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Applied Biosystems® ViiA™ 7 Real-Time PCR System

Getting Started Guides

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Getting Started Guides

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Roadmap

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BOOKLET 3 **Running Relative Standard Curve and Comparative C_T Experiments**

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BOOKLET 4 **Running Genotyping Experiments**

BOOKLET 5 **Running Presence/Absence Experiments**

BOOKLET 6 **Running Melt Curve Experiments**

BOOKLET 7 **ViiA™ 7 System Experiments - Appendixes**

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BOOKLET 1

Getting Started with ViiA™ 7 System Experiments

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About This Guide

How to use these guides

These guides function as both a tutorial and as a guide for performing your own experiments.

Note: First-time users of the ViiA™ 7 System, please read this booklet, *Getting Started with ViiA™ 7 System Experiments* thoroughly. The booklet provides information and general instructions that are applicable to all the experiments described in this binder.

How to use these booklets as tutorials

Each booklet in this guide provides a tutorial for running an example experiment using ViiA™ 7 Software and the example data provided on the installation CD. The following booklets are provided:

- *Getting Started with ViiA™ 7 System Experiments* – introductory information and experiment workflow common to all experiments.
- *Running Standard Curve Experiments* – designing, running, and analyzing a Standard Curve experiment.
- *Running Relative Standard Curve and Comparative C_T Experiments* – designing, running, and analyzing Relative Standard Curve and Comparative C_T experiments.

  Note: This booklet also provides information on setting up, running, and analyzing a gene expression study of two Comparative C_T experiments.

- *Running Genotyping Experiments* – designing, running, and analyzing a Genotyping experiment.
- *Running Presence/Absence Experiments* – designing, running, and analyzing a Presence/Absence experiment.
- *Running Melt Curve Experiments* – designing, running, and analyzing a Melt Curve experiment.
- *ViiA™ 7 System Experiments - Appendixes* – common information such as ordering information, additional documentation, and glossary.

  Note: In all booklets, the term "experiment" refers to the entire process of performing an experiment, including setup, run, and analysis.

How to use the guides with your own experiments

Each booklet contains instructions specific to an example experiment provided on the installation CD. However, you can use the booklets as guides for your own experiments; tips for running your own experiments are provided at various points in each booklet.

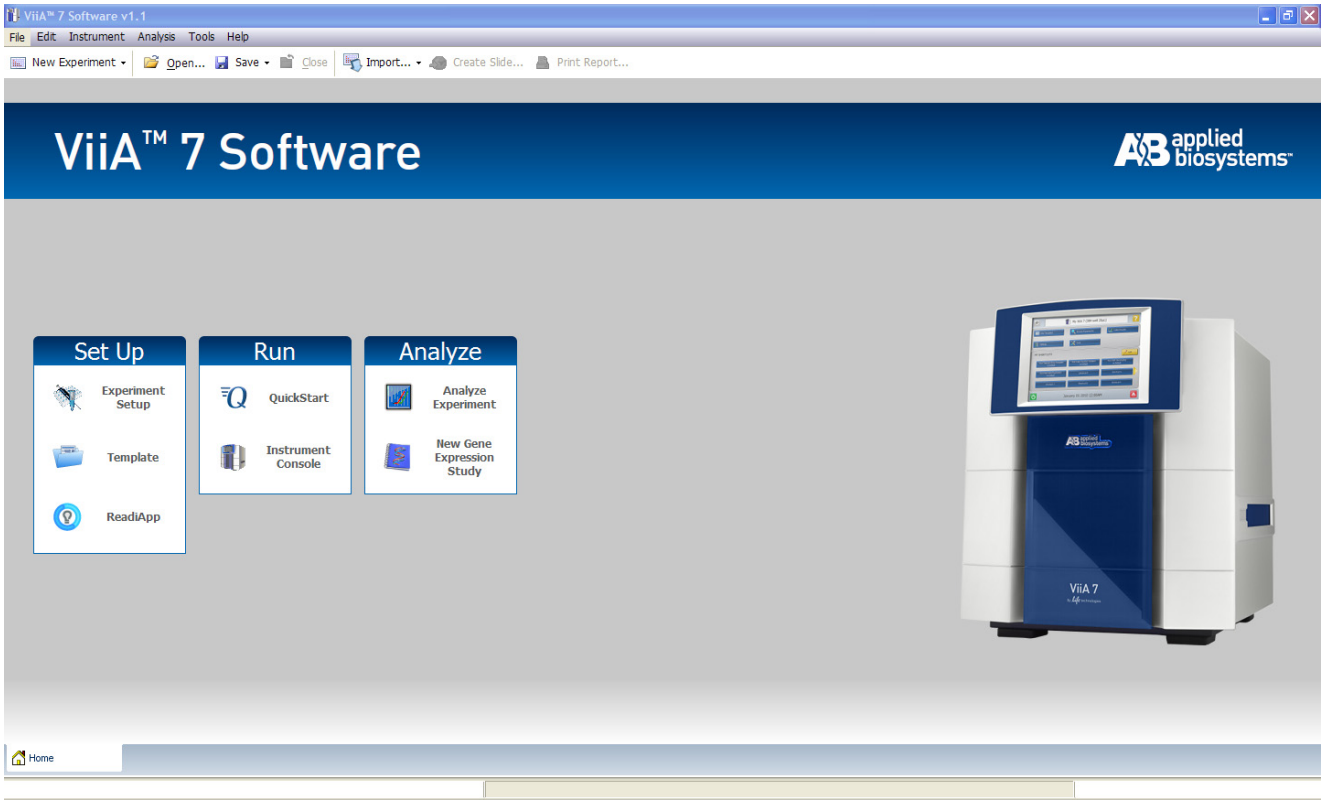
Assumptions

This guide assumes that you have access to the example experiments provided with the software.

How to access an example experiment

Start the ViiA™ 7 Software

Double-click (ViiA™ 7 Software shortcut) to access the Home screen, shown below.



Open an example experiment

From the Home screen, click **Open**, to navigate to the **experiments** folder (default):

<drive>:\Applied Biosystems\ViiA7 Software v1.1\experiments\examples, and open the example experiment file.

Data files in the Examples folder

- ViiA7 96-Well Comparative Ct Example.eds
- ViiA7 96-Well Multiplex Example.eds
- ViiA7 96-Well Presence-Absence Example.eds
- ViiA7 96-Well SNP Genotyping Example.eds
- ViiA7 96-Well Standard Curve Example.eds
- ViiA7 96-Well SYBR Green PCR w Melt Example.eds
- ViiA796-Well Relative Standard Curve Example 2.eds

- ViiA796-Well Relative Standard Curve Example.eds
- ViiA7_384-Well_Comparative_Ct_Example.eds
- ViiA7_384-Well_Comparative_Ct_Example_1.eds
- ViiA7_384-Well_Comparative_Ct_Example_2.eds
- ViiA7_384-Well_Comparative_Ct_Study_Example.edm
- ViiA7_384-Well_Comparative_Ct_with_Bioreplicates_Study_Example.edm
- ViiA7_384-Well_Melt_ Example.eds
- ViiA7_384-Well_Multiplex_Example.eds
- ViiA7_384-Well_Presence-Absence_Example.eds
- ViiA7_384-Well_Relative_Standard_Curve_Example.eds
- ViiA7_384-Well_Relative_Standard_Curve_Example_2.eds
- ViiA7_384-Well_SNP_Genotyping_Example.eds
- ViiA7_384-Well_Standard_Curve_Example.eds
- ViiA7_384-Well_SYBR_Green_Melt_Example.eds
- ViiA7_384-Well_SYBR_Green_PCR_with_Melt_Example.eds
- ViiA7_TaqMan_Array_Comparative_Ct_Example.eds
- ViiA7_TaqMan_Array_RNaseP_Example.eds
- ViiA7_TaqMan_Array_Standard_Curve_Example.eds

In addition to the above, the experiments folder also contains the following user sample files:

- BarCode_Template 384Wb.txt
- ViiA 7 Custom Sample Properties_example.xls

A note on system security

The Security, Auditing, and e-Signature (SAE) feature in ViiA™ 7 Software enables role-based access control to enforce data integrity and authentication of users logging into the system, to strengthen system security. The feature tracks actions performed by users on experiments, templates, and studies, and it tracks changes to the SAE settings. You can enable and later disable this feature to accommodate your security needs.

