R) and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe. **Step 1** (continued): When both dyes are attached to the probe, reporter dye emission is quenched. **Step 2**: During each extension cycle, the AmpliTaq Gold® DNA polymerase cleaves the reporter dye from the probe. **Step 3**: After being separated from the quencher, the reporter dye emits its characteristic fluorescence.

For more information about the TaqMan probe-based chemistry, refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4378658).

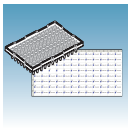
Chemistry Kits for Plus/Minus Assay

The following reagents are available from Applied Biosystems for designing and running plus/minus assays.

Kit	Part Number
TaqMan® Exogenous Internal Positive Control Reagents with TaqMan Universal PCR Master Mix	4308323
TaqMan® Universal PCR Master Mix	4304437

Note: The IPC DNA, primers, and probe supplied in these reagents can be used with all sample target systems. Refer to the *TaqMan® Universal PCR Master Mix Protocol* (PN 4304449) for instructions on optimizing amplification of your target.

Notes



Designing the Probe and Primers

Design a probe and primer set for your target sequence. Applied Biosystems provides the Primer Express® software for this purpose. For more information about using this software, refer to the *Primer Express Software v3.0 Getting Started Guide* (PN 4362460).

Sample Experiment

In the example experiment, we extracted DNA from 84 batches of hamburger meat and tested them for the presence of *E. coli* using the plus/minus assay on the 7300/7500 Real Time PCR System. Six no IPC/no target template controls, six IPC/no target template controls, and 84 unknown samples were run.

For the example experiment, the TaqMan® Exogenous Internal Positive Control Reagents Kit supplies one 1-mL tube of 10× Exo IPC Mix. This mix contains the IPC primers and VIC®-labeled probe. The primers/probe set for *E. coli* was designed by Applied Biosystems Primer Express software, and contained a FAM™-labeled probe with TAMRA™ as the quencher.

Notes

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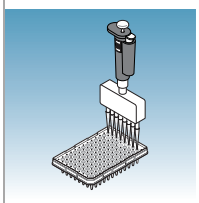
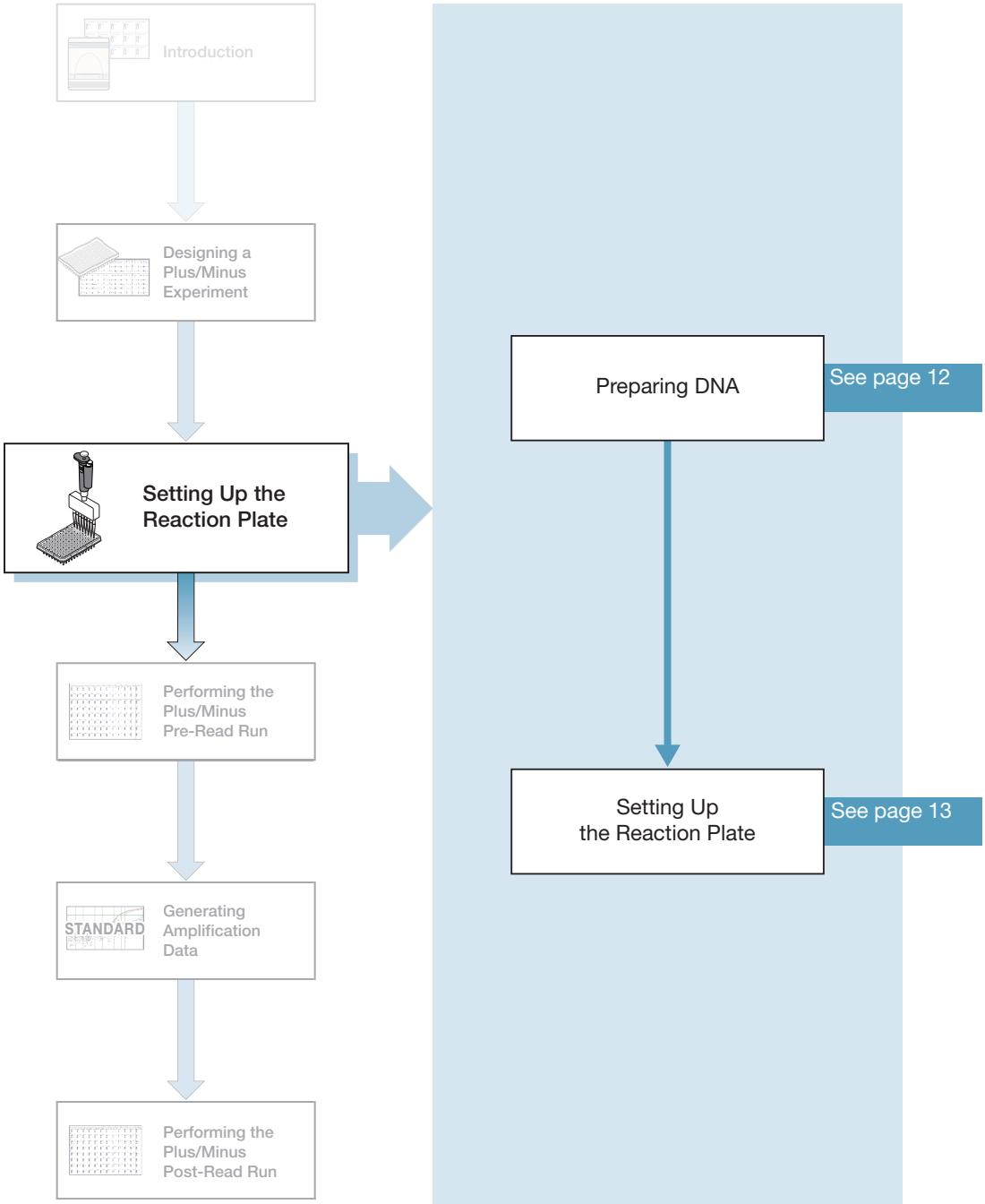


Setting Up the Reaction Plate



Notes

Preparing DNA

Systems and Chemistries for DNA Isolation

Applied Biosystems supplies several instrument systems and chemistries for isolating DNA from a variety of starting materials, such as blood, tissue, cell cultures, plant material, and food.

System	Part Number
BloodPrep™ Chemistry	4346860
NucPrep® Chemistry	4340274
PrepMan™ Ultra Sample Preparation Reagent Kit	4322547
ABI PRISM® 6100 Nucleic Acid PrepStation	6100-01

For more information, refer to:

- *DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol* (PN 4343586)
- *NucPrep® Chemistry Isolation of Genomic DNA from Animal and Plant Tissue Protocol* (PN 4333959)

Quality of DNA

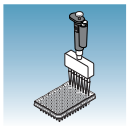
Ensure that the DNA you use for a plus/minus experiment:

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

Sample Experiment

The meat samples are frozen with liquid nitrogen and ground to a fine powder with a pre-chilled mortar and pestle. DNA is extracted using the PrepMan™ Ultra Sample Preparation Reagent Kit (PN 4322547) and protocol (PN 4318925) to obtain a final concentration of 10 ng/µL of DNA for each sample.

Notes

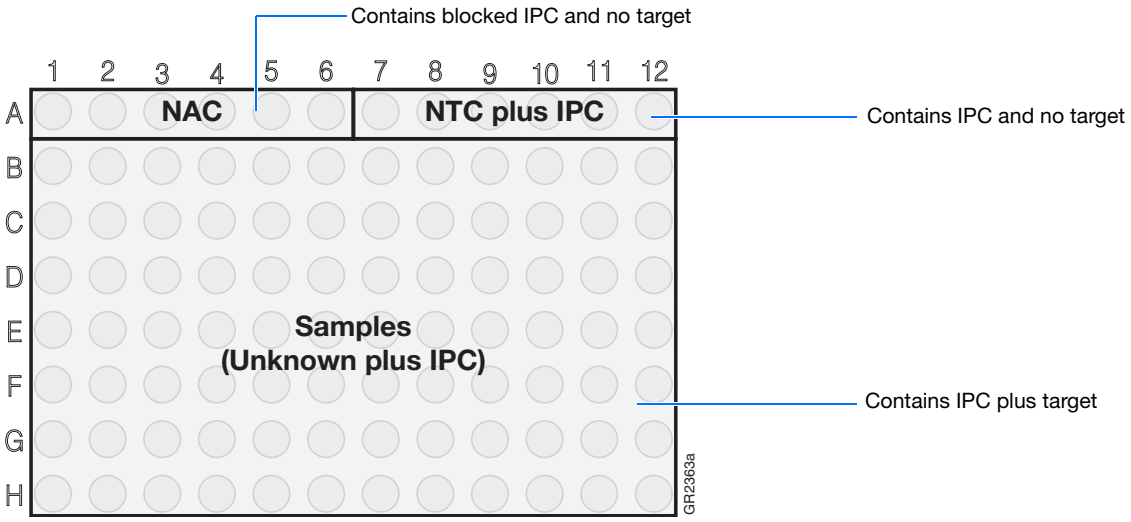


Setting Up the Reaction Plate

This section describes how to set up a 96-well plate for a plus/minus run with samples and reaction mix. The reagents, volumes, and final concentrations in "Preparing the PCR Reaction Mix" on page 14 were taken from the *TaqMan® Exogenous Internal Positive Control Reagents Protocol* (PN 4308323).

Sample Experiment

Extracted DNA samples are pipetted onto a 96-well plate along with negative and positive controls. Wells A1-A6 contained blocked IPC and no target template, wells A7-A12 contained IPC template (IPC+), but no target template, and wells B1-H12 contained both IPC and target template.



Notes

Preparing the PCR Reaction Mix

Make a volume of reaction mix sufficient to provide 45 µL (18 µL for the Fast system) for each well you use on the plate.

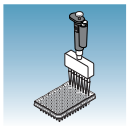
CAUTION **CHEMICAL HAZARD. TaqMan® Universal PCR Master Mix (2×) No AmpErase UNG** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Item	Volume for one Reaction (Standard)	Volume for one Reaction (Fast)	Final Concentration
TaqMan® Universal PCR Master Mix (2×)	25.0	10.0	1×
10× Exo IPC Mix (IPC kit)	5.0	2.0	50 to 900 nM
50× Exo IPC DNA (IPC kit)	1.0	0.4	50 to 900 nM
Target primers, probe, and deionized water	14.0	5.6	50 to 250 nM
Total	45.0	18.0	—

If preparing	Then add (Standard)	Then add (Fast)
NAC‡	5 µL of 10× Exo IPC Block (IPC kit)	2 µL of 10× Exo IPC Block (IPC kit)
NTC§	5 µL of 1× TE or H_2O	2 µL of 1× TE or H_2O

‡ No Amplification Control – well contains no target template and blocked IPC.
§ No Template Control – well contains no target template, only IPC.

Notes

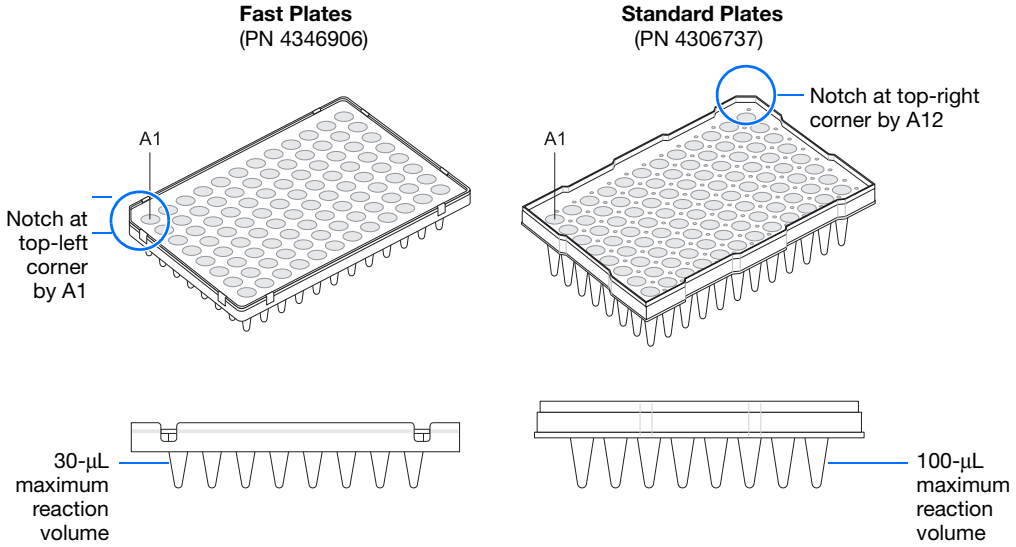


Preparing the Plate

Standard vs. Fast Plates

IMPORTANT! Ensure you use the standard Optical 96-Well Plate on the 7500 Real-Time PCR system. Optical 96-Well Fast Plates will *not* fit into the standard block correctly and will result in loss of data.

IMPORTANT! Ensure you use the Optical 96-Well Fast Plate on the 7500 Fast Real-Time PCR system. Standard plates will not function properly and they may be crushed when using the 96-Well Fast Block.



Notes

To prepare the plate:

1. Into each well, pipette 45 µL (18 µL for the Fast system) of the reaction mix.
2. Pipette 5 µL (2 µL for the Fast system) of sample (NAC, NTC, or unknowns) into each well of a 96-well plate.

   Note: The final reaction volume in each well should be 50 µL (20 µL for the Fast system).