To enable or disable the feature, from the toolbar select **Tools ▸ Security ▸ Settings**.

For more information on the SAE feature, please refer to *Applied Biosystems ViiA™ 7 Real-Time PCR System User Guide* (PN 4442661).

Note: If you have not purchased the SAE module, the security feature is disabled for experiments, templates, and studies.

Safety information

Note: For general safety information, see this Preface and Appendix B, Safety. When a hazard symbol and hazard type appear by a chemical name or instrument hazard, see the "Safety" Appendix for the complete alert on the chemical or instrument.

Safety alert words

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

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

CAUTION! – 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.

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

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

Except for IMPORTANTs, each safety alert word in an Applied Biosystems document appears with an open triangle figure that contains a hazard symbol. These hazard symbols are identical to the hazard symbols that are affixed to Applied Biosystems instruments (see "Safety symbols" in Appendix B, Safety).

Safety Data Sheets (SDSs)

The SDSs for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining SDSs, see "SDSs" in **Appendix B, Safety**.

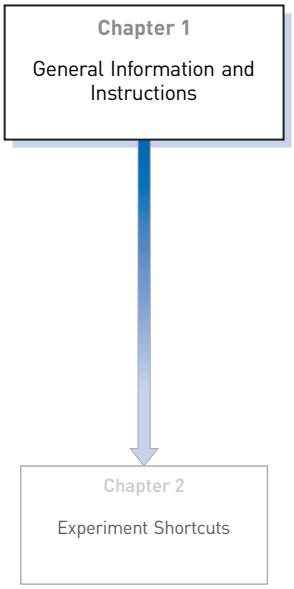
IMPORTANT! For the SDSs of chemicals not distributed by Applied Biosystems, Ambion or Invitrogen contact the chemical manufacturer.

Safety labels on instruments

The following CAUTION, WARNING, and DANGER statements may be displayed on Applied Biosystems instruments in combination with the safety symbols described in the preceding section.

Hazard symbol	English	Français
	CAUTION! Hazardous chemicals. Read the Safety Data Sheets (SDSs) before handling.	ATTENTION! Produits chimiques dangereux. Lire les fiches techniques de sûreté de matériels avant toute manipulation de produits.
	CAUTION! Hazardous waste. Refer to SSDS(s) and local regulations for handling and disposal.	ATTENTION! Déchets dangereux. Lire les fiches techniques de sûreté de matériels et la régulation locale associées à la manipulation et l'élimination des déchets.
	WARNING! Hot lamp.	AVERTISSEMENT! Lampe brûlante.
	WARNING! Hot. Do not remove lamp until **15** min after disconnecting supply.	AVERTISSEMENT! Lampe brûlante, après avoir déconnecté le câble d'alimentation de l'appareil, attendre environ 15 minutes avant d'effectuer un remplacement de la lampe.
	WARNING! Hot. Replace lamp with an Applied Biosystems lamp.	AVERTISSEMENT! Composants brûlants. Remplacer la lampe par une lampe Applied Biosystems.
	CAUTION! Hot surface.	ATTENTION! Surface brûlante.
	DANGER! High voltage.	DANGER! Haute tension.
	WARNING! To reduce the chance of electrical shock, do not remove covers that require tool access. No user-serviceable parts are inside. Refer servicing to Applied Biosystems qualified service personnel.	AVERTISSEMENT! Pour éviter les risques d'électrocution, ne pas retirer les capots dont l'ouverture nécessite l'utilisation d'outils. L'instrument ne contient aucune pièce réparable par l'utilisateur. Toute intervention doit être effectuée par le personnel de service qualifié venant de chez Applied Biosystems.
	CAUTION! Moving parts. Crush/pinch hazard.	ATTENTION! Pièces en mouvement, risque de pincement et/ou d'écrasement.

1 General Information and Instructions



This chapter covers:

- Order materials for the experiment 12
- Set up an experiment 13
- Prepare reactions 24
- Start the experiment 38
- Review experiment results 52
- Export an experiment 57

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems ViiA™ 7 Software by pressing F1, clicking in the toolbar, or selecting **Help ▸ ViiA™ 7 Software Help**.

Order materials for the experiment

There are two ways to order materials required for the experiment:

- Order directly from the Applied Biosystems store.
- Access the Applied Biosystems store from the ViiA™ 7 Software.

About ordering from the Applied Biosystems store

Note: Product availability and pricing may vary according to your region or country. Online ordering through the Applied Biosystems Store is not available in all countries. Contact your local Applied Biosystems representative for help.

Note: See *Applied Biosystems ViiA™ 7 Real-Time System User Guide* (PN 4442661) for a complete list of instrument parts and accessories, consumables, and reagents or Booklet 7, *ViiA™ 7 System Experiments - Appendixes* of this binder for a complete list of compatible consumables and reagents.

Applied Biosystems recommends the following browsers and Adobe® Acrobat® Reader® versions to use the Applied Biosystems web site:

Note: Confirm that your computer has an Internet connection.

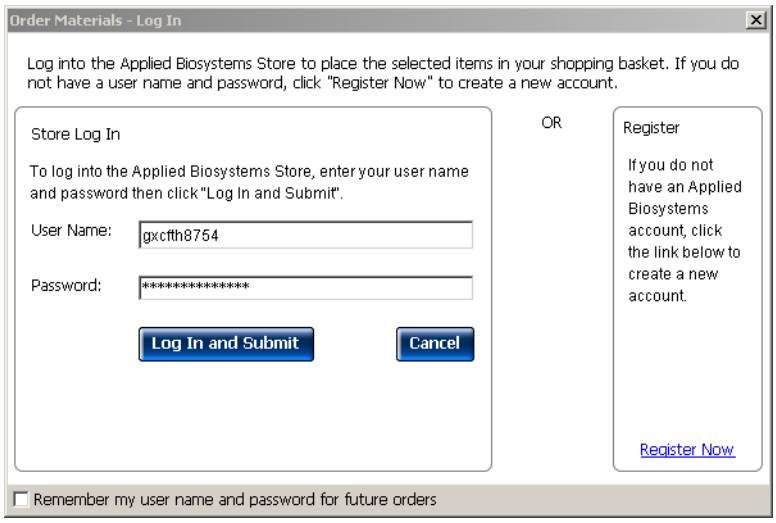
Operating System	Mozilla® Firefox®	Microsoft® Internet Explorer®	Adobe® Reader®
Windows® XP/ Windows® 7	Firefox® v2.0 or later	Internet Explorer® v6.0 or later	Adobe® Reader® v4.0 or later

Note: Make sure that cookies and JavaScript are turned on for the web site to function correctly.

Access the store from ViiA™ 7 Software

1. To find your assay on the Applied Biosystems Store, complete the Find Assay pane in the ViiA™ 7 Software:
   a. Enter a gene name in the Enter Gene Name field, then click **Find Assay**.
   b. In the Find Assay Results dialog box, select your assay.
   c. Click **Apply Assay Selection**. The selected assay gets added to your shopping list.
2. Check that the Experiment Shopping List contains the desired materials, other than the assay selected in the previous step, and that the quantities are correct, then click **Order Materials in List**.

3. In the Order Materials - Login dialog box, enter your user name and password for the Applied Biosystems Online Store, then click **Log In and Submit**.



Note: If you do not have an account with the Applied Biosystems Online Store, click **Register Now** to create an account.

4. When you are connected to the Applied Biosystems Store, follow the prompts to complete your order.

Set up an experiment

Define experiment properties

All experiments require the same general setup tasks; individual booklets supply specific parameters. The following procedures outline general steps to take to set up an experiment.

Access ViiA™ 7 Software and click the Experiment Setup icon. Click **Experiment Properties** to access the Experiment Properties screen.

Define experiment name and type

1. Enter a unique experiment name in the Experiment Name field. The default is a date and time stamp, which you can change. For example, 2010-04-12 173730.
   - Enter a name that is descriptive and easy to remember. You can enter up to 100 characters.
   - You can only use the alpha-numeric, period (.), hyphen (-), underscore (_) and spaces ( ) characters.

Note: Make sure each experiment name is unique. If you have named two different experiments with the same name, you cannot run them on the same instrument. You will receive the following error message:

If you do not want to delete the existing experiment, rename the duplicate experiment and then proceed to the run.

2. *(Optional)* Enter or scan the barcode on the reaction plate. You can enter up to 100 characters in the Barcode field.

3. *(Optional)* Enter a user name to identify the owner of the experiment. You can enter up to 100 characters in the User Name field.

4. *(Optional)* Enter comments to describe the experiment.

5. Select the block type you are using to run the experiment
   - 384-Well Block
   - Array Card Block
   - 96-Well Block (0.2mL)
   - Fast 96-Well Block (0.1mL)

6. Select the experiment type:
   - Standard Curve
   - Relative Standard Curve
   - Comparative C_T ($\Delta\Delta C_T$)
   - Melt Curve
   - Genotyping
   - Presence/Absence

Select the reagent

Select the reagent you are using to detect the target sequence:

- TaqMan® Reagents
- SYBR® Green Reagents
- Other

Note: If you select SYBR® Green as the reagent, then you have the option of including a melt curve for that experiment.

Define the instrument run properties

1. Select the ramp speed for the experiment:
   - Standard
   - Fast

2. For Genotyping and Presence/Absence experiments, select the options for the data collection to include in the experiment run:
   - **Pre-PCR Read** - to include data before amplification occurs. Use the data collected during pre-PCR read to normalize florescence data collected during post-PCR read.
   - **Amplification** - to include real-time data.
   - **Post-PCR Read** - to include data after amplification has taken place.

3. *(Optional)* For real-time data collection, you can change the default analysis settings in the Preferences for the following:
   - Automatic analysis
   - Automatic save
   - Baseline settings

   Go to **Tools** ▸ **Preferences**. Click the Experiment tab. Select the Auto Analysis and Auto Save check boxes for the **ViiA™ 7 Software** to automatically analyze and save experiment results. You can also edit the following default baseline settings:

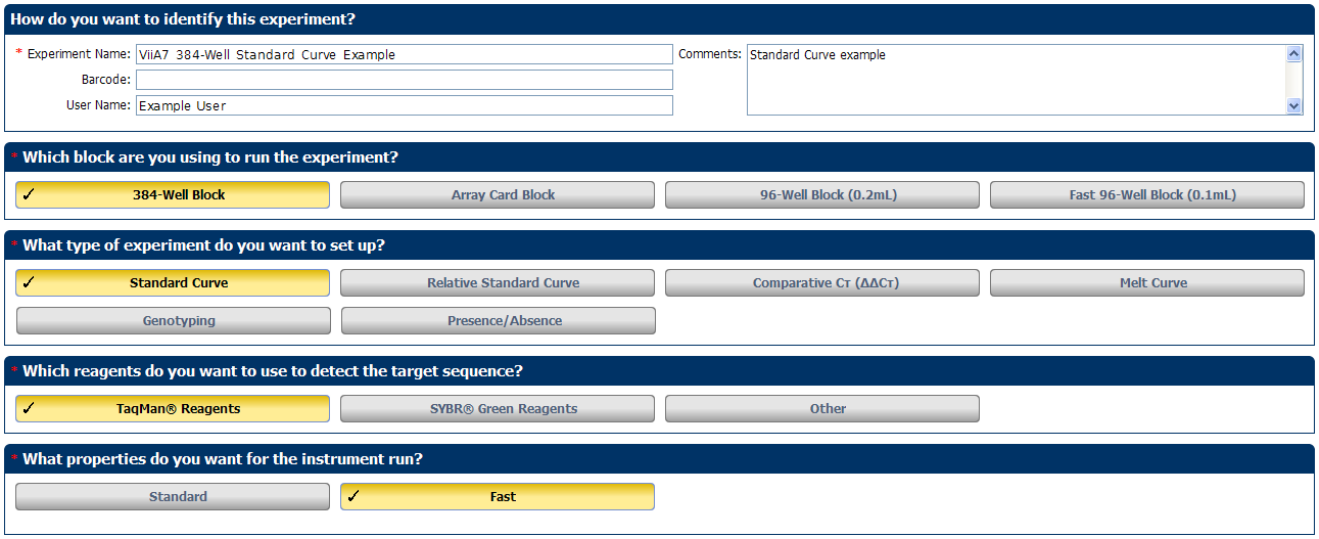
Field	Entry
Start Cycle Number	3 (default)
End Cycle Number	15 (default)

   Note: By default, the Auto Analysis and Auto Save check boxes are selected.

4. For the Melt Curve experiment, select the **Include PCR** check box, to include PCR.

5. Save the experiment. The default file name (.eds extension) is the experiment name that you entered when you set up the experiment and saved it for the first time. Changes to the experiment name after the first save do not update the file name. To change the file name, select **File** ▸ **Save As**.

The Experiment Properties screen for a Standard Curve experiment looks like this:



Define targets, samples, and biological replicate groups

Use the Define screen to define targets, samples and biological replicates for your experiment. For Genotyping experiments, use this screen to specify the number of SNP assays to include in the experiment.

Note: You can start a run without these definitions, but there will be no real-time data in the amplification plots (the amplification plots can be seen only after you have set up the plate).

1. Click **Define** to access the Define screen.
2. Define targets.
   a. Click **New** to add targets and define them.
   b. In the target table, click a cell in the Target Name column for the target, then enter your target name. The default name is Target 1.
   c. Select the **Reporter** and **Quencher** from the respective drop-down menu.

      Note: The default reporter and quencher dyes used depend on the reagent selected during experiment setup. For example, if TaqMan® is the selected reagent, the default reporter FAM and default quencher is **NFQ-MGB**.

   d. Select the target **Color** from the drop-down menu.
   e. (*Optional*) Click **Save to Library** to save the newly added or existing edited targets to the target library.

      Note: Use the targets from the Target Library to avoid re-entering the information. See "(Optional) Use libraries when designing your own experiments" on page 23 for information on target libraries.

   f. Click **Import from Library** to add targets from the target library.

3. Define samples.
   a. Click **New** to add samples and name them.
   b. In the samples table, click a cell in the Sample Name column for the sample to define and enter your sample name. The default sample name is Sample 1.
   c. Select the sample **Color** from the drop-down menu.
   d. (*Optional*) Click **Save to Library** to save the newly added or existing edited samples to the sample library.

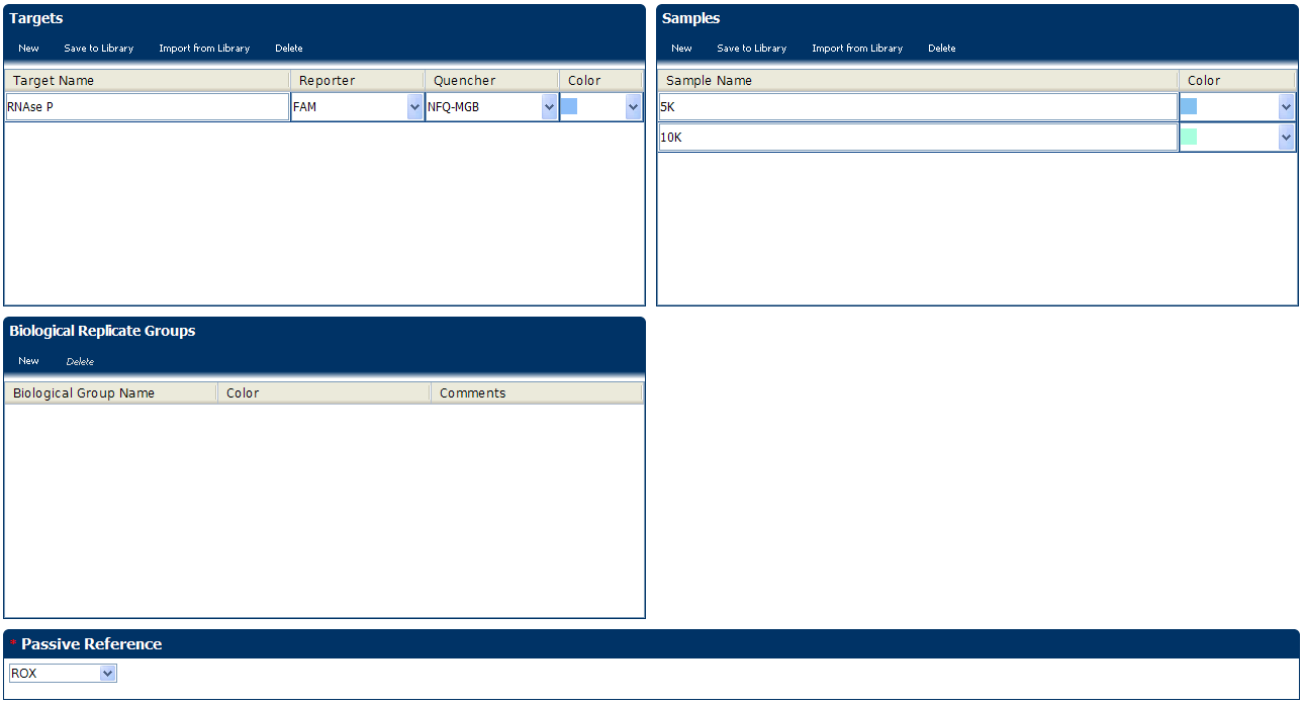
      Note: Use the samples from the Sample Library to avoid re-entering the information. See "(Optional) Use libraries when designing your own experiments" on page 23 for information on sample libraries.