3. Keep the reactions on ice until the plate is loaded into the 7300/7500/7500 Fast instrument.

Sample Experiment

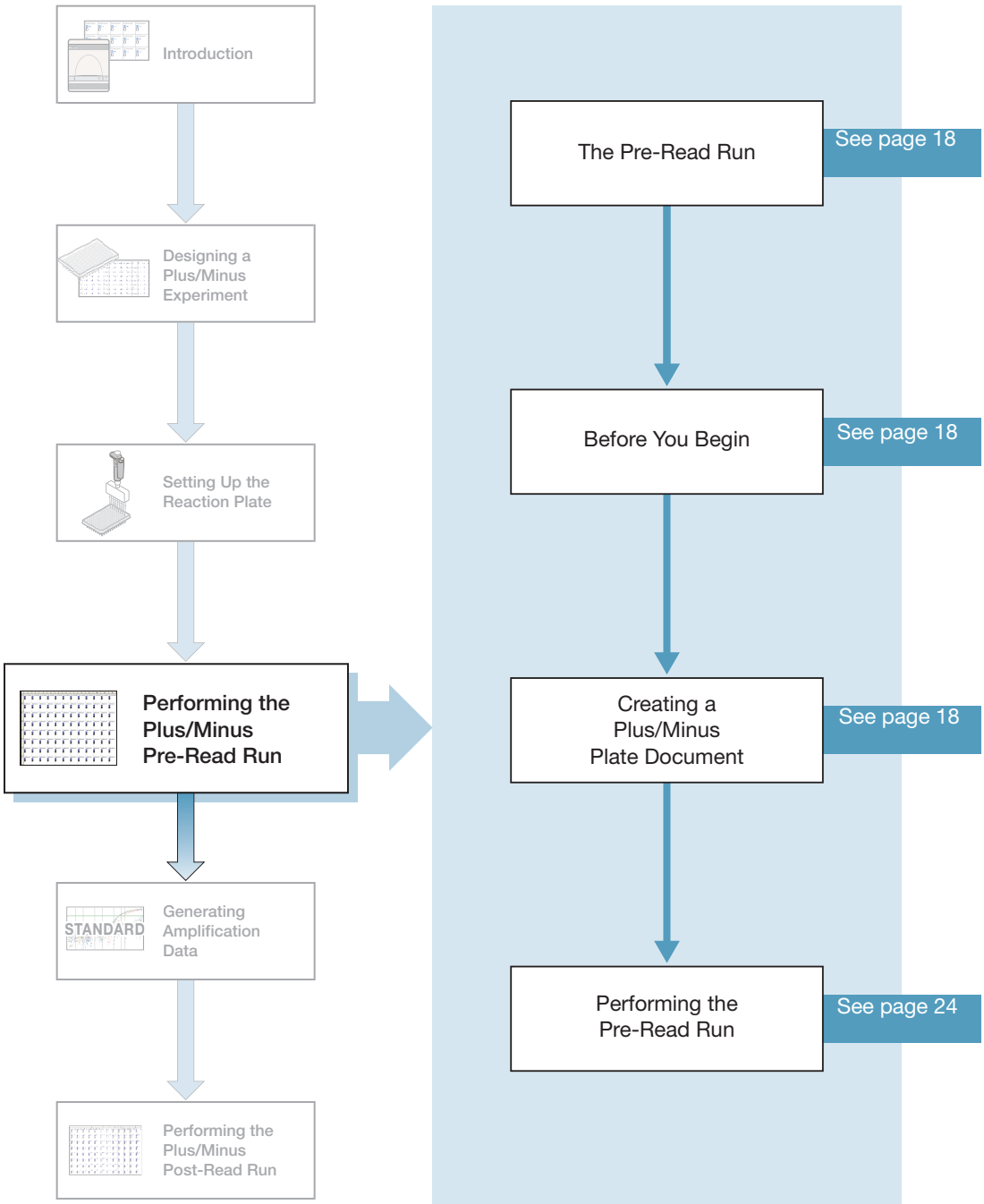
For the example experiment, 45 µL of reaction mix is pipetted into each well of a 96-well plate, and 5 µL of the following is added as specified in the table below.

Wells	To prepare	Add to each well (Standard)	Add to each well (Fast)
A1 to A6	NAC	5 µL of 10× Exo IPC Block (IPC kit)	2 µL of 10× Exo IPC Block (IPC kit)
A7 to A12	NTC	5 µL of 1× TE or H_2O	2 µL of 1× TE or H_2O
B1 to H12	U‡	5 µL of sample DNA	2 µL of sample DNA

‡ Unknown – well contains both target template and IPC.

Notes

Performing the Plus/Minus Pre-Read Run



Notes

The Pre-Read Run

A pre-read run records the background fluorescence of each well of the plus/minus plate before PCR. During the post-read run, the pre-read fluorescence is subtracted from the post-read fluorescence to account for pre-amplification background fluorescence. Subtracting pre-read from post-read fluorescence ensures accurate results.

Before You Begin

Check that background and pure-dye runs have been performed regularly to ensure optimal performance of the 7300/7500/7500 Fast system. For more information about calibrating the system, see the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Online Help*.

Creating a Plus/Minus Plate Document

A plus/minus plate document is an SDS Software 1.3.1 document that stores data collected from a plus/minus run for a single 96-well plate. Plus/Minus plate documents also store other information about the run, including sample names and detectors.

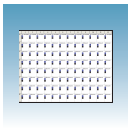
Plate Document Parameters

When you create a plus/minus plate document with an IPC, you define specific parameters for each plus/minus reaction plate:

- **Detectors** – A virtual representation in the SDS Software 1.3.1 of a TaqMan® probe and primer set and an associated fluorescent dye that detects a single target nucleic acid sequence. Appendix A explains how to create detectors.
- **Task** – A setting that you apply to each well of a plate document that determines the way the SDS Software 1.3.1 uses the data collected from the well during analysis.

Note: Applied Biosystems recommends you run six replicates of each control (NAC and NTC) to accurately define plus/minus thresholds and obtain plus/minus calls with a 99.7% confidence level.

Notes



Detector Tasks

You assign a task to each detector in each well of a plate document.

For plus/minus plate documents, there are four types of tasks:

Task	Symbol	Apply to...
Unknown	U	All detectors of wells that contain target sequence.
IPC	I	All detectors of wells that contain IPC.
IPC+	I+	All detectors of control wells that contain IPC but no target template.
NTC	N	All detectors of negative control wells that contain PCR reagents, but no target template and no IPC.

Notes

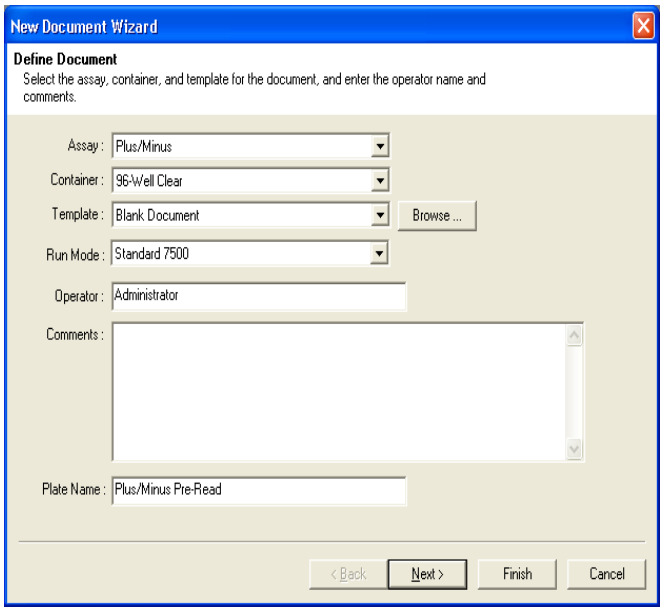
Creating a New Plus/Minus Plate Document

You can enter sample information into a new plate document, import sample information from existing plate documents, or use a template document to set up new plate documents.

This section describes setting up new plate documents. Refer to the Online Help for information about importing sample information or using template documents.

To create a new plus/minus plate document:

1. Select **Start > Programs > 7300/7500/7500 Fast System > 7300/7500/7500 Fast System Software** ( ) to start the 7300/7500/7500 Fast SDS Software 1.3.1.
2. Select **File** > **New**.
3. In the New Document Wizard, click the assay drop-down list, then select **Plus/Minus** assay. Accept the default settings for the Container and Template fields (**96-Well Clear** and **Blank Document**).
4. In the Plate Name field, type **Plus/Minus Pre-Read**.
5. Click **Next>**.

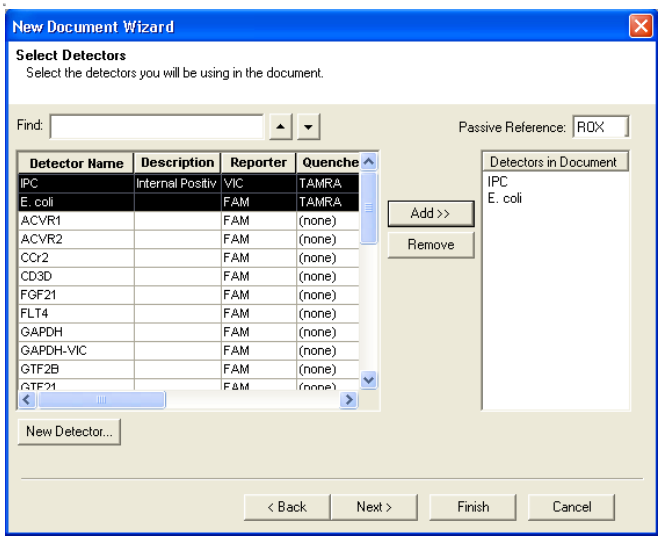


Notes

6. Select the detectors to add to the plate document.

   a. Click to select a detector. (Ctrl-click to select multiple detectors.) If no detectors are listed, click **New Detector** to open the New Detector dialog box. For more information about creating new detectors, refer to Appendix A, "Creating Detectors," on page 45.

   b. Click **Add>>**. The detector(s) are added to the Detectors in Document list box.



IMPORTANT! Ensure that the reporter dye for the target is different from the reporter dye for the IPC, which is VIC®.

Note: The TaqMan® Exogenous Internal Positive Control Reagents Kit (PN 4308323) uses an IPC VIC-labeled probe with TAMRA™ dye-labeled quencher.

Note: To remove a detector in the Detectors in Document window, select the detector, then click **Remove**.

   c. Click **Next**.

7. Select six wells on the plate document for the no amplification controls (blocked IPC and no target template).

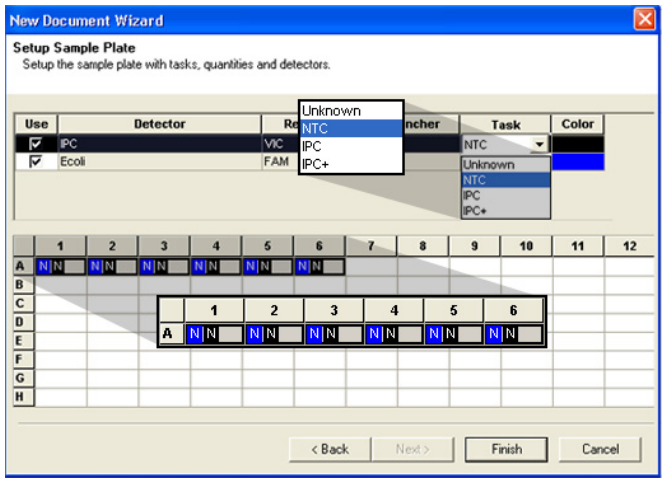
   a. Select wells A1 to A6.

   b. Select the target detector by checking the **Use** box next to it.

   c. Select **Task > NTC**. An N appears in the well.

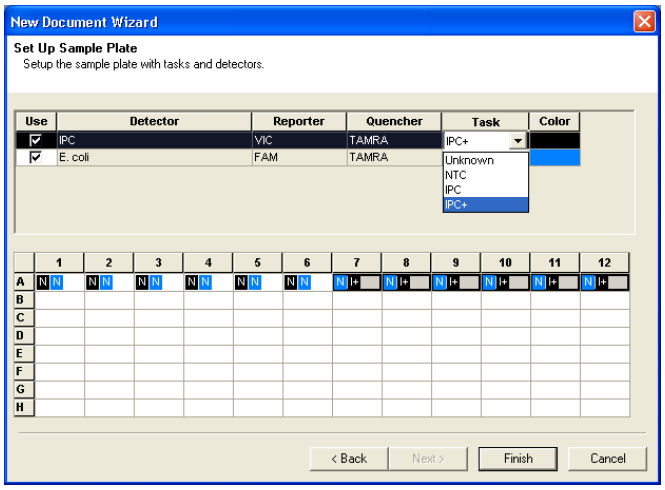
   d. Select the IPC detector by checking the **Use** box next to it.

   e. Select **Task > NTC**. A second N appears in the well.

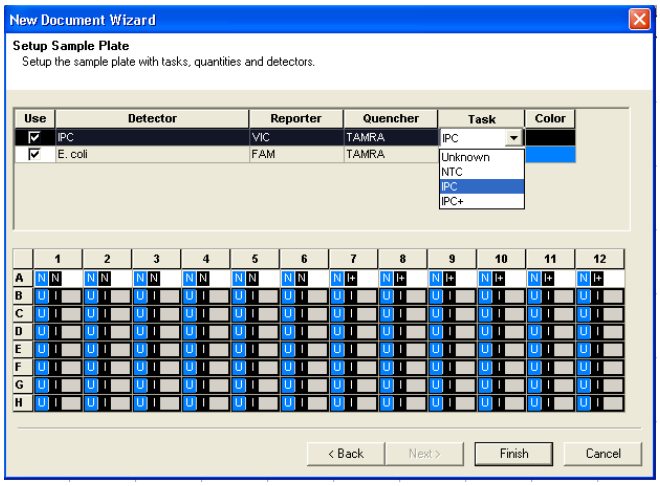


Notes

8. Select six more wells on the plate document for the no template controls (IPC but no target template).
   a. Select wells A7 to A12.
   b. Select the target detector by checking the **Use** box next to it.
   c. Select **Task > NTC**. An N appears in the well.
   d. Select the **IPC** detector by checking the **Use** box next to it.
   e. Select **Task > IPC**$^{+}$. An I+ appears in the well next to the N.

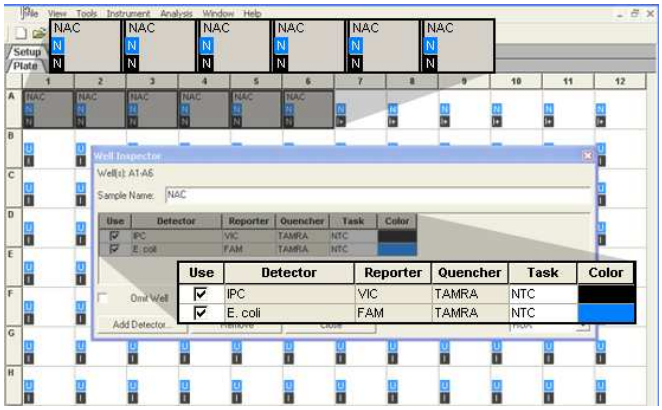


9. Select all remaining wells for the unknown samples (IPC and target template).
   a. Select wells B1 to H12 by click-dragging across all empty wells.
   b. Select the target detector by checking the **Use** box next to it.
   c. Select **Task > Unknown**. A U appears in the boxes.
   d. Select the **IPC** detector by checking the **Use** box next to it.
   e. Select **Task > IPC**. An I appears next to the U.



10. Click **Finish**.

11. Enter sample names for each well.
    a. Click or select **View > Well Inspector** from the menu.
    b. Click-drag to select replicate wells.
    c. Type the sample name.

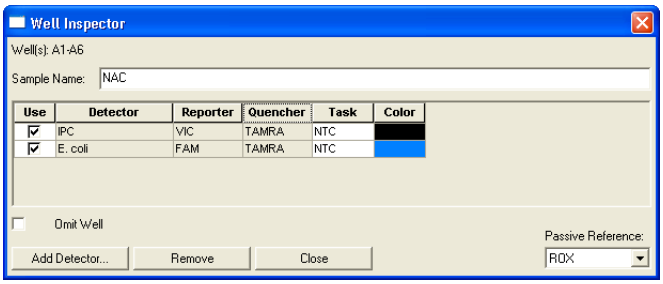


Notes

d. Accept the default setting (ROX™) for the Passive Reference, ROX dye.

e. Repeat steps b through c until you name all wells.

f. Click ✕ to close the Well Inspector.



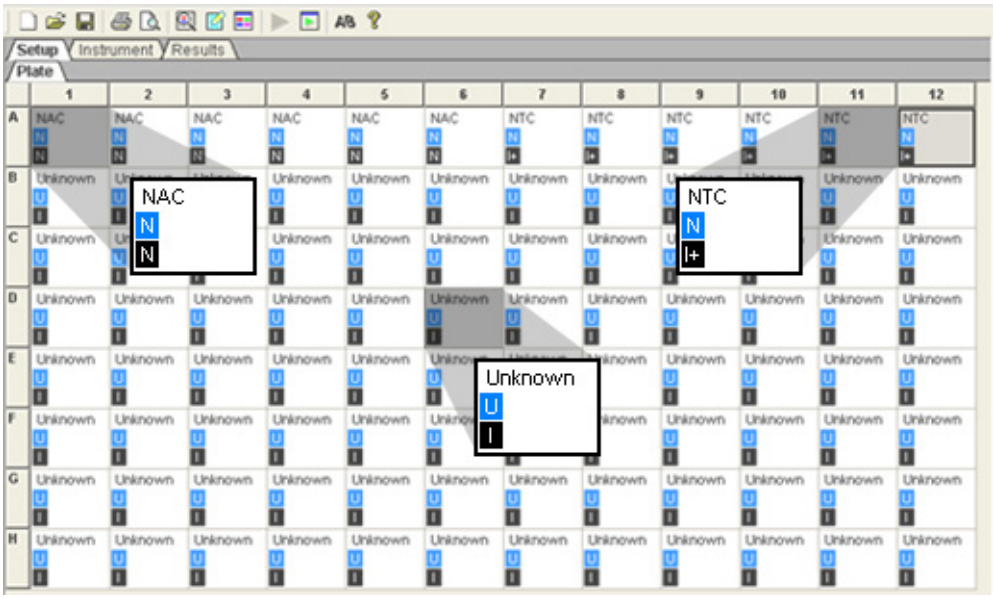
IMPORTANT! If your experiment does not use all the wells on a plate, do not omit the wells from use at this point. You can omit unused wells after the run is completed. For more information about omitting wells, refer to Online Help.

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

g. Verify the information on each well in the Setup tab.

Sample Experiment

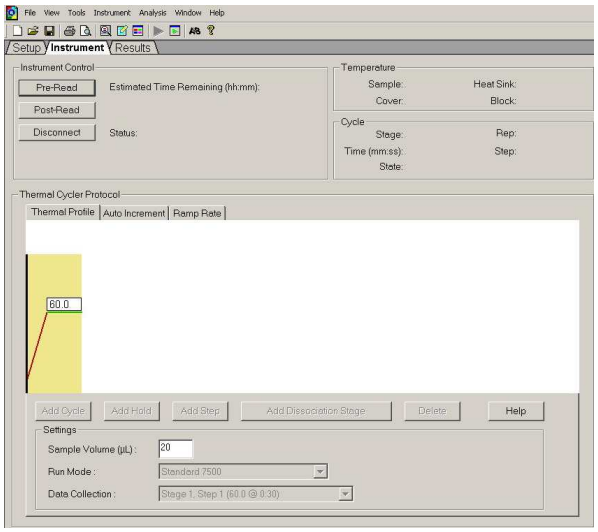
The pre-read plate document we created with controls and samples is shown in the picture below. We selected two detector tasks for each well, one for the target and one for the IPC. There are three possible combinations shown below: the NN box is the NAC (no target template, plus blocked IPC), the NI+ box seen below is the NTC (no target template plus IPC), and the UI box is the unknown sample plus IPC.



Notes

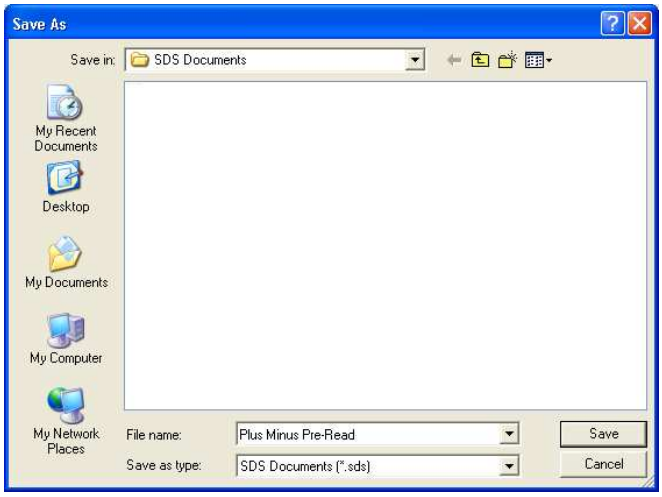
Performing the Pre-Read Run

1. Select the **Instrument** tab.
2. Accept the default value for sample volume.

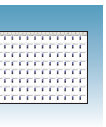


3. Select **File** > **Save As**, type **Plus/Minus Pre-Read** for the plus/minus plate document, then click **Save**.

   (Optional) If you want to use this plate setup again, you can save it as a template. Select **File** > **Save As**, type a File Name, then select **Save As type**: (*.sdt).

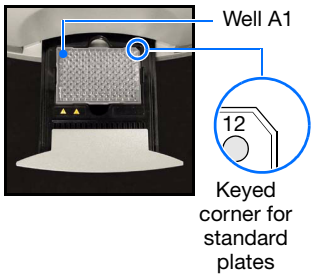


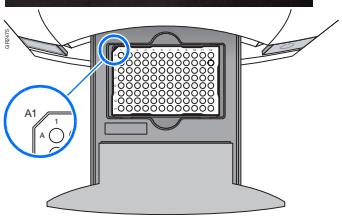
Notes



4. Load the reaction plate into the instrument.

   Note: The A12 position is notched in the top-right side of the instrument tray for the 7300/7500 system. The A1 position is notched on the top-left side of the instrument tray for the 7500 Fast system.



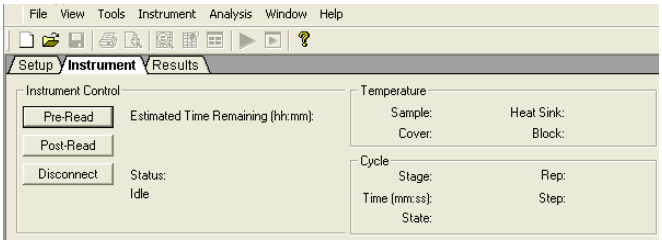


Keyed corner for Fast plates

5. Click **Pre-Read**.

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

   As the instrument performs the run, it displays status information in the Instrument tab. After the run is finished, the status values and the buttons are greyed out, and a message indicates whether or not the run is successful.

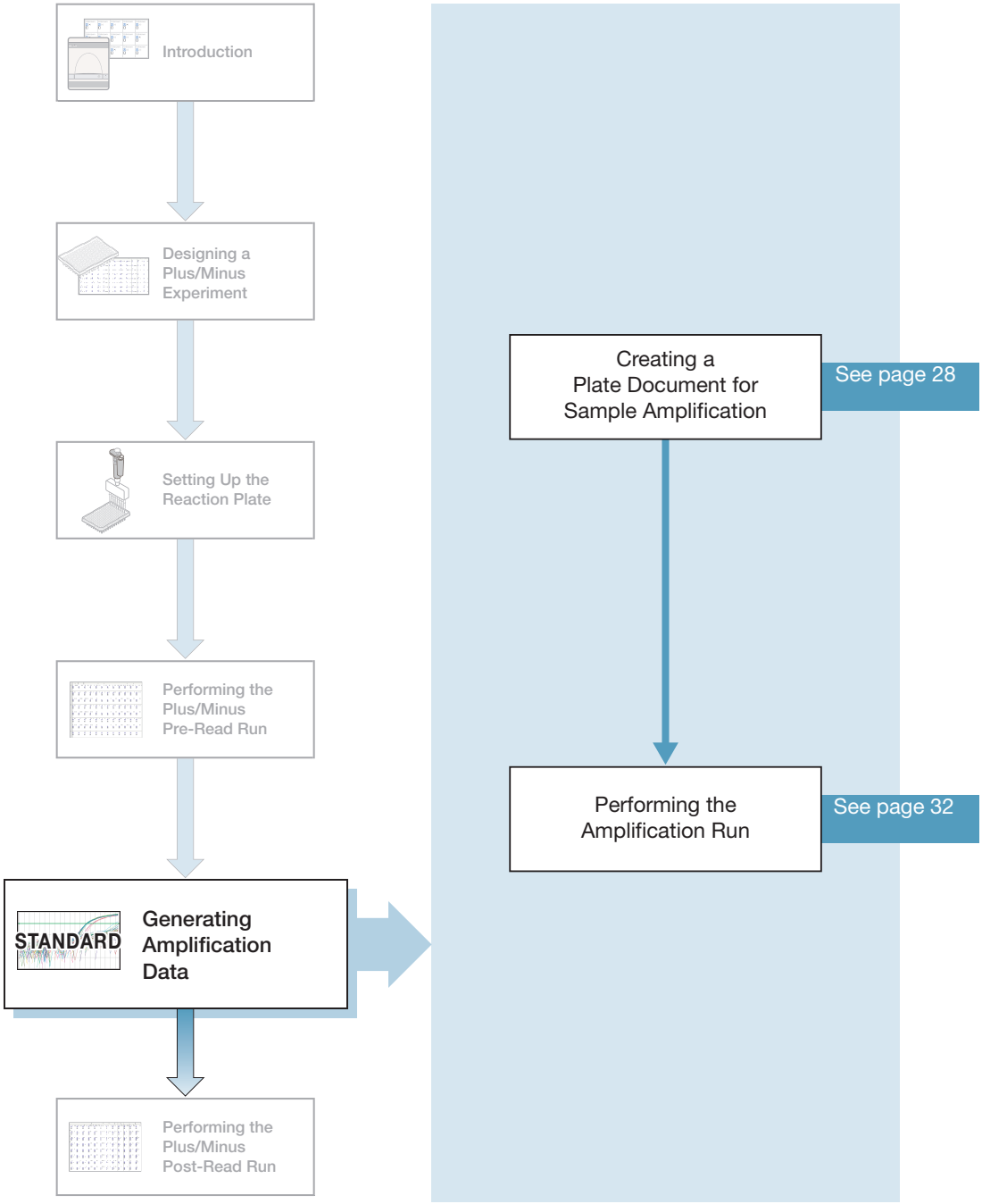


6. Select **File > Close**.

Notes

Notes

Generating Amplification Data



Notes

Creating a Plate Document for Sample Amplification

Benefits of Real-Time Amplification

Because the plus/minus assay is an end-point assay, you can amplify the target sequences offline using any thermal cycler. However, using the 7300/7500/7500 Fast system to amplify the target sequences provides real-time PCR data. After the plus/minus samples are analyzed, you can study the amplification plots if you observe questionable calls or observe no data for a well.

Using AQ Plate Documents for Amplification

You create and use absolute quantification (AQ) plate documents to store real-time data for plus/minus assays. Because the AQ plate document is used only to amplify target sequences (not to quantify the PCR data), you do not need a standard curve for the AQ plate.

Detector Tasks

For AQ plate documents, there are three types of tasks:

Task	Symbol	Apply to...
Unknown	U	Detectors of wells that contain target sequences.
Standard	S	Detectors of wells that contain samples of known quantities.
NTC	N	Detectors of negative control wells that contain no template.

The task label “unknown” is used for both IPC and the target samples.

Notes

To create a new AQ plate document:

1. Select **Start > Programs > 7300/7500/7500 Fast System > 7300/7500/7500 Fast System Software** ( ) to start the 7300/7500/7500 Fast SDS instrument software.

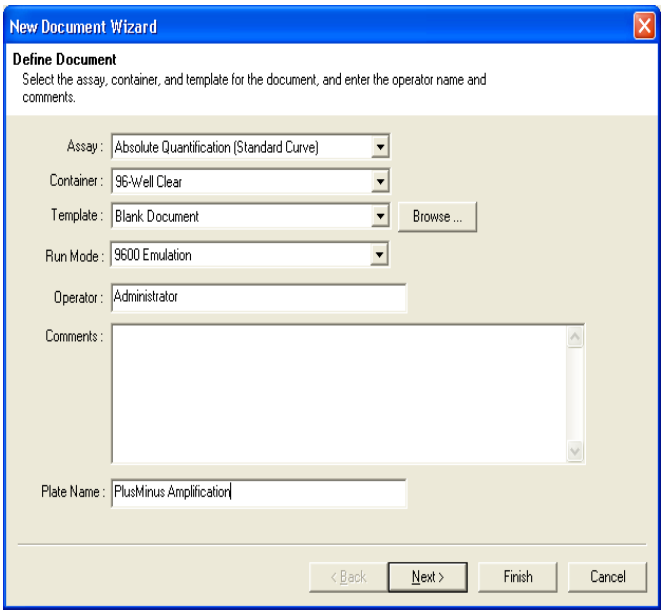
2. Select **File** > **New**. The New Document dialog box opens.

3. In the Assay drop-down list, select **Absolute Quantification (Standard Curve)**. Accept the default settings for the Container and Template (**96-Well Clear** and **Blank Document**).

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

4. In the Plate Name field, type **PlusMinus Amplification**.

5. Click **Next>**. A list of detectors is displayed in the New Document Wizard.

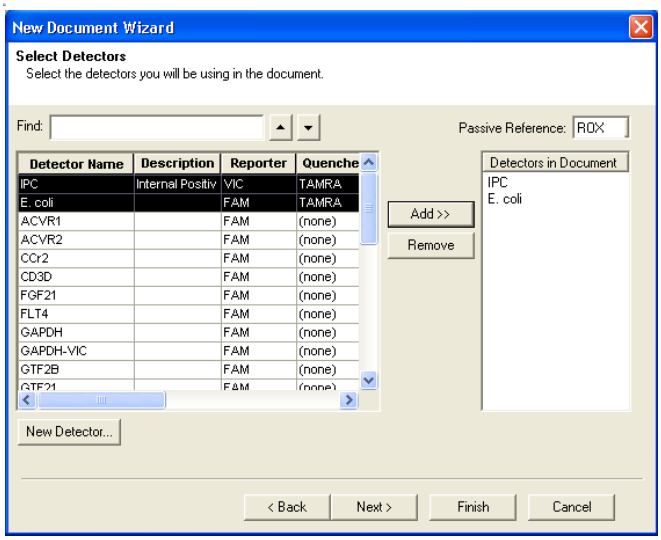


6. Select the detectors to add to the plate document, then click **Add>>**.

   IMPORTANT! Ensure that the reporter dye for the target detector is different from the reporter dye for the IPC detector, which is VIC®.

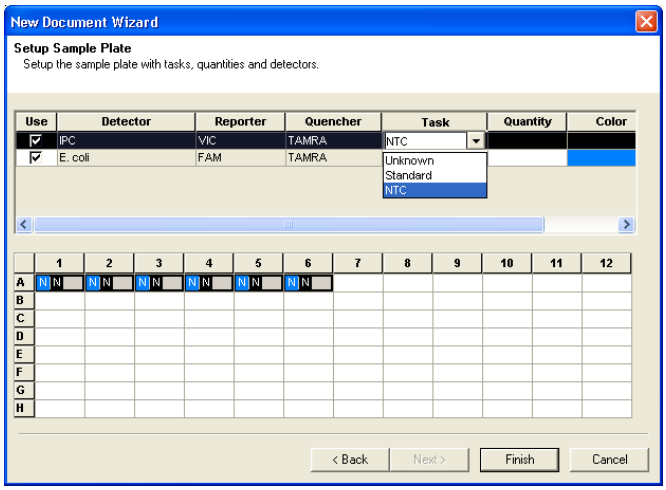
   Note: The TaqMan® Exogenous Internal Positive Control Reagents kit uses an IPC VIC-labeled probe with TAMRA™ dye-labeled quencher.