   e. Click **Import from Library** to add samples from the sample library.

4. Define biological replicates.
   a. In the Define Biological Replicates Groups table, click **New** to add biological replicate group and name them. You can enter up to 100 characters in this field.
   b. Select the **Color** from the drop-down menu.
   c. Click in the **Comments** column to add comments for that biological replicate group.

5. Select the Passive Reference from the drop-down menu.

   The Define screen for a Standard Curve experiment looks like this:



Assign targets, samples, and biological replicate groups

Use the Assign screen to assign targets, samples, and biological replicate groups to wells in the reaction plate. For Genotyping experiments, use this screen to assign SNP assays.

Note: You can start a run without these assignments, but there will be no real-time data in the amplification plots (the amplification plots can be seen only after you have set up the plate).

1. Click **Assign** to access the Assign screen.
2. Assign targets.
   a. Select wells using the plate layout or the well table on the Assign screen.
   b. Select a target and assign its task, in the plate, from the drop-down menu. Depending on the experiment type, options are:

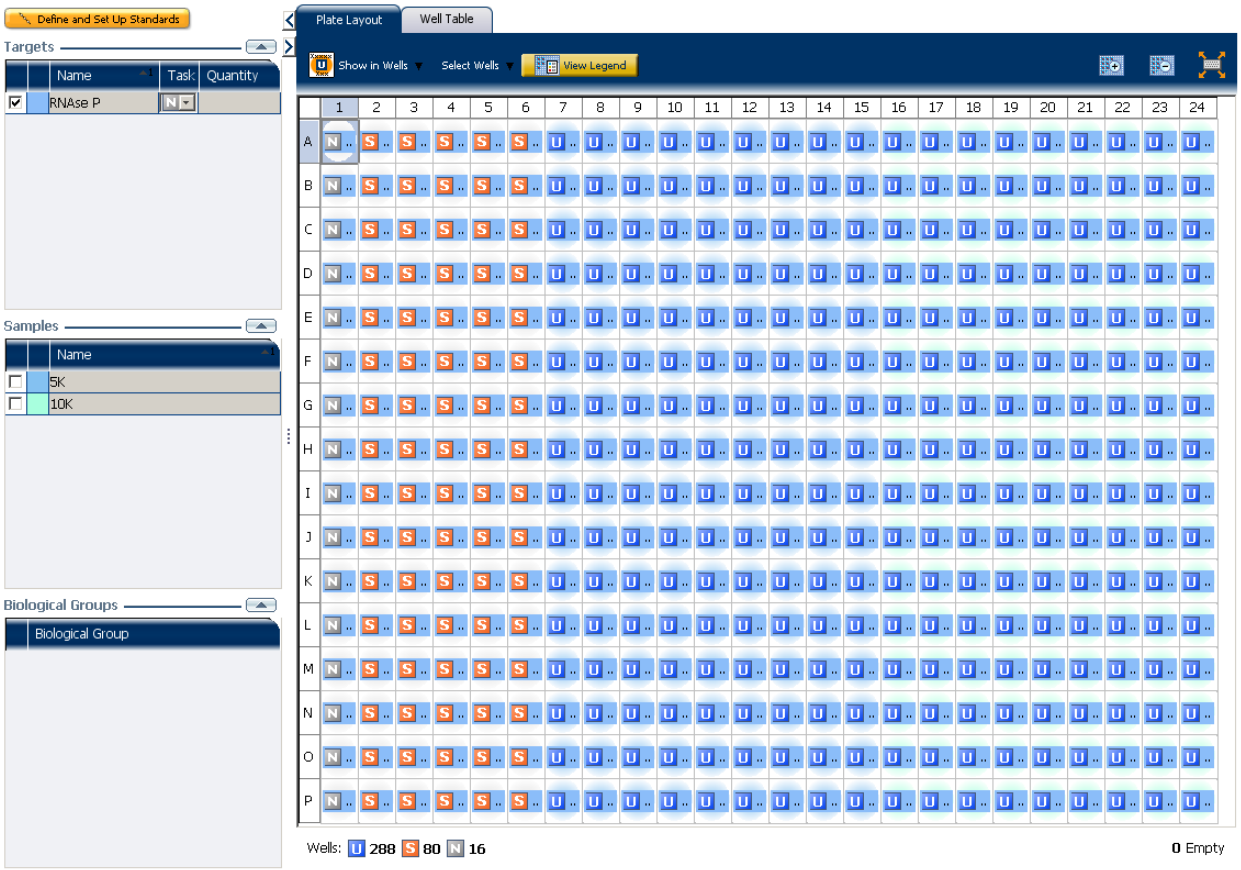
Experiment type	Legend	Tasks
Standard Curve	U	Unknown
	S	Standard
	N	Negative Control
Relative Standard Curve	U	Unknown
	S	Standard
	N	Negative Control
Comparative CT	U	Unknown
	N	Negative Control
Genotyping	U	Unknown
	1/1	Positive Control Allele 1/ Allele 1
	2/2	Positive Control Allele 2/ Allele 2
	1/2	Positive Control Allele 1/ Allele 2
	N	Negative Control
Presence/ Absence	U	Unknown
	I	Internal Positive Control
	N	Negative Control
Melt Curve	U	Unknown
	N	Negative Control

3. Assign Samples.
   a. Select wells using the plate layout or the well table on the Assign screen.
   b. Select the check box next to the sample to assign to the selected wells.

      Note: You can assign only one sample to a well. If the selected wells contain mixed assignments (indicated by a), remove existing sample assignments before you make the new sample assignment.

4. Assign Biological Replicate Groups.
   a. Select wells using the plate layout or the well table on the Assign screen.
   b. Select the check box next to the biological replicate group to assign to the selected wells.

The Assign screen for a Standard Curve experiment looks like this:

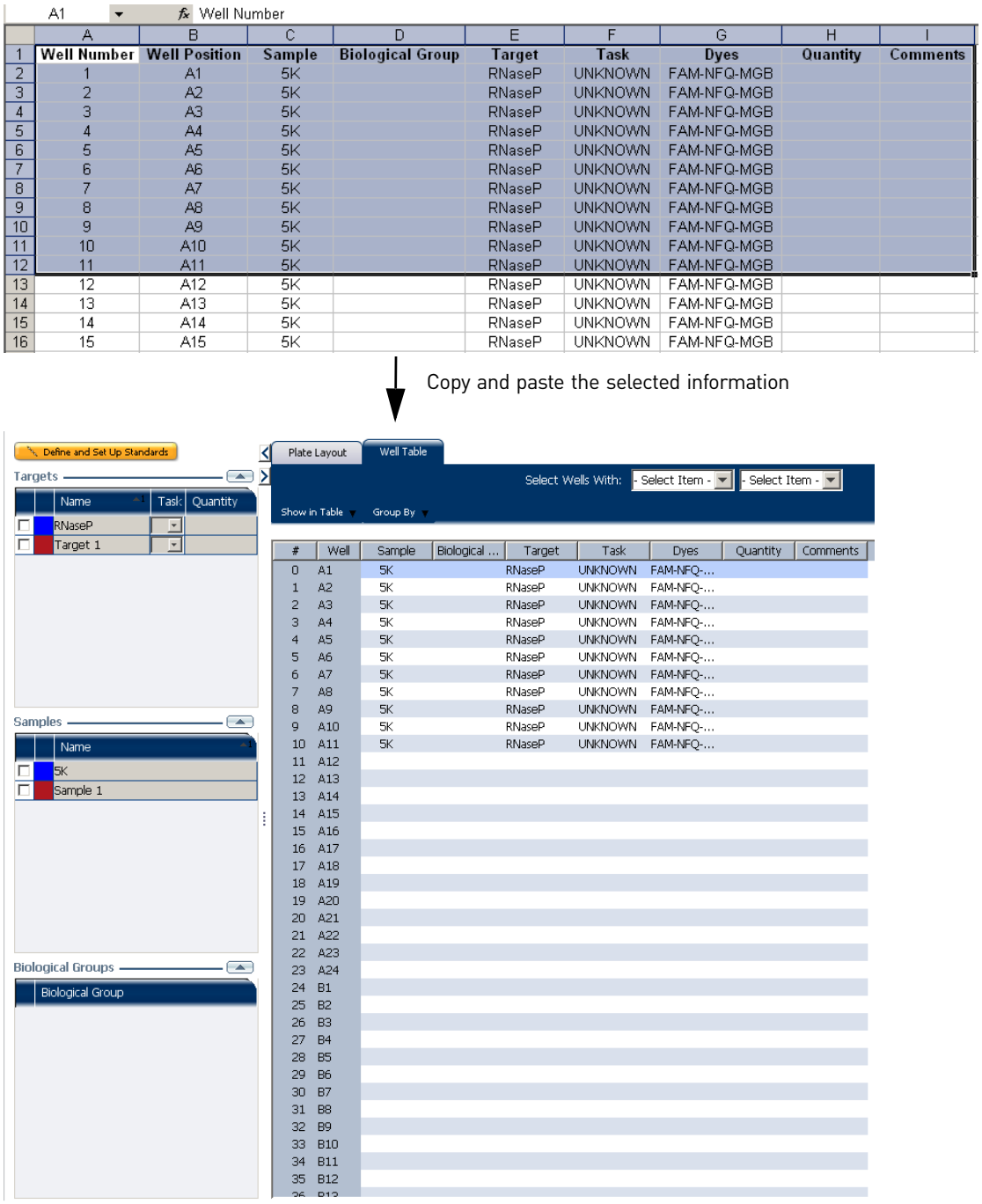


Alternate procedure for assigning targets, samples, and biological replicate groups

As shown below, you can also paste assignment information from an ***.xls** file into the plate layout of the ViiA™ 7 Software for wells with single targets.

Note: You must select the header, and the Well Number and Well Position columns while copying information from the ***.xls** file.

Note: Any of the columns not copied are treated as NULL values for those columns.



	A	B	C	D	E	F	G	H	I
1	Well Number	Well Position	Sample	Biological Group	Target	Task	Dyes	Quantity	Comments
2	1	A1	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
3	2	A2	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
4	3	A3	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
5	4	A4	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
6	5	A5	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
7	6	A6	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
8	7	A7	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
9	8	A8	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
10	9	A9	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
11	10	A10	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
12	11	A11	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
13	12	A12	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
14	13	A13	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
15	14	A14	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
16	15	A15	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		

Define the run method

Use the Run Method screen to set up the run method for your own experiments in the ViiA™ 7 Software.

1. Click **Run Method** to access the Run Method screen.

   Note: You can save multiple run methods to the Run Method Library for later use. See "(Optional) Use libraries when designing your own experiments" on page 23 for information on run method libraries.

2. Enter a number from 1 to 20 for the reaction volume per well. The ViiA™ 7 Instrument supports the following maximum reaction volumes for the consumables listed below:
   - MicroAmp® Optical 384-Well Reaction Plate - 30µL
   - Applied Biosystems Array Card - 1µL
   - MicroAmp® Optical 96-Well Reaction Plate (0.2mL)- 200µL
   - MicroAmp® Optical 96-Well Reaction Plate (0.1mL)- 100µL
   - MicroAmp® Optical 8-Tube Strip with cap (0.2µL)- 200µL
   - MicroAmp® Fast 8-Tube Strip with cap(0.1µL)- 100µL
   - MicroAmp® Optical Reaction Tube without cap (0.2µL)- 200µL
   - MicroAmp® Fast Reaction Tube without cap (0.1µL)- 100µL

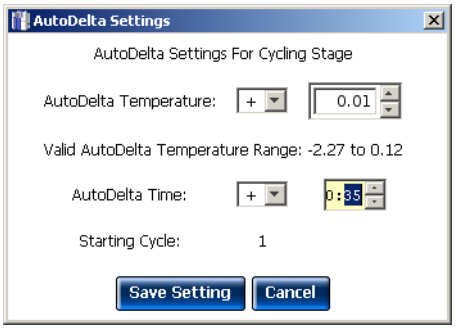
3. In the **Graphical View** tab, review and, if necessary, edit the run method.
   - Make sure that the thermal profile is appropriate for your reagents.
   - Edit the default run method or replace it with one from the run method library included in the ViiA™ 7 Software.
   - Enable data collection by clicking .

     Note: Enabling data collection is especially useful when you later need to analyze data collected in real-time during the various stages.

   - Edit the ramp rate. You can increase or decrease the ramp rate for a stage.

     Note: Ramp rates are decimal numbers from 0.015—3.4.

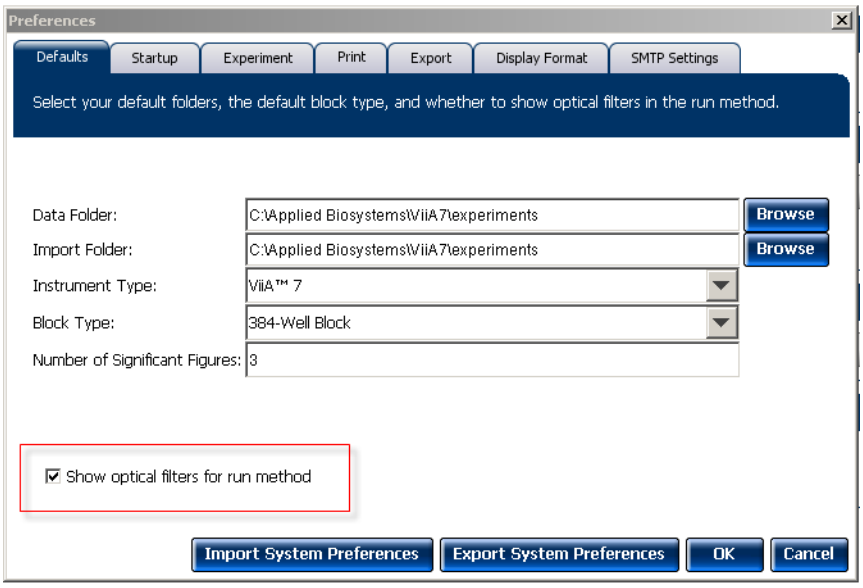
- Edit the PCR Stage.
- Change the Number of Cycles for the PCR stage.
- Select the **Enable AutoDelta** check box, to increase or decrease the temperature and/or hold time for each subsequent cycle or to change the Starting Cycle for AutoDelta. Enabling AutoDelta displays the ▲ icon. Click the AutoDelta Off ▲ icon to change the AutoDelta settings for the cycling stage in the AutoDelta Settings dialog box. Then, click **Save Setting** to display the AutoDelta On ▲ icon.