   Note: To remove a detector in the Detectors in Document window, select the detector, then click **Remove**.

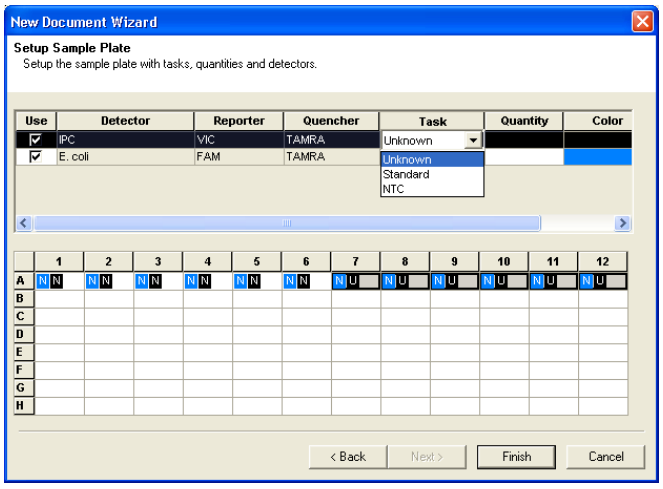


Notes

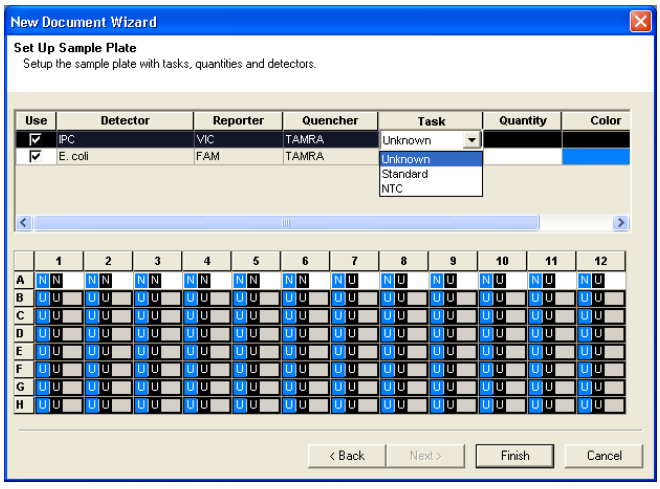
7. Select six wells on the plate document for the no amplification control (blocked IPC and no target template).
   a. Select wells A1-A6.
   b. Select the target detector by checking the **Use** box next to it.
   c. Select **Task > NTC**. An N appears in the well.
   d. Select the **IPC** detector by checking the **Use** box next to it.
   e. Select **Task > NTC**. A second N appears in the well.



8. Select six more wells on the plate document for the no template control (IPC but no target template).
   a. Select wells A7-A12.
   b. Select the target detector by checking the **Use** box next to it.
   c. Select **Task > NTC**. An N appears in the well.
   d. Select the **IPC** detector by checking the **Use** box next to it.
   e. Select **Task > Unknown**. A U appears in the well next to the N.



9. Select all remaining wells for the unknown samples (IPC template and target template).
   a. Select wells B1-H12 by click-dragging across all empty wells.
   b. Select your **Target** detector by checking the **Use** box next to it.
   c. Select **Task > Unknown**. A U appears in the boxes.
   d. Select the **IPC** detector by checking the **Use** box next to it.
   e. Select **Task > Unknown**. Another U appears next to the target U.

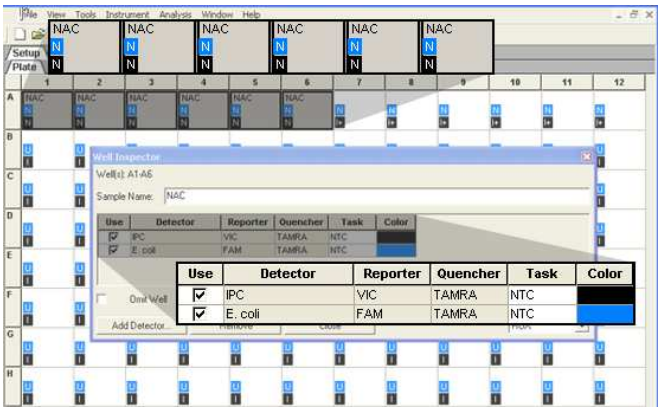


Notes

10. Click **Finish**.

The 7300/7500/7500 Fast SDS software creates the plate document.

11. Enter sample names for each well.



a. Double-click one well to open the Well Inspector, or select **View > Well Inspector**.

b. Click-drag to select all replicate wells for that sample.

c. Type the sample name in the Well Inspector. The information appears in the selected well(s).

d. Accept the default setting (ROX™) for the Passive Reference, ROX dye. Optionally, you can change the detector task and Passive Reference dye.

e. Repeat steps b through d until all wells have names.

f. Click ⊠ to close the Well Inspector.

IMPORTANT! If your experiment does not use all the wells on a plate, do not omit the wells from use at this point. You can omit unused wells after the run. For information about omitting unused wells, refer to the Online Help.

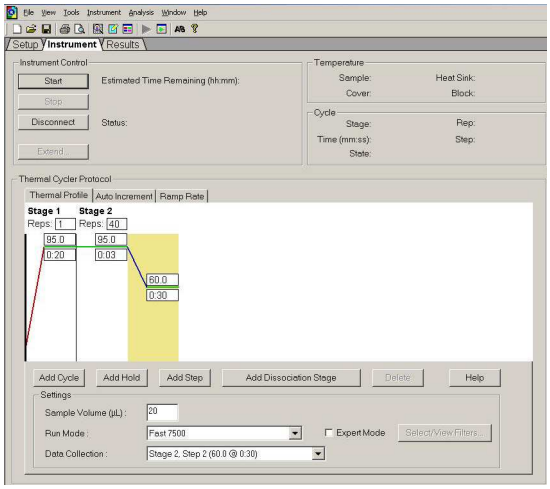
g. Verify the information about each well in the Setup tab.

Notes

Performing the Amplification Run

1. Select the **Instrument** tab.

   By default, the standard PCR conditions for the PCR step are displayed.



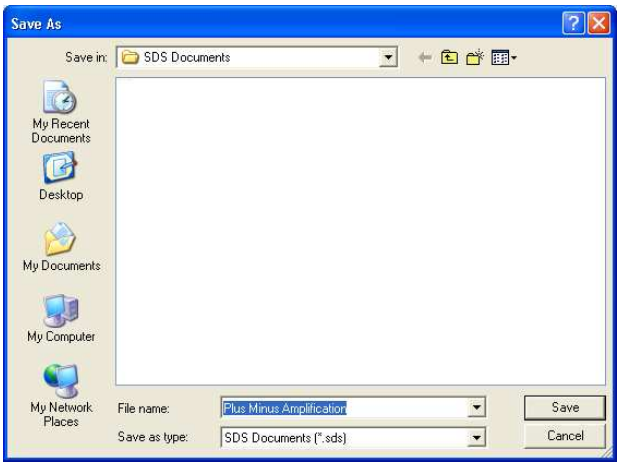
Times and Temperatures			
Initial Steps		PCR (Each of 40 cycles)	
AmpErase® UNG Activation	AmpliTaq Gold® DNA Polymerase Activation	Melt	Anneal/Extend
HOLD	HOLD	CYCLE	CYCLE
2 min @ 50 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

2. Accept default values for:
   - Sample volume – 50 µL (20 µL for the Fast system).
   - 9600 Emulation – selected.

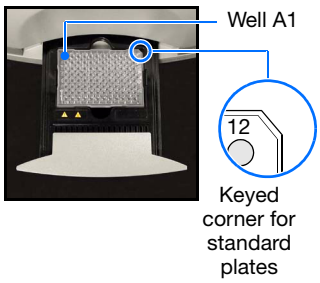
   Note: The 9600 Emulation feature is not available for the 7300 instrument.

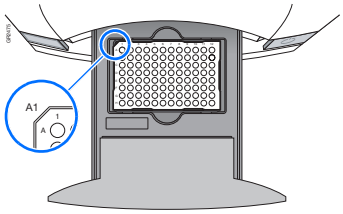
Notes

3. Select **File** > **Save**, then click **Save** to retain the name you assigned when you created the plate document.



4. Load the reaction plate into the instrument.





Keyed corner for Fast plates

Note: The A12 position is notched in the top-right side of the instrument tray for the 7300/7500 system. The A1 position is notched on the top-left side of the instrument tray for the 7500 Fast system.

5. Click **Start**.

   As the instrument performs the PCR run, it displays real-time status information in the Instrument tab and records the fluorescence resulting from cleavage of TaqMan® probes in the presence of the target sequences.

   After the run is finished, the status values and the buttons are grayed-out, the Analysis button is enabled (▶), and a message indicates whether or not the run is successful.

   All data generated during the run are saved to the plate document that you specified in step 3, and this data can be analyzed later for troubleshooting purposes.

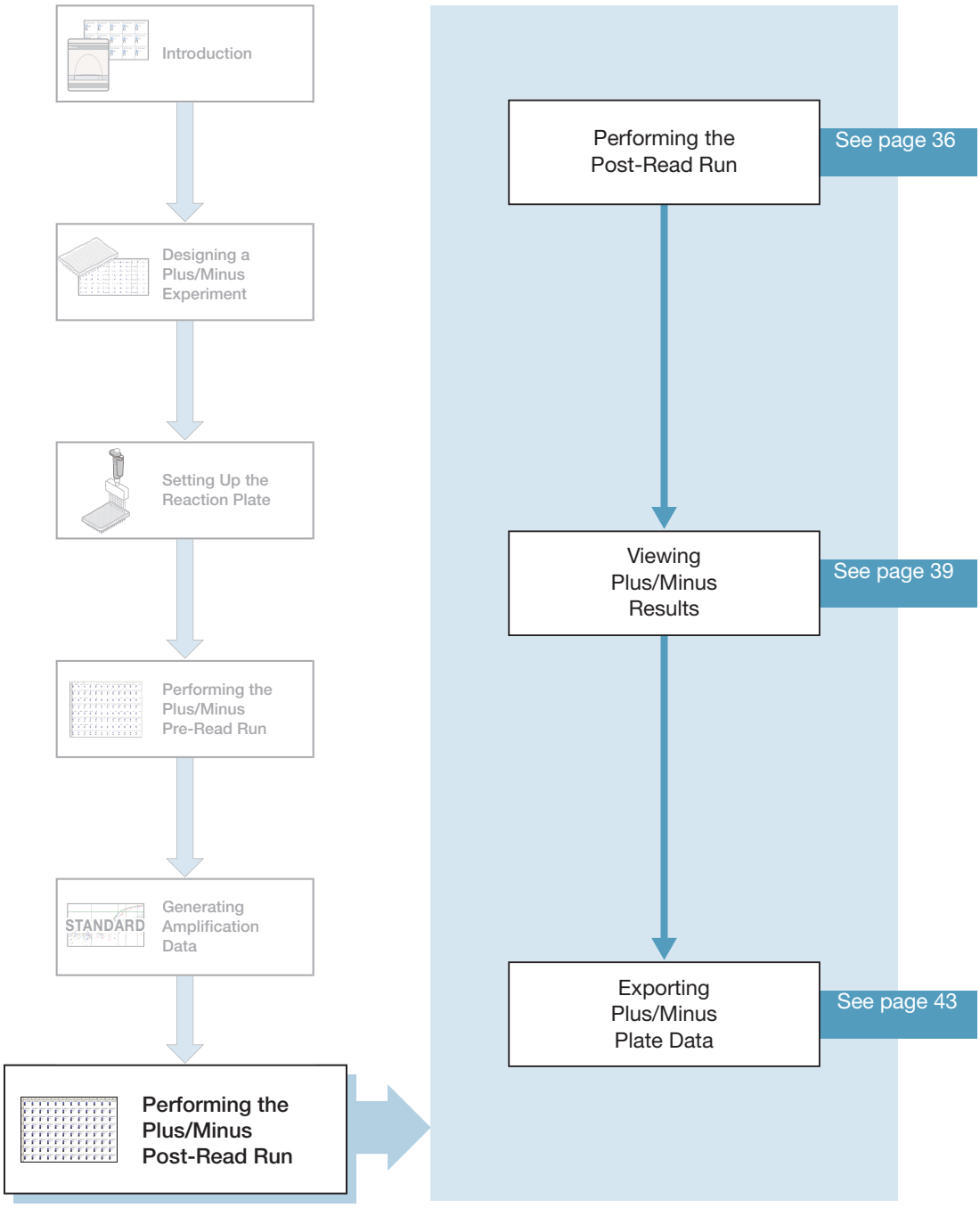
6. To view real-time PCR after the run is finished, click the Analysis button ▶, select the **Results** tab, select the **Amplification Plot** tab, then select all wells in the upper left box (next to A1).

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

Notes

Notes

Performing the Plus/Minus Post-Read Run

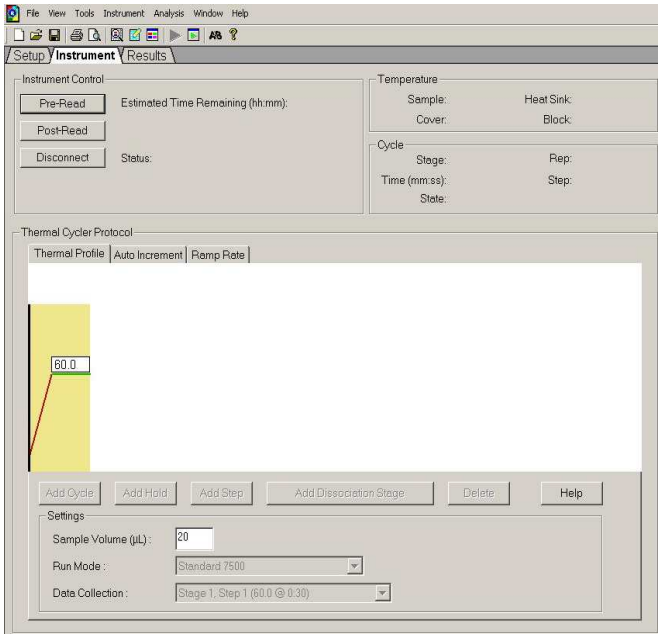


Notes

Performing the Post-Read Run

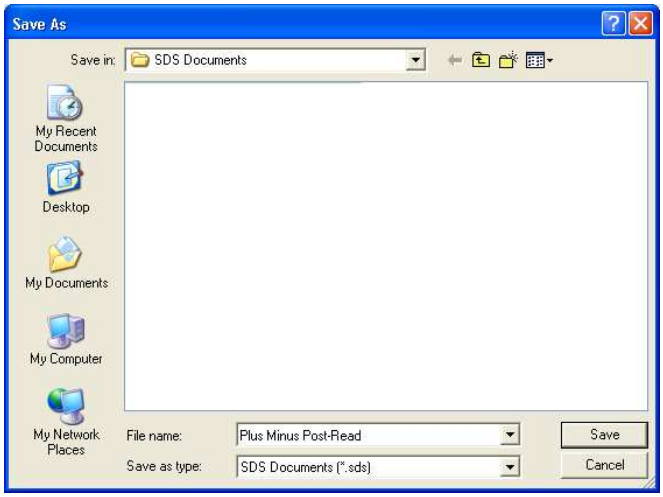
Open the pre-read plate document.