Note: If you selected SYBR® Green as the reagent, the Melt Curve stage automatically appears in the Run Method screen. If you delete the Melt Curve Stage section from the protocol, then the melt curve is active in the Add Stage drop-down menu.

4. Complete the tasks on the Optical Filters tab:

   By default, the Optical Filters tab is not visible. To show the Optical Filters tab, go to **Tools** ▸ **Preferences**, and select the Show optical filters for run method check box under the Default tab.



- To add a new filter set to the filter set library, click **Save**.
- To load a saved filter set, click **Load**.
- To go back to the original filter set combinations, click **Revert to Defaults**.

Note: Select the filter set that matches the profile of the dye you have added to the plate. Refer to the *Applied Biosystems ViiA™ 7 Real-Time PCR System User Guide* (PN 4442661) for information on the emission spectrum for each dye, and the filter at which each dye is read.

(Optional) Use libraries when designing your own experiments

The ViiA™ 7 Software allows you to save information to libraries, so you can easily use the information again when setting up an experiment. The libraries include:

- Targets library
- Samples library
- SNP Assay library (only available for Genotyping experiments)
- Run Method library

Target, Sample, and SNP Assay libraries

You can access the Targets, Samples, and SNP Assay libraries from the Tools menu to add, edit, delete, and import or export items. You can also access a library by clicking **Import from Library** in the Define screen when you are setting up an experiment.

Run Method library

You can use the Run Method library from the Run Method screen to:

- Save a new run method for later use.
- To select an existing run method for an experiment.

To add a run method to the Run Method Library:

1. Click **Save Run Method** in the toolbar of the Graphical View tab on the Run Method screen.
2. Enter a name and description (*optional*) for the run method, then click **Save**.

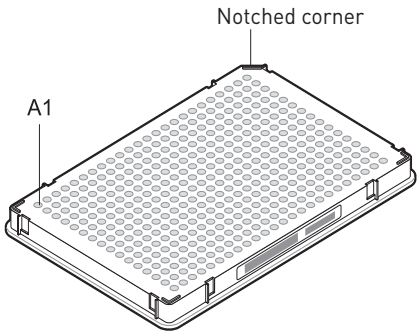
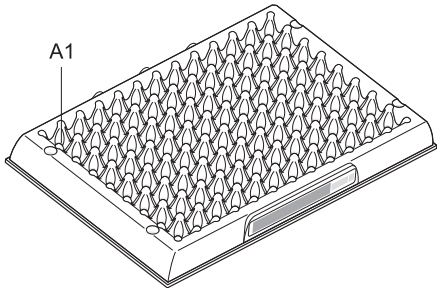
To select a run method from the Run Method Library

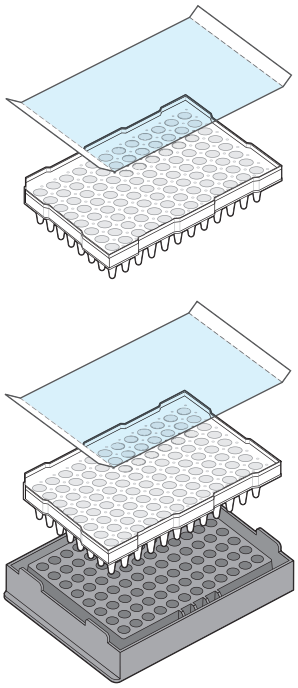
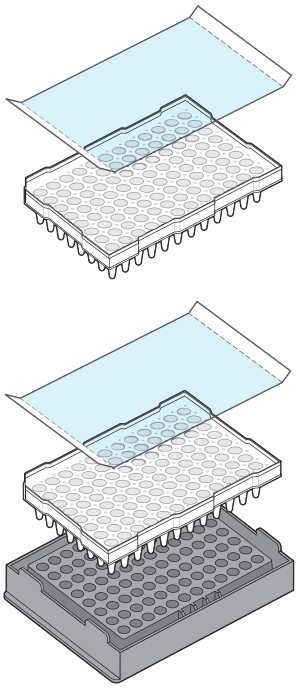
Click **Open Run Method** on the Run Method screen, and select one from the saved run methods.

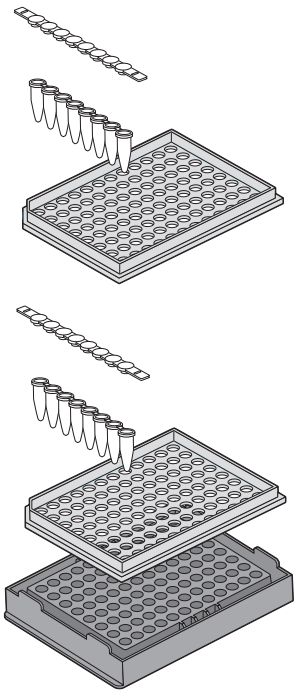
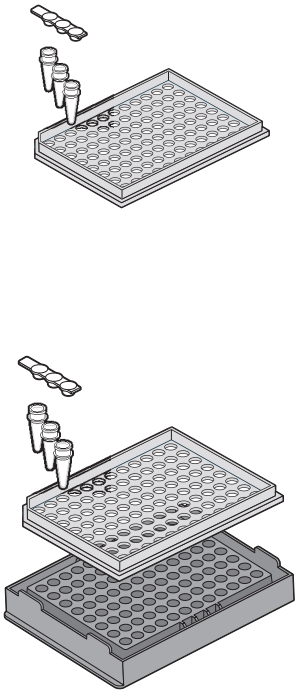
Prepare reactions

Supported consumables

The ViiA™ 7 Instrument is optimized for Applied Biosystems consumables. These can be ordered from the Applied Biosystems website. Use the consumables appropriate for the sample block of your instrument.

Sample block	Consumable		Maximum reaction volume (µL) supported	Recommended reaction volume (µL)
384-Well Plate		• MicroAmp® Optical 384-Well Reaction Plate • MicroAmp® Optical Adhesive Film	30	5 - 20
Array Card		Applied Biosystems Array Card	1	1

Sample block	Consumable		Maximum reaction volume (µL) supported	Recommended reaction volume (µL)
96-Well Plate (0.2mL)		• MicroAmp® Optical 96-Well Reaction Plate • MicroAmp® Optical Adhesive Film • MicroAmp® 96-Well Support Base (only used during sample preparation) • ViiA™ 7 System 96-Well Plate Adaptor	200	10-100
Fast 96-Well Plate (0.1mL)		• MicroAmp® Fast Optical 96-Well Reaction Plate • MicroAmp® Optical Adhesive Film • MicroAmp® 96-Well Support Base (only used during sample preparation) • ViiA™ 7 System Fast 96-Well Plate Adaptor	100	10-30

Sample block	Consumable		Maximum reaction volume (µL) supported	Recommended reaction volume (µL)
96-Well Plate (0.2µL) and Fast 96-Well plate (0.1µL)		• MicroAmp® Optical 8-Cap Strip • MicroAmp® Optical 8-Tube Strip (0.2µL)/ MicroAmp® Fast 8-Tube Strip (0.1µL) • MicroAmp® 96-Well Tray/ Retainer Set (Blue) (0.2µL)/ MicroAmp® 96-Well Tray (Black) (0.1µL) • MicroAmp® 96-Well Support Base (only used during sample preparation) • ViiA™ 7 System 96-Well Tube Adaptor/ ViiA™ 7 System Fast 96-Well Tube Adaptor	• 100 for Fast 96-well plate • 200 for 96-well plate	• 10-30 for Fast 96-well plate • 10-100 for 96-well plate
96-Well Plate (0.2µL) and Fast 96-Well plate (0.1µL)		• MicroAmp® Optical Tube without cap (0.2µL)/ MicroAmp® Fast Reaction Tube without Cap (0.1µL) • MicroAmp® Optical 8-Cap Strip • MicroAmp® 96-Well Support Base (only used during sample preparation) • MicroAmp® 96-Well Tray/ Retainer Set (Blue) (0.2µL)/ MicroAmp® 96-Well Tray (Black) (0.1µL) • ViiA™ 7 System 96-Well Tube Adaptor/ ViiA™ 7 System Fast 96-Well Tube Adaptor	• 100 for Fast 96-well plate • 200 for 96-well plate	• 10-30 for Fast 96-well plate • 10-100 for 96-well plate

WARNING! Make sure that you use the flat caps for 0.2µL tubes and 0.1µL tubes. Use of rounded caps damages the heated cover.