1. Select the **Instrument** tab.



2. Accept the default value for sample volume.

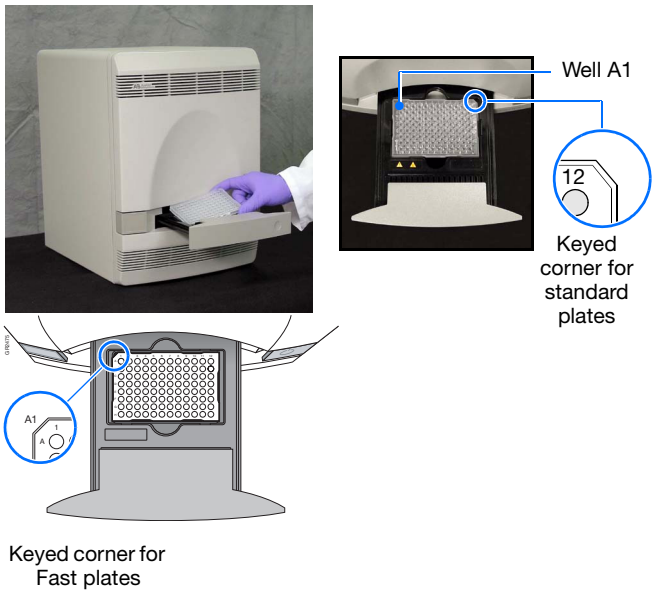
3. Select **File** > **Save As**, type the name **Plus/Minus Post-Read** for the plus/minus plate document, then click **Save**.



Notes

4. Load the reaction plate into the instrument.

   Note: The A12 position is notched in the top-right side of the instrument tray for the 7300/7500 system. The A1 position is notched on the top-left side of the instrument tray for the 7500 Fast system.



5. Click **Post-Read**.

   After the run is finished, the status values and buttons are grayed-out, and a message indicates whether or not the run is successful.

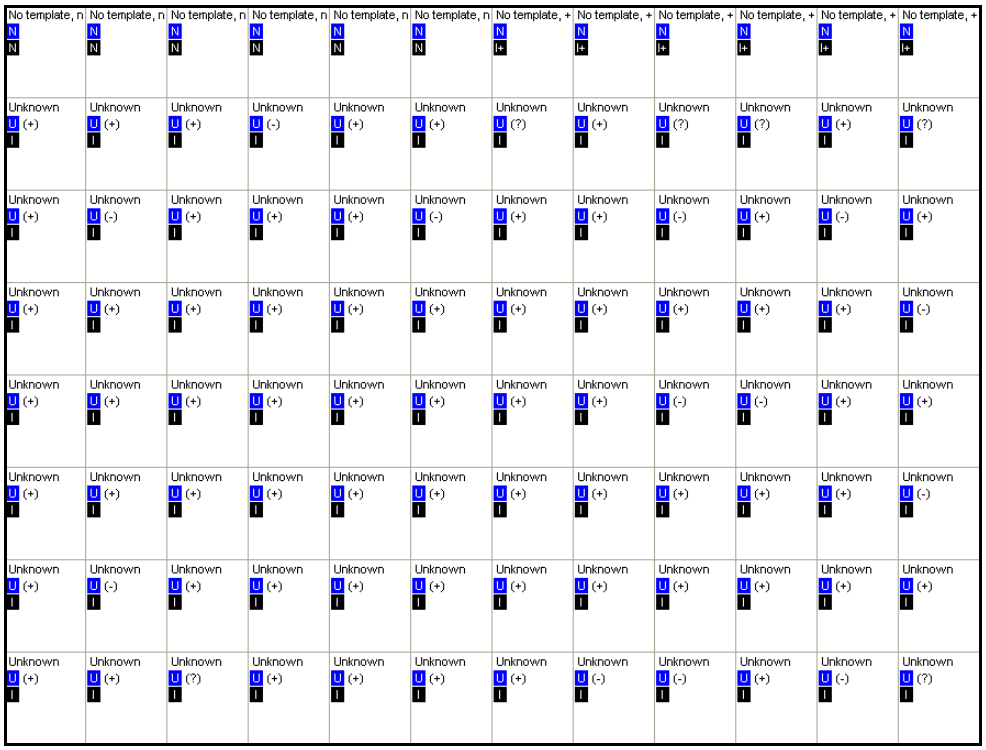
6. Click the green analysis button ( ) to start analysis.

   All data generated during the run are saved to the plus/minus plate document that you specified in step 3.

Notes

Sample Experiment

In the example plus/minus experiment using an IPC, the pre-read run was subtracted from the post-read run to account for background fluorescence. Post-read results for the presence of *E. coli* are displayed in the Results > Plate tab. For an explanation of results see "Viewing Plus/Minus Results" on page 39.



Notes

Viewing Plus/Minus Results

Results

After completing the plus/minus post-read run, the SDS software compares the relationship between the spectral changes in the unknown samples and the control reactions defined previously, NAC and NTC. An IPC threshold is calculated from the NAC control reactions, and the target threshold is calculated from the NTC control reactions. The target threshold is used to determine amplification of the unknown sample signal for each well. The IPC threshold is used to determine amplification of the IPC signal in each unknown sample well.

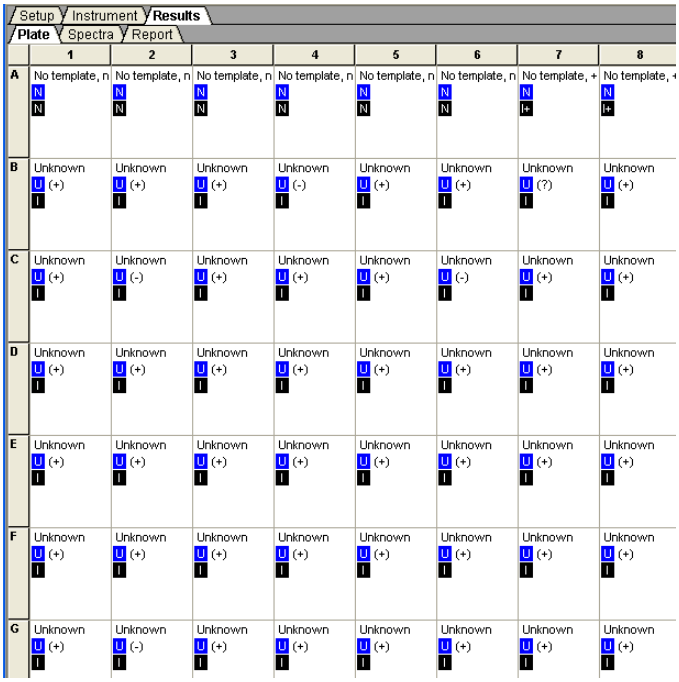
Calling Unknowns Using IPC

To call the unknown samples, the SDS software compares the normalized reporter signal of each unknown sample to the target threshold and IPC sample to IPC threshold. The results are determined as follows:

- If the unknown sample signal is above the target threshold, then the call is positive (+).
- If the unknown sample signal is below the target threshold, the SDS software compares the IPC sample signal to the IPC threshold as follows:
  - If IPC sample signal is above the IPC threshold, then the call is negative (–).
  - If IPC sample signal is below the IPC threshold, then the call is undetermined (**?**).

The Plate Tab

When the post-read is complete, select the Plate tab on the Results page to display the plus/minus calls for the presence or absence of the target sequence for each well.



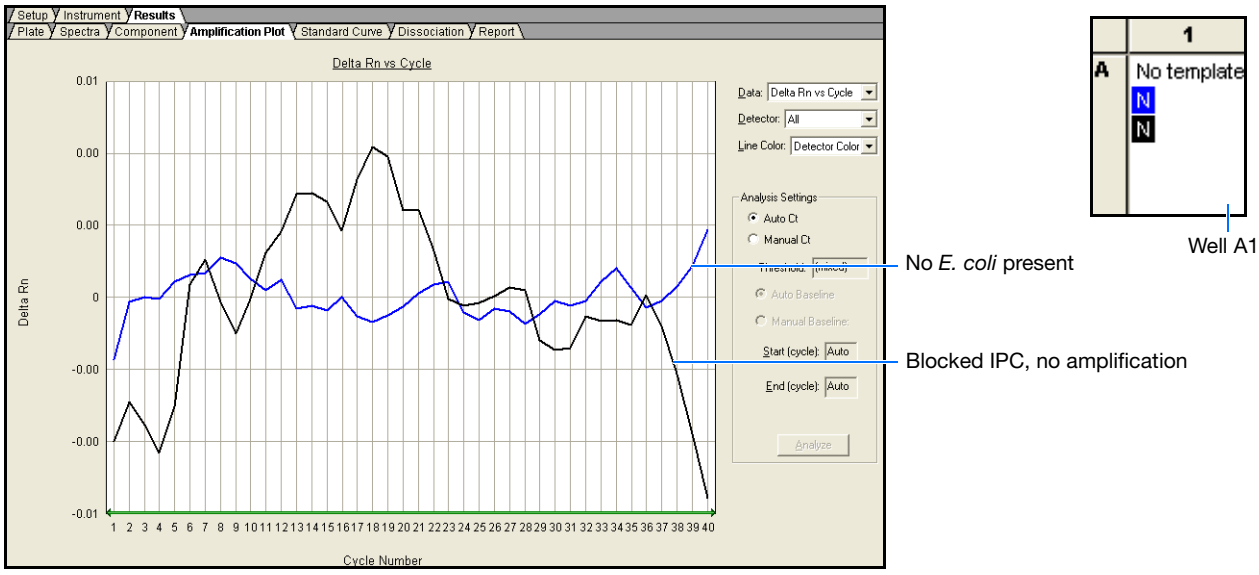
Notes

The following Example Experiment boxes show the amplification plots of each type of result (NAC, NTC, plus (+), minus (–), and undetermined (**?**)).

Note: The amplification plots are obtained from the AQ plate document used to amplify the samples in the 96-well plate.

Sample Experiment

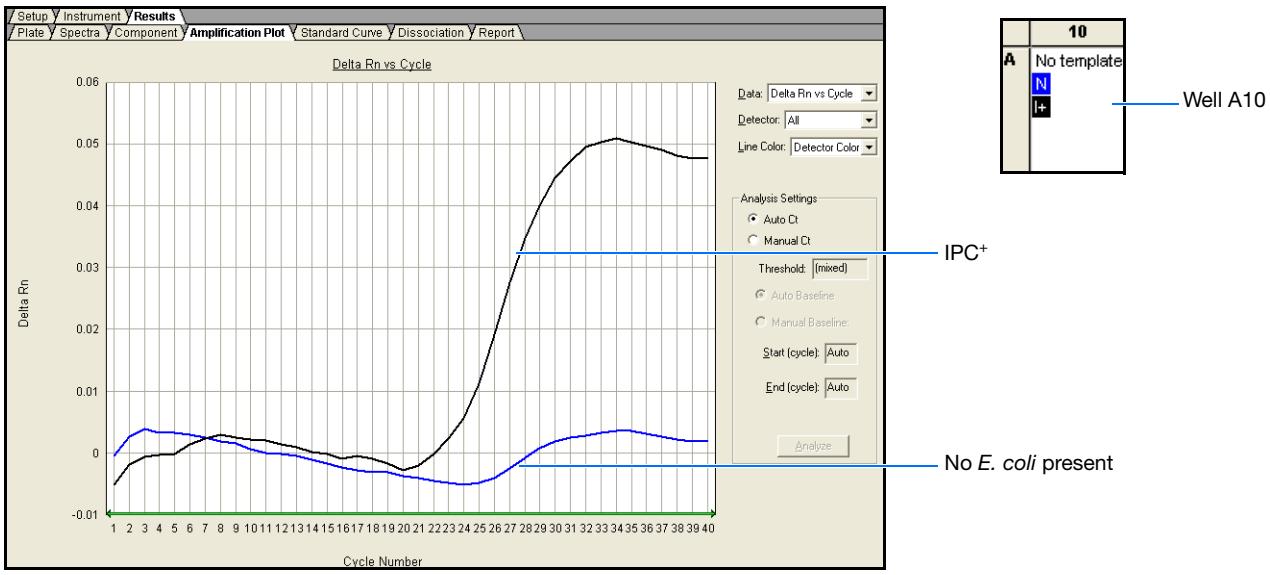
The image below displays the amplification plot for well A1, the No Amplification Control (NAC), which contains blocked IPC and no target template. This plot demonstrates that there is no amplification for IPC or the *E. coli* target sequence.



Notes

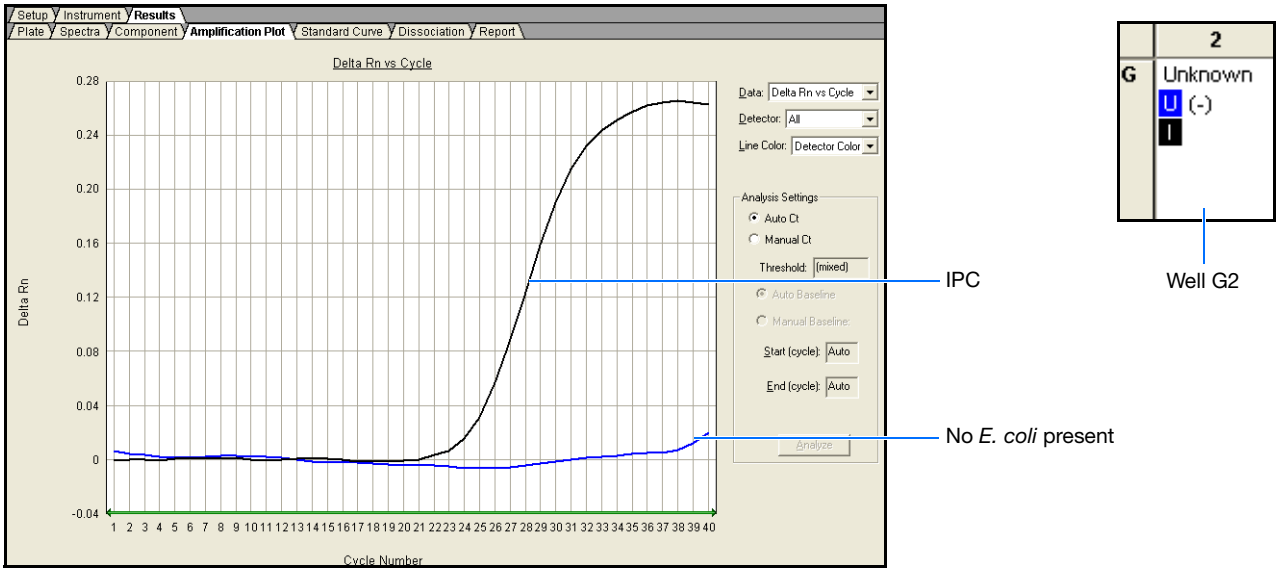
Sample Experiment

The image below shows the amplification plot for well A10, the No Template Control (NTC), which contains IPC but no target template. This plot demonstrates a positive amplification curve for IPC and no amplification for the *E. coli* target sequence.



Sample Experiment

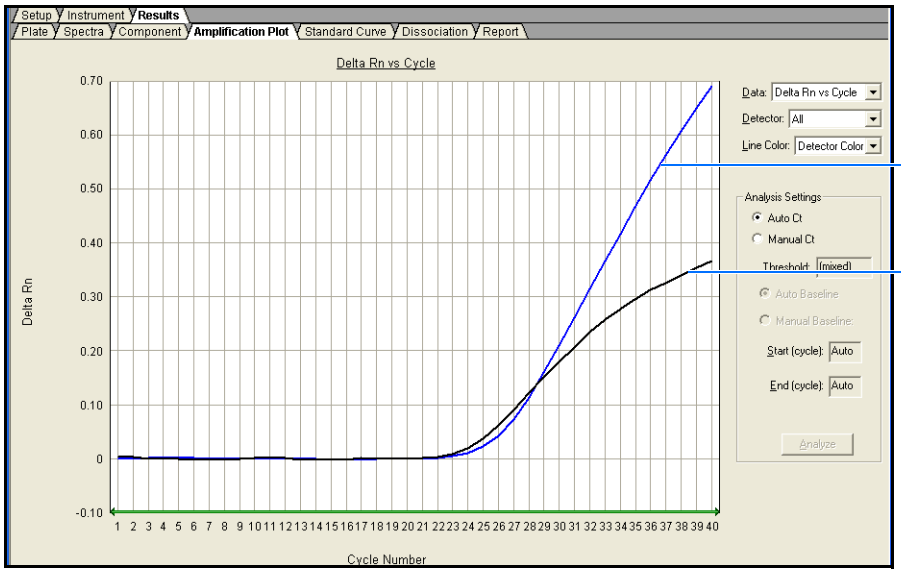
The image below shows the amplification plot for unknown sample well G2, which displays a negative (-) result. The sample is negative because the amplification for the *E. coli* target sequence is less than the target threshold, while the IPC sample signal is above the calculated IPC threshold defined by the SDS software.

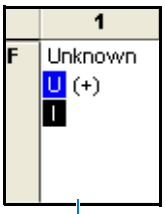


Notes

Sample Experiment

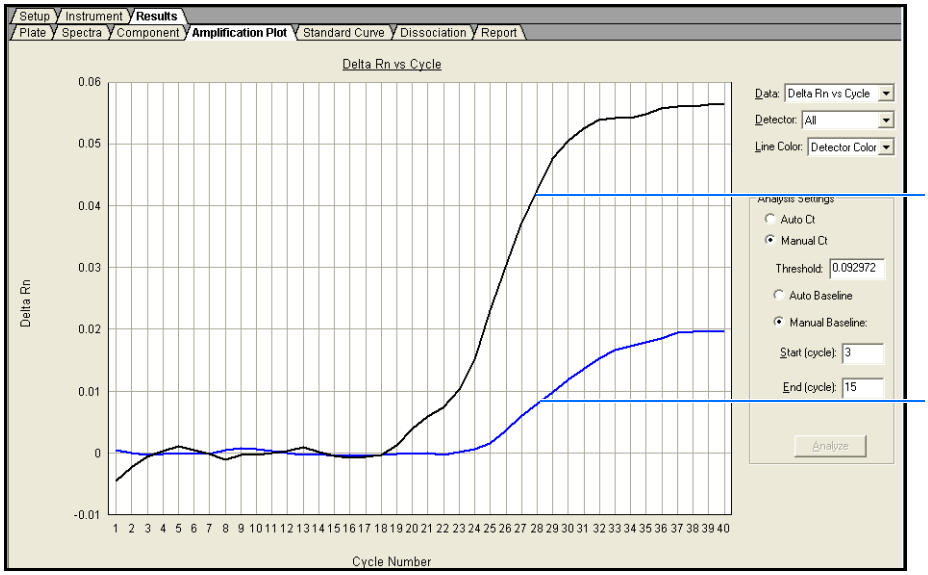
The image below shows the amplification plot for unknown sample well F1, which displays a positive (**+**) result. The sample is positive for the *E. coli* target sequence because it has amplification above the target threshold.

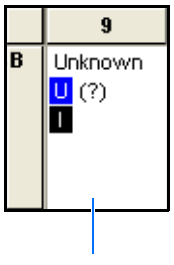




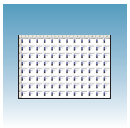
Sample Experiment

The image below shows the amplification plot for unknown sample well B9, which displays a questionable (**?**) result. The unknown sample is labeled with a question mark (**?**) because the unknown sample signal is below the target threshold and the IPC sample signal is below the calculated IPC threshold defined by the SDS software.





Notes



Note: For more information on analyzing the amplification data and your plus/minus results, see Appendix B, "Viewing Amplification Data," on page 47.

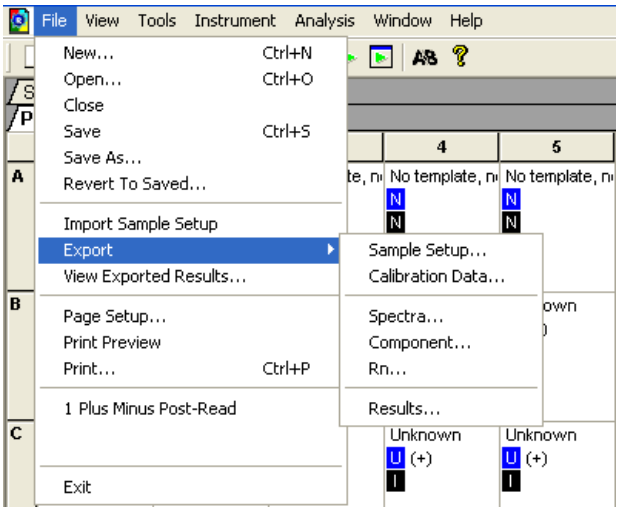
Exporting Plus/Minus Plate Data

You can export numeric data from plus/minus plates into text files, which can then be imported into spreadsheet applications such as Microsoft® Excel.

1. Select **File** > **Export**, then select the data type to export:

   - **Sample Setup** (*.txt)
   - **Calibration Data** (*.csv)
   - **Spectra** (*.csv)
   - **Component** (*.csv)
   - **Rn** (*.csv)
   - **Results** (*.csv)

   Refer to the Online Help for information about the export file types.

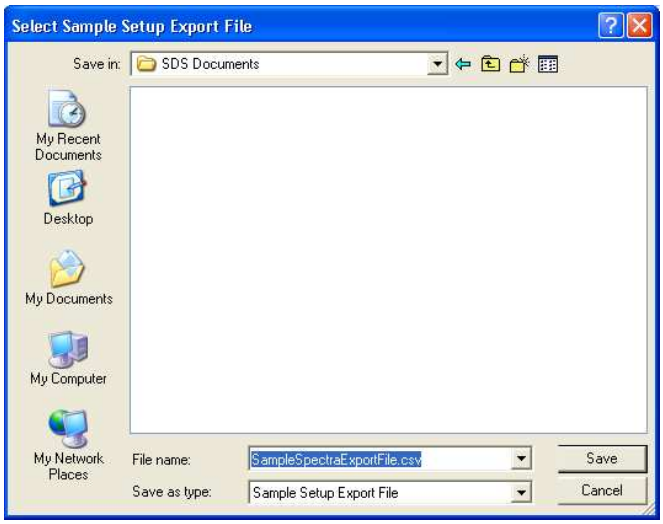


Notes

2. Type a file name for the export file.

   Note: The name of the dialog box depends on the type of data you selected to export.

3. Click **Save**.



Notes

Creating Detectors

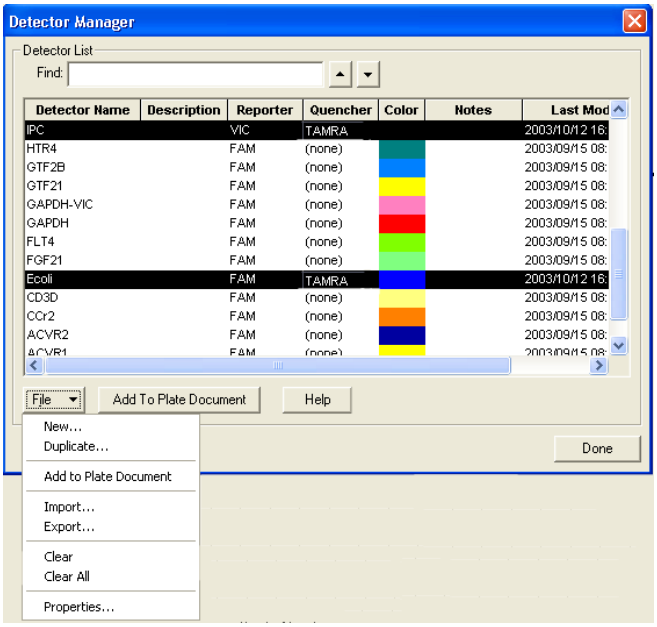
Before you can use a plate document to run a plate, create and apply detectors for all samples on the plate. A detector is a virtual representation of a gene- or allele-specific nucleic acid probe reagent used for analyses performed on instruments.

To create a detector:

1. Select **Tools > Detector Manager**.

   Note: A plate document (any type) must be open before you can access the Tools menu.

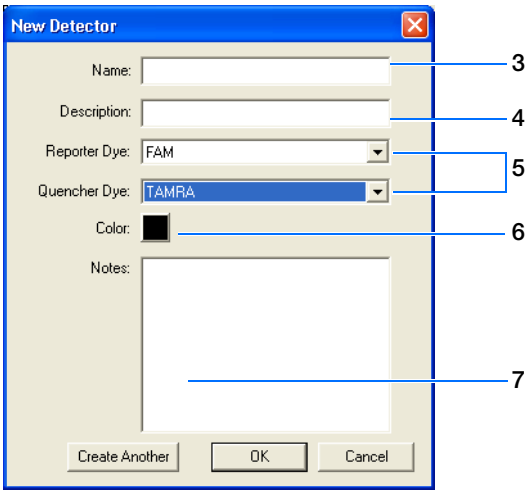
2. Select **File > New**.



3. Enter a name for the detector.

   IMPORTANT! The name of the detector must be unique and should reflect the target locus of the assay (such as IPC or *E. coli*). Do not use the same name for multiple detectors.

4. Optionally, click the **Description** field, then enter a brief description of the detector.



Notes

5. In the Reporter Dye and Quencher Dye drop-down lists, select the appropriate dyes for the detector.

   Note: The dyes that appear in the Reporter and Quencher Dye lists are those that have been previously entered using the Dye Manager. If the dye that you want to use does not appear in a list, use the Dye Manager to add the dye and then return to step 5 in this procedure. Refer to the Online Help for more information.

6. Click the **Color** box, select a color to represent the detector using the Color dialog box, then click **OK**.

7. Optionally, click the **Notes** field, then enter any additional comments for the detector.

8. Click **OK** to save the detector and return to the Detector Manager.

9. Repeat steps 2 to 8 for the remaining detectors.

10. In the Detector Manager, click **Done** when you finish adding detectors.

Sample Experiment

In the example plus/minus experiment, a detector was created for the *E. coli* target, and another was created for the IPC. The *E. coli* detector was assigned a blue color and IPC a black color.

Note: When creating detectors, you use the reporter dye and quencher information (and optionally, the gene name or symbol for the sample name). You can view the contents in a spreadsheet program, such as Microsoft® Excel.

Notes

Viewing Amplification Data

Specifying Analysis Settings

Before you analyze, specify parameters to enable auto-baseline and auto-threshold calculations.

Unless you have already determined the optimal baseline and threshold settings for your experiment, analyze data twice: first using the automatic baseline and threshold feature of the SDS software (**Auto C_T**), and again after determining the optimal baseline and threshold for your data.

To specify analysis settings:

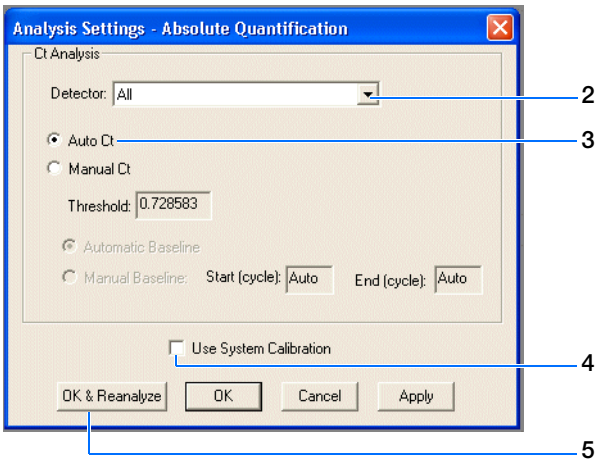
1. Click or select **Analysis** > **Analysis Settings**.
2. In the Detector drop-down list, select **All**.
3. Select **Auto Ct** to set the SDS software to automatically generate baseline and threshold values for each well in the study.

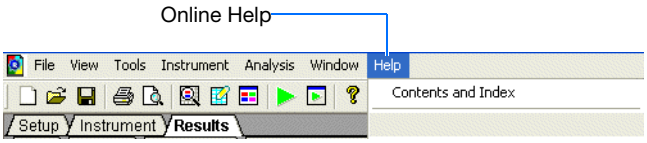
   Note: After analysis, you must verify that the baseline and threshold were called correctly for each well. Alternatively, you can select Manual Ct and specify the threshold and baseline manually. For more information about manually adjusting C_T, refer to the Online Help.

4. Select **Use System Calibration** to use the calibration files (Background and Pure Dye) that are stored on the computer rather than the calibration information that is stored in the plate document itself.

   For more information about system calibration files, refer to the Online Help.

5. Click **OK & Reanalyze**.





Notes

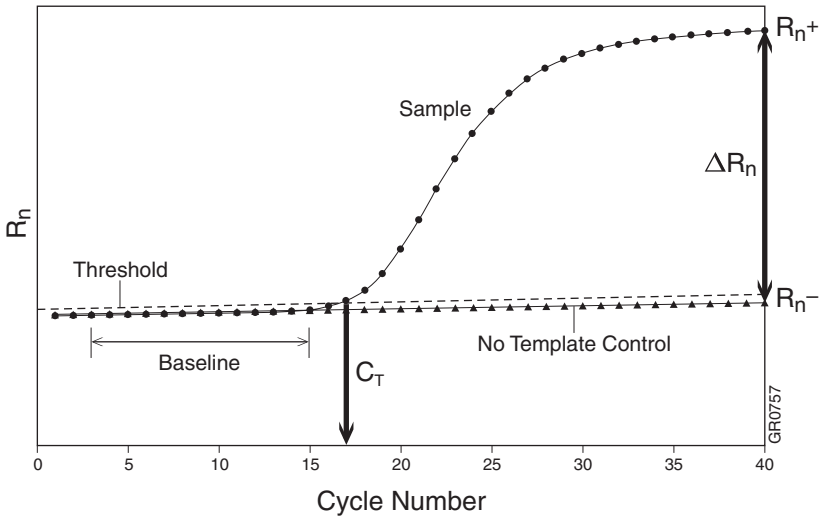
Analyzing the Plus/Minus Amplification Data (AQ Plate)

Terms Used in Quantification Analysis

The following are terms commonly used in quantification analysis.