Supported reagents

Applied Biosystems supports the reagents listed below for experiments performed on the ViiA™ 7 System.

Experiment	Reagent
Standard Curve	• Applied Biosystems TaqMan® Reagents • Applied Biosystems SYBR® Green reagents • Other reagents
Relative Standard Curve	• Applied Biosystems TaqMan® Reagents • Applied Biosystems SYBR® Green reagents • Other reagents
Comparative C_T ($\Delta\Delta C_T$)	• Applied Biosystems TaqMan® Reagents • Applied Biosystems SYBR® Green reagents • Other reagents
Melt Curve	• Applied Biosystems SYBR® Green reagents • Other reagents
Genotyping	• Applied Biosystems TaqMan® Reagents • Other reagents
Presence/Absence	• Applied Biosystems TaqMan® Reagents • Other reagents

Note: Fast Universal Master Mix is not recommended to be used with the 96-well (0.2 µL) reaction plates or reaction tubes and tube strips sealed with caps.

Reagent detection process

Applied Biosystems TaqMan® Reagents

Description

TaqMan® reagents use a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles.

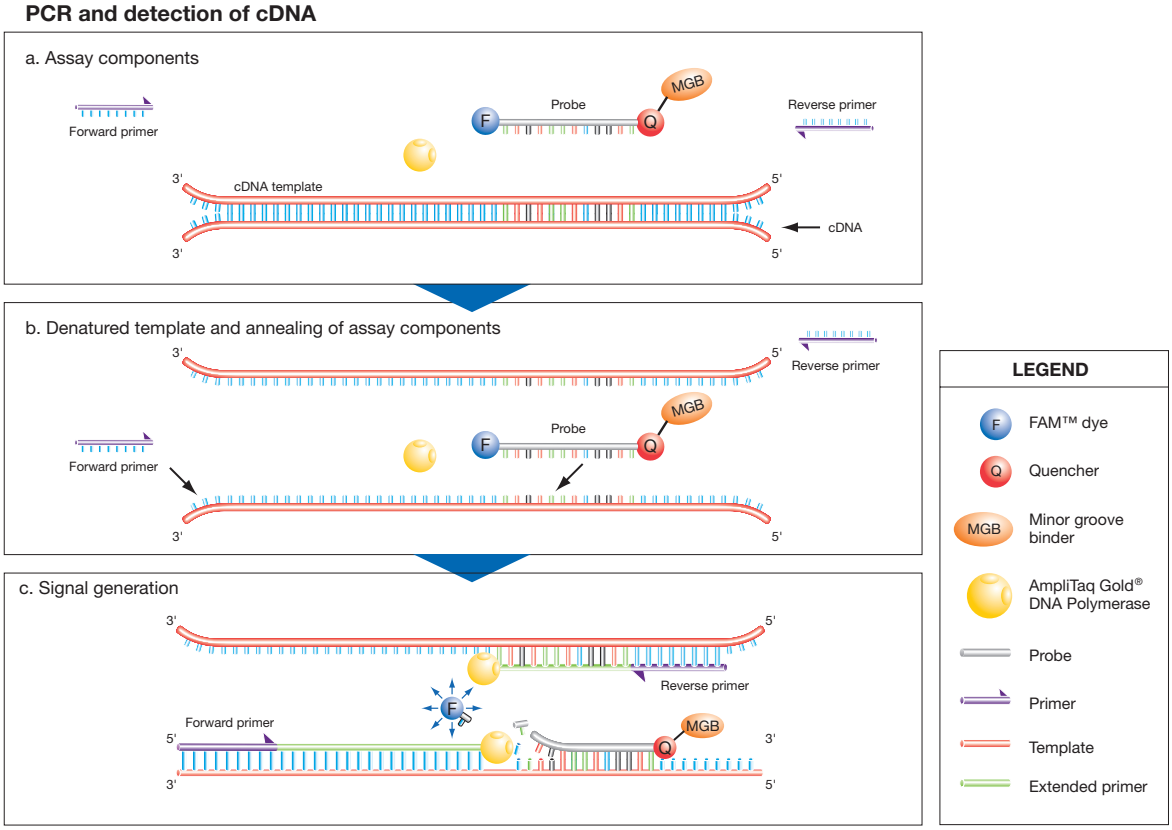
Advantages

- Increased signal specificity with the addition of a fluorogenic probe.
- Multiplex capability.
- Optional preformulated assays, optimized to run under universal thermal cycling conditions, are available.
- Can be used for either 1- or 2-step RT-PCR.

Limitations

Require synthesis of a unique fluorogenic probe.

TaqMan® Reagents detection process



Applied Biosystems SYBR® Green reagents

Description

SYBR Green reagents use SYBR® Green I dye, a double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles.

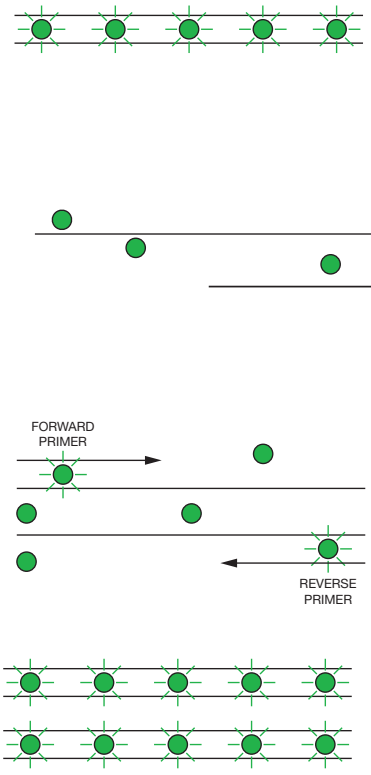
Advantages

- Economical (no probe needed).
- Allow for melt curve analysis to measure the Tm of all PCR products.
- Can be used for either 1- or 2-step RT-PCR.

Limitations

Bind nonspecifically to all double-stranded DNA sequences. To avoid erroneous information signals, check for nonspecific product formation using melt curve or gel analysis.

SYBR® Green detection process



Step 1: Reaction setup
The SYBR® Green I dye fluoresces when bound to double-stranded DNA.

Step 2: Denaturation
When the DNA is denatured into single-stranded DNA, the SYBR® Green I dye is released and the fluorescence is drastically reduced.

Step 3: Polymerization
During extension, primers anneal and PCR product is generated.

Step 4: Polymerization completed
SYBR® Green I dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the instrument.

Precautions while preparing reactions

- Make sure that you do not prepare the reactions on a wet table. Wet surfaces lead to contamination of your reactions.
- Wear appropriate protective eyewear, clothing, and powder-free gloves.
- Make sure that you use the appropriate consumables. The quality of pipettors and tips and the care used in measuring and mixing dilutions affect data accuracy.
- Make sure that you perform dilutions exactly as instructed. Mistakes or inaccuracies in making the dilutions directly affect the quality of results.
- Use a permanent marker or pen to mark a tube and the side of a plate or array card. Do not use fluorescent markers.
- Make sure that the arrangement of the PCR reactions matches the plate layout displayed in the ViiA™ 7 Software.

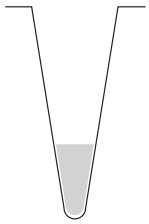
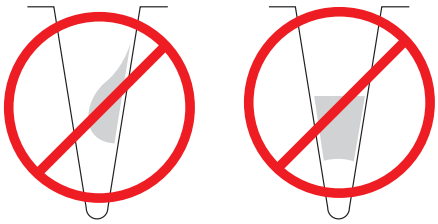
Materials required while preparing the dilutions

- DI water or DEPC water
- Microcentrifuge tubes
- Pipettors

- Pipette tips
- Vortex mixer
- Centrifuge
- Sample stock
- Standard stock
- Reaction mix components
- Plate or array card

Guidelines for preparing the dilutions, reaction mix, and plate

- Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers.
- Use TE buffer or water to dilute the standards and samples.
- Prepare the reagents according to the manufacturer's instructions.
- Keep the dilutions and assay mix protected from light, in the freezer, until you are ready to use it. Excessive exposure to light may affect the fluorescent probes or dyes.
- Prior to use:
  - Mix the master mix thoroughly by swirling the bottle.
  - Resuspend the assay mix by vortexing, then centrifuge the tube briefly.
  - Thaw any frozen samples by placing them on ice. When thawed, resuspend the samples by vortexing, then centrifuge the tubes briefly
- Do not allow the bottom of the reaction plate to become dirty. Fluids and other contaminants that adhere to the bottom of the reaction plate can contaminate the sample block(s) and cause an abnormally high background signal.

Correct	Incorrect
	
Liquid is at the bottom of the well.	Not centrifuged with enough force *Or* Not centrifuged for enough time

- For Genotyping experiments, prepare the reactions for each SNP separately.
- Place the reaction plate or array card at 4 °C and in the dark until you are ready to load it into the instrument

Seal the reaction plate

If you use optical adhesive film to seal your reaction plates, seal each reaction plate as follows:

Note: The sealing instructions are applicable to 384-well and 96-well reaction plates.

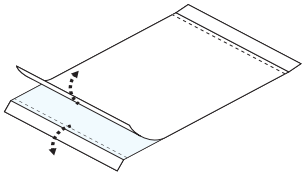
1. Load the reaction plate using the plate layout described in "Assign targets, samples, and biological replicate groups" on page 18.

   Note: For 96-well reaction plates, place the reaction plate onto the center of the 96-well base, then perform this step. Be sure that the reaction plate is flush with the top surface of the 96-well base.

   Note: You can also use the MicroAmp® Optical 8-Cap Strip to seal the 96-well reaction plates.

2. Remove a single optical adhesive film (film) from the box. Bend both end-tabs upward. Hold the film backing side up.

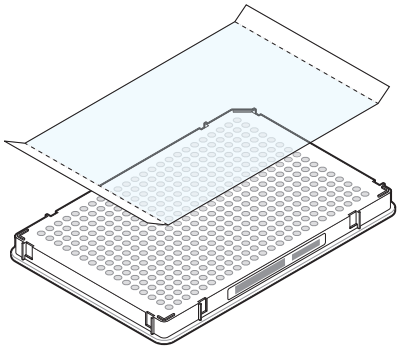
3. In one swift movement, peel back the white protective backing from the center sealing surface. Do not touch the center sealing surface.

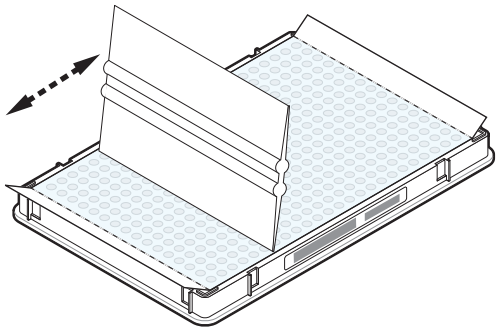


IMPORTANT! Improper peeling of the optical adhesive film may result in haziness, but it will not affect results. Haziness disappears when the film comes into contact with the heated cover in the instrument.

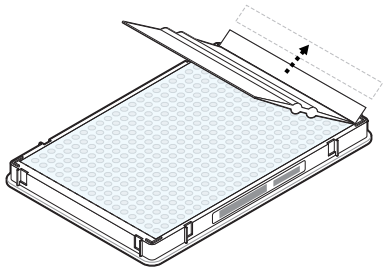
4. Holding the film by the end-tabs, lower the film onto the reaction plate (adhesive side facing the reaction plate). Make sure that the film completely covers all wells of the reaction plate.

5. Applying firm pressure, move the applicator slowly across the film, horizontally and vertically, to ensure good contact between the film and the entire surface of the reaction plate.





6. Using the applicator to hold the edge of the film in place, grasp one end of the end-tab and pull up and away sharply. Repeat for the other end-tab.

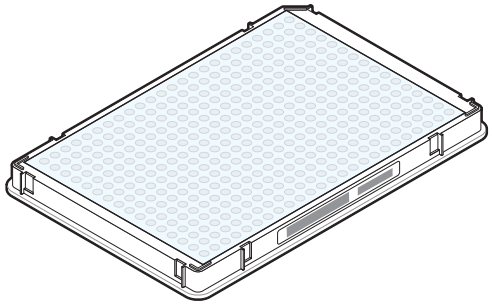


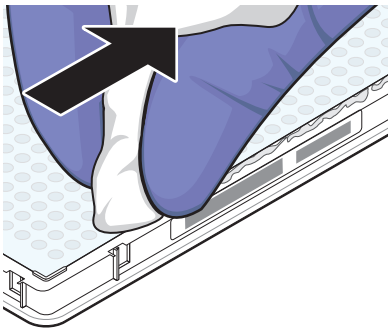
Note: Ensure clean removal of both end-tabs from the dotted line. Improper peeling of the end-tab can cause sticking of plate on the heated cover assembly.

7. To ensure a tight, evaporation-free seal, repeat step 5. Applying firm pressure, run the edge of the applicator along all four sides of the outside border of the film.

Note: Optical adhesive films do not adhere on contact. The films require the application of pressure to ensure a tight, evaporation-free seal.

8. Inspect the reaction plate to be sure that all wells are sealed. You should see an imprint of all wells on the surface of the film. Check for the perforated tab to be completely torn off to avoid plates from sticking to the instrument after a run.





IMPORTANT! Remove all excess adhesive from the perimeter of the optical adhesive cover. When the film is applied, the glue from the optical adhesive cover can adhere to the edges of the plate. If the excess glue is not removed, the plate may adhere to the gripper of the Twister® II Robot or to the sample block of the ViiA™ 7 Instrument.

Fill and seal the array card

Fill and spin the array card

IMPORTANT! Wear powder-free gloves while preparing the Arrays.

1. Remove an array card from its box and place it on a clean, dry surface.
2. Using a permanent marker, mark the side of the empty array cards.
3. Transfer the experiment-related chemistries and solutions into the port of the array card.

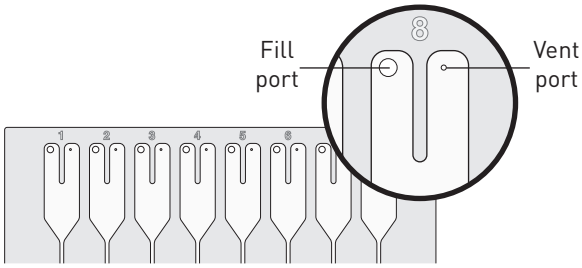
   For each transfer:

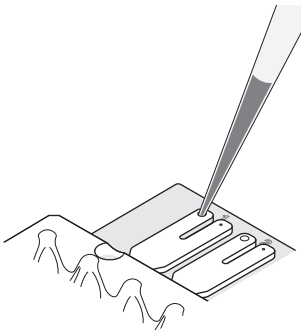
   a. Place the array card on a lab bench, with the foil side down.

   b. Load 100 µL of fluid into a pipette.

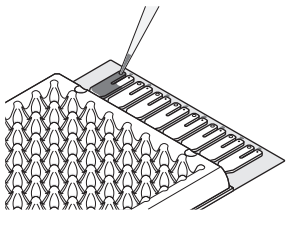
   c. Hold the pipette in an angled position (~45 degrees) and place the tip into the fill port. There is a fill port on the left arm of each fill reservoir – it is the larger of the two holes.

      Do not allow the tip to contact and possibly damage the coated foil beneath the fill port.



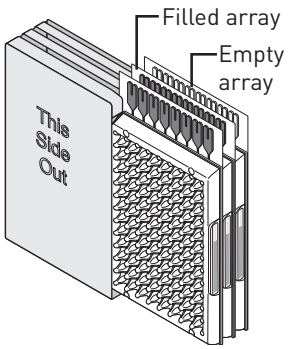


   d. Dispense the fluid so that it sweeps in and around the fill reservoir toward the vent port. Pipette fluid into the fill reservoir, but **do not** go past the first stop of pipettor plunger when pipetting