Term	Definition
Baseline	A line fit to fluorescence intensity values during the initial cycles of PCR, in which there is little change in fluorescence signal.
Threshold cycle (C_T)	The fractional cycle number at which the fluorescence intensity exceeds the threshold intensity.
Passive reference	A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations caused by changes in concentration or volume.
Reporter dye	The dye attached to the 5′ end of a TaqMan® probe. The dye provides a signal that indicates specific amplification.
Normalized reporter (R_n)	The ratio of the fluorescence intensity of the reporter dye signal to the fluorescence intensity of the passive reference dye.
Delta R_n (ΔR_n)	The magnitude of the signal generated by a set of PCR conditions ($\Delta R_n = R_n$ –baseline).

The figure below is a representative DNA amplification plot and includes some of the terms defined above.



Starting the Analysis

To analyze the amplification data (AQ plate), click ▶ or select **Analysis > Analyze**. The software generates several types of result views, as described in the following section.

Notes

Viewing the Amplification Data

About the Results Tab

In the Results tab, you can view the results of the amplification run, change the parameters, and reanalyze the data.

The Results tab has seven secondary tabs. Details about each tab are provided in the Online Help.

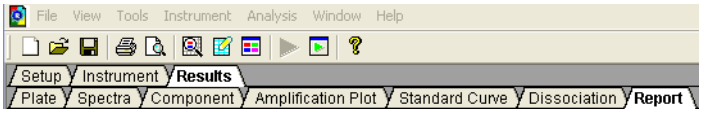
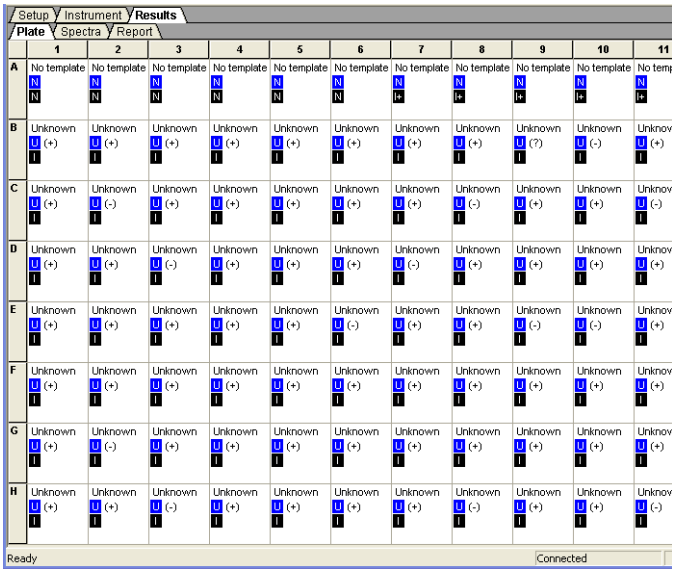


Plate Tab

Displays the results data of each well, including:

- The sample name and detector task and color for each well.
- A calculated value – quantity (default; displays are not determined for runs without standard curves), ΔRn, or C_T. Select **Analysis > Display** to select the value to display.

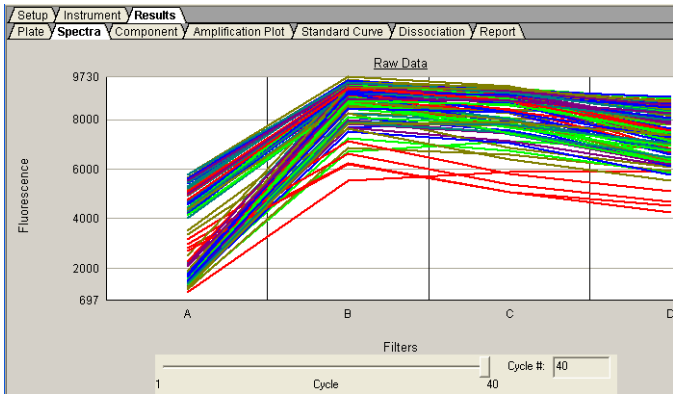


Notes

Spectra Tab

Displays the fluorescence spectra of selected wells.

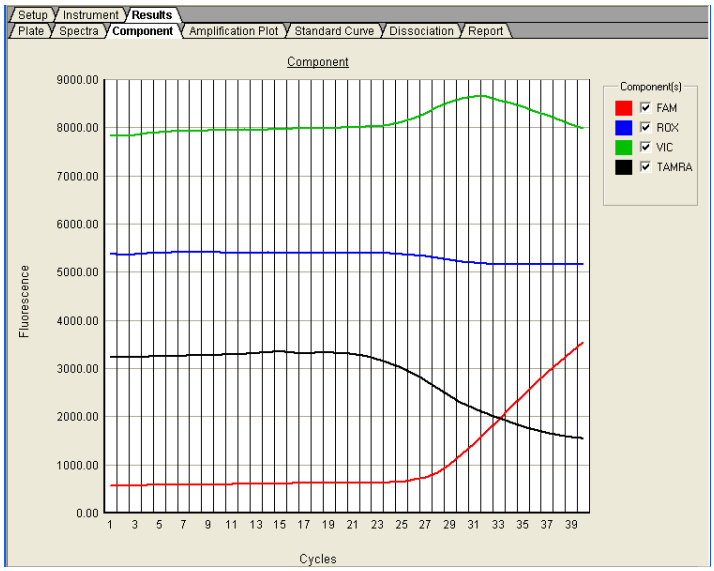
- The Cycles slider allows you to see the spectra for each cycle by dragging it with the pointer.
- The Cycle # text box shows the current position of the slider.



Double-clicking the y-axis opens the Graph Settings dialog box where you can reset the y- and x-axes or allow autoscaling.

Component Tab

This tab displays the complete spectral contribution of each dye in a selected wells over the duration of the PCR run. Only the first selected well is shown at one time.



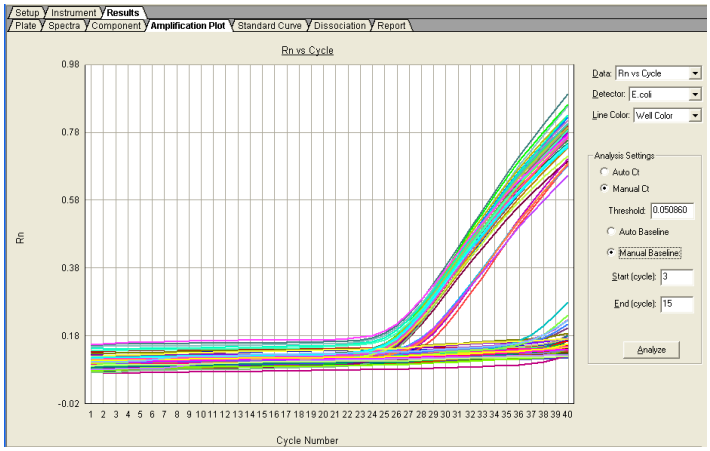
Double-clicking the y-axis displays the Graph Settings dialog box.

Notes

Amplification Plot Tab

The Amplification Plot tab allows you to view both real-time and post-run amplification of specific samples. The Amplification Plot tab displays all samples in the selected wells.

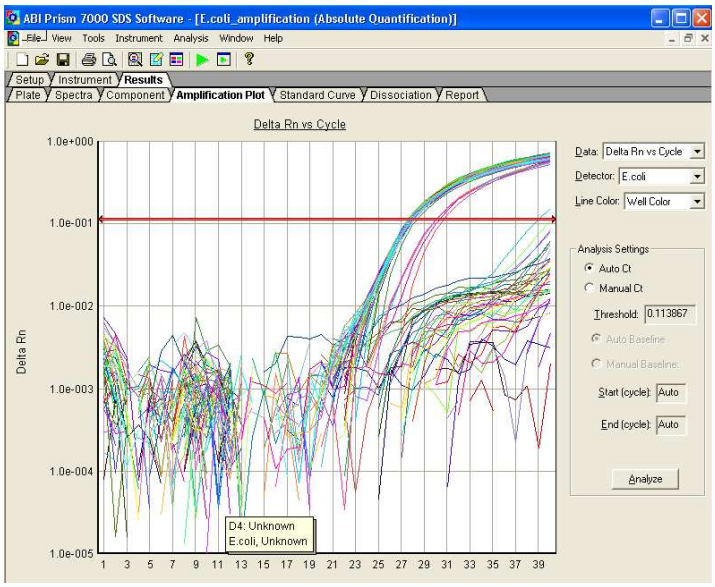
Rn vs. Cycle (Linear)



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

For more information about R_n, refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4378658).

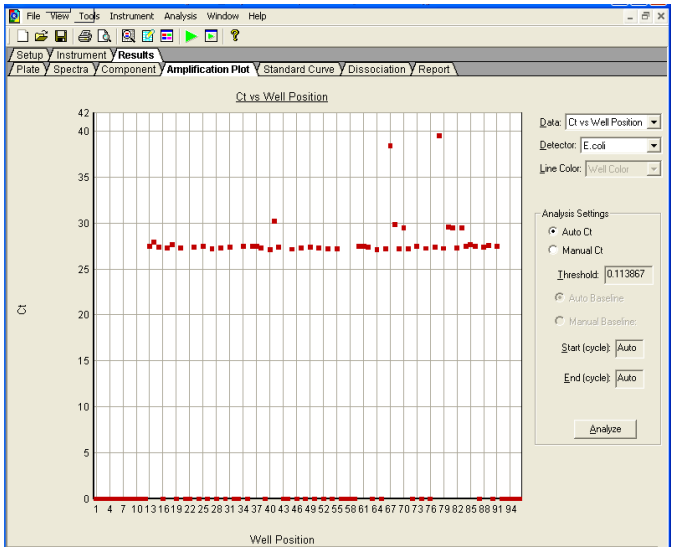
ΔRn vs.Cycle (Log)



This plot displays the Rn dye fluorescence as a function of cycle. You can use this plot to identify and examine irregular amplification and to manually set the threshold and baseline parameters for the run.

Notes

Ct vs. Well Position Plot



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

Report Tab

This tab displays data for selected wells in a table format. The data columns associated with the report are determined by the assay type. For plus/minus assays, the following data columns are available: Well, Sample Name, Detector, Task, C_T, and Std Dev C_T.

You can format the display of the report and how the report is printed. Refer to the Online Help for more information.

Setup | Instrument | **Results**
Plate | Spectra | **Report**

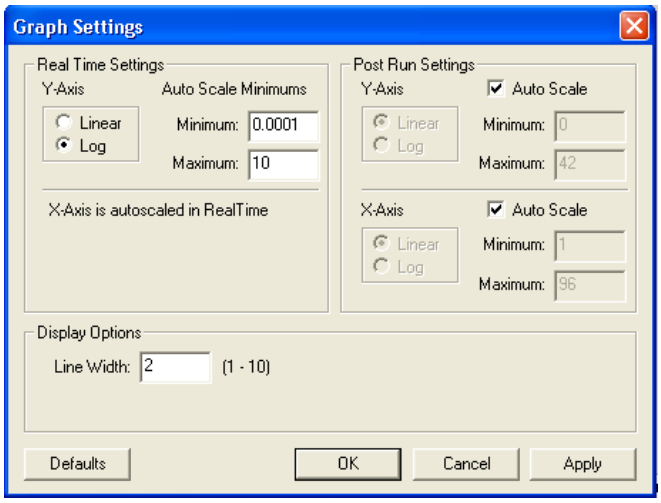
Well	Sample Name	Detector	Task	Call	Rn	Mean Rn	Std Dev Rn
A1	No template, no IPC	E.coli	NTC		0.150	0.1373	0.0113
		IPC	NTC		1.272	1.3710	0.0606
A2	No template, no IPC	E.coli	NTC		0.120	0.1373	0.0113
		IPC	NTC		1.344	1.3710	0.0606
A3	No template, no IPC	E.coli	NTC		0.149	0.1373	0.0113
		IPC	NTC		1.396	1.3710	0.0606
A4	No template, no IPC	E.coli	NTC		0.138	0.1373	0.0113
		IPC	NTC		1.417	1.3710	0.0606
A5	No template, no IPC	E.coli	NTC		0.133	0.1373	0.0113
		IPC	NTC		1.357	1.3710	0.0606
A6	No template, no IPC	E.coli	NTC		0.132	0.1373	0.0113
		IPC	NTC		1.441	1.3710	0.0606
B1	Unknown	E.coli	Unknown	(+)	0.743	0.5025	0.3030
		IPC	IPC		1.329	1.4925	0.2040
B2	Unknown	E.coli	Unknown	(+)	0.759	0.5025	0.3030
		IPC	IPC		1.199	1.4925	0.2040
B3	Unknown	E.coli	Unknown	(+)	0.744	0.5025	0.3030
		IPC	IPC		1.126	1.4925	0.2040
B4	Unknown	E.coli	Unknown	(-)	0.133	0.5025	0.3030
		IPC	IPC		1.731	1.4925	0.2040

Notes

Adjusting Graph Settings

Clicking on the Spectra, Component, Amplification Plot, and Standard Curve displays the Graph Settings dialog box, which allows you to adjust the plot settings.

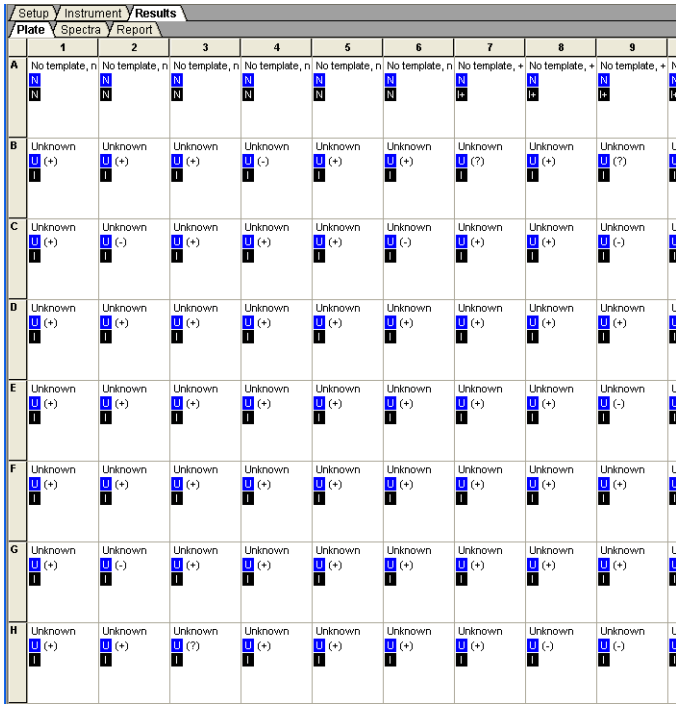
The adjustable settings depend on which plot you are viewing. Refer to the Online Help for more information.



Notes

Overview of Result Calls

Go to the plate Results tab to view the plus and minus calls for the presence or absence of the target sequence for each well. For further determination of the results, check the plots for each well.



Notes

Example Plus/Minus Experiment

Overview

To better illustrate how to design, perform, and analyze plus/minus experiments, this section provides an example experiment. The example experiment represents a typical plus/minus experiment that you can use as a quick-start procedure to familiarize yourself with the plus/minus workflow. Details about the plus/minus workflow are described in the subsequent chapters of this guide. Example Experiment boxes appear in subsequent chapters to illustrate workflow details.

Description

The objective of the example plus/minus experiment is to determine if an *E. coli* target sequence is present or not present in each batch of hamburger meat. The experiment uses duplex PCR where a set of primers and a VIC®-labeled probe for the IPC plus a set of primers and a FAM™-labeled probe for the target *E. coli* sequence are run together in each reaction. The set of primers/probe for detecting *E. coli* was custom designed by Applied Biosystems Primer Express® software.

Reactions were set up for PCR using the TaqMan® Universal PCR Master Mix and appropriate primers and probes.

The example plus/minus experiment data and results were generated using a 7300/7500 system by performing:

- **A pre-read run** on a plus/minus plate to determine the baseline fluorescence associated with primers and probes before amplification.
- **An amplification run** using an AQ plate document to generate real-time PCR data, which can be used to analyze and troubleshoot the PCR data for the plus/minus assay, if needed.
- **A post-read run** using the original plus/minus plate document, which automatically subtracts the pre-read baseline fluorescence determined during the pre-read run, then assigns positive or negative calls using the amplified data.

Notes

Example Plus/Minus Experiment Procedure

Design the experiment and prepare DNA:

1. Design the experiment as explained in Chapter 2.
   a. Order the TaqMan® Exogenous Internal Positive Control Reagents kit and the TaqMan® Universal PCR Master Mix.
   b. Design the primers and FAM™-labeled probe set for *E. coli* detection with Applied Biosystems Primer Express software.
2. Extract DNA from samples (see "Preparing DNA" on page 12) using the PrepMan Ultra Sample Preparation Reagent Kit (PN 4322547) and protocol (PN 4318925) to obtain a final concentration of 10 ng/µL of DNA for each sample.
3. Prepare sufficient reaction mix (see "Preparing the PCR Reaction Mix" on page 14) by using the volumes as listed in the table on the right

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

Item	Volume for one Reaction (Standard)	Volume for one Reaction (Fast)	Final Concentration
TaqMan® Universal PCR Master Mix (2×)	25.0	10.0	1×
10× Exo IPC Mix (IPC kit)	5.0	2.0	50 to 900 nM
50× Exo IPC DNA (IPC kit)	1.0	0.4	50 to 900 nM
Target primers, probe, and deionized water	14.0	5.6	50 to 250 nM
Total	45.0	18.0	—

Notes

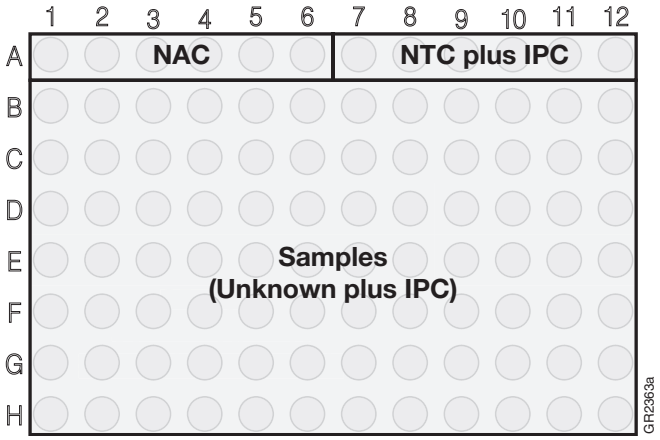
4. Prepare the reaction plate:

   a. Pipette 45 µL (18 µL for the Fast system) of the reaction mixture into each well of a 96-well reaction plate.

   b. Pipette 5 µL (2 µL for the Fast system) of IPC block, TE or water, or unknown sample into the designated wells of a 96-well plate such as the example indicated in the table to the right. (see "Setting Up the Reaction Plate" on page 13).

   Note: The final reaction volume in each well is 50 µL (20 µL for the Fast system).

   c. Keep the reaction plate on ice until you are ready to load it into the 7300/7500/7500 Fast system.



Wells	If preparing...	Add (Standard)	Add (Fast)
A1 to A6	NAC‡	5 µL of 10× Exo IPC Block	2 µL of 10× Exo IPC Block
A7 to A12	NTC§	5 µL of 1× TE or H_2O	2 µL of 1× TE or H_2O
B1 to H12	U#	5 µL of sample being tested for *E. coli*	2 µL of sample being tested for *E. coli*

‡ No Amplification Control – Well contains no target template and no IPC.
§ No Template Control – Well contains no target template, only IPC.
# Unknown – Well contains both target template and IPC.

C

Notes

Perform the pre-read run:

1. Create a plus/minus plate document:

   Follow the instructions as described in Chapter 4. Briefly, in the New Document Wizard,

   a. Select **File > New**.

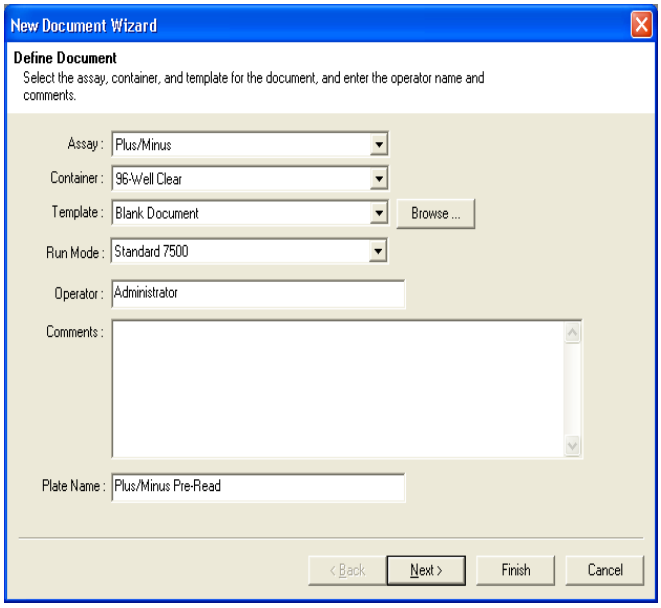
   b. Select **Plus/Minus** in the Assay drop-down list.

   c. In the Plate Name field, type **Plus/Minus Pre-Read**, then click **Next**.

   d. Add detectors to the plate document (see Appendix A, Creating Detectors on page 45) then click **Next**.

   e. Specify detectors and tasks for each well, then click **Finish**.

   f. Double-click each well to type the sample name, then save the document.



2. Enter the sample names and specify tasks in the Well Inspector (**View > Well Inspector**).

   IMPORTANT! If your experiment does not use all the wells on a plate, do not omit the wells from use at this point. You can omit unused wells after the run is completed. For more information about omitting wells, refer to the Online Help.

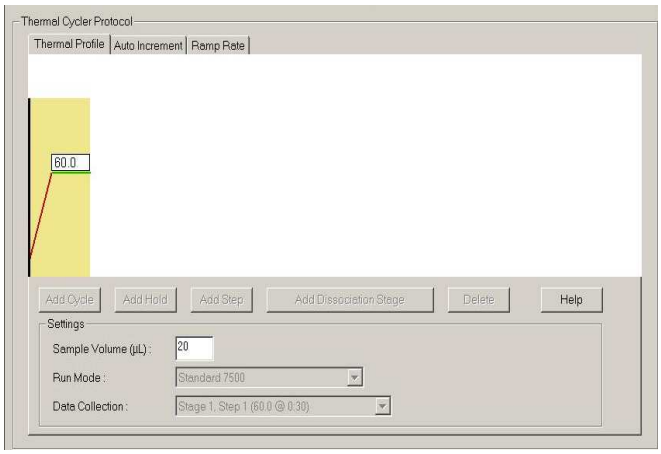
3. Perform the plus/minus pre-read run.

   a. Select the **Instrument** tab. By default, the standard PCR conditions are displayed.

   b. Select **File > Save As**, type a name for the plus/minus plate document, then click **Save**.

   c. Load the reaction plate into the instrument.

   d. Click **Pre-Read**.



Notes

Amplify the DNA:

1. Create an AQ plate document for amplification:

   Follow the instructions as described in Chapter 5. Briefly, in the New Document Wizard,

   a. Select **File > New**.

   b. Select **Absolute Quantification (Standard Curve)** in the Assay drop-down list.

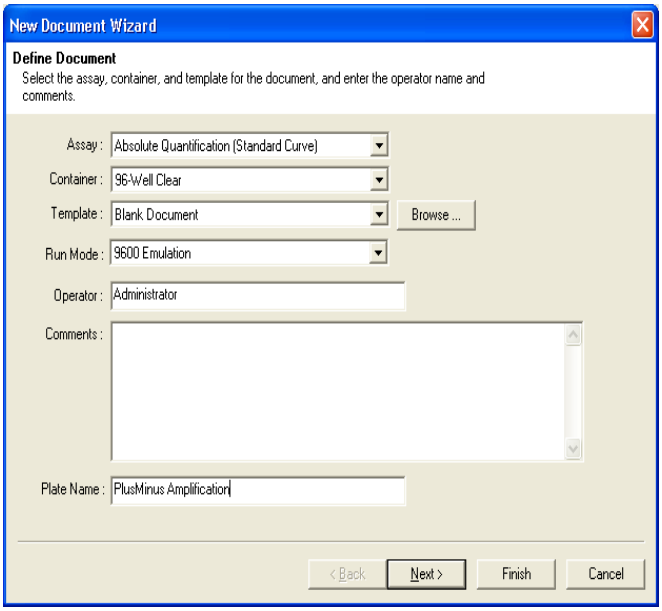
   c. In the Plate Name field, type **PlusMinus Amplification**, then click **Next**.

   Note: A standard curve is not needed for a non-quantification amplification run.

   d. Add detectors to the plate document (see Appendix A, "Creating Detectors," on page 45), then click **Next**.

   e. Specify the detectors and tasks for each well, then click **Finish**.

   f. Type the sample names, then save the document.



2. Perform the plus/minus amplification run.

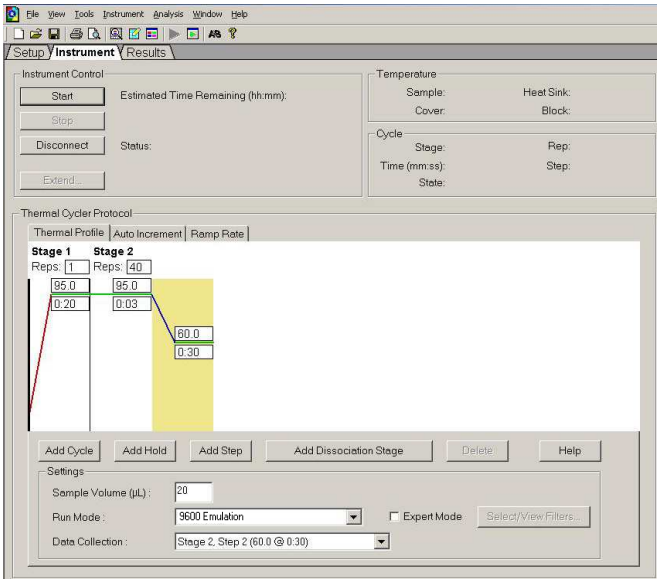
   a. Select the **Instrument** tab.

   b. Select **File > Save As**, type a name for the AQ Plate document, then click **Save**.

   c. Load the reaction plate into the instrument, then click **Start**.

   By default, the standard PCR conditions for the PCR step are displayed.

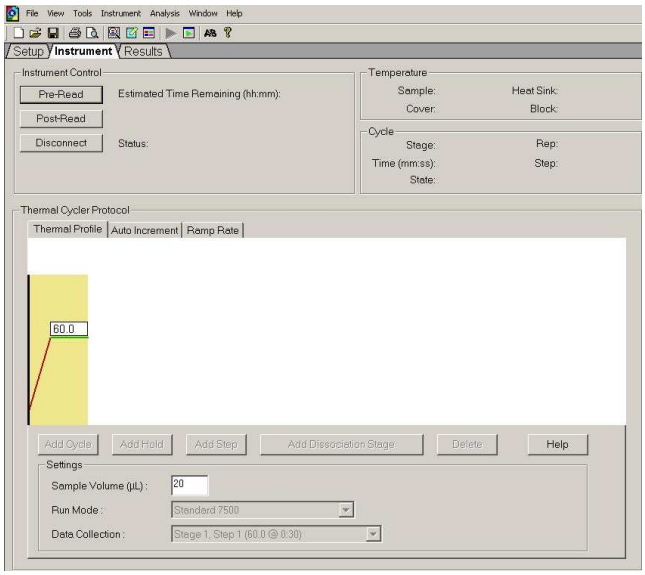
   After the run, a message indicates if the run is successful, or if errors were encountered.



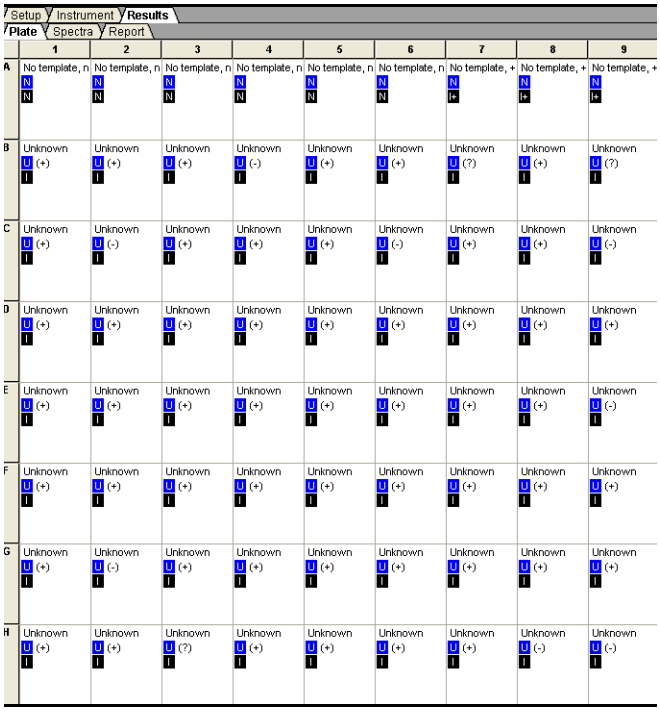
Notes

Perform the plus/minus post-read run:

1. Open the plus/minus pre-read plate document, then use it to perform the post-read run. (See "Performing the Post-Read Run" on page 36.)
   a. Select the **Instrument** tab.
   b. Select **File > Save As**, type a name for the plus/minus post-read plate document, then click **Save**.
   c. Load the reaction plate into the instrument.
   d. Click **Post-Read**.



   e. Click or select **Analysis > Analyze**. Click the **Results** tab to view results for each well.



2. If you need to troubleshoot the plus/minus results, see "Viewing the Amplification Data" on page 49.

Notes

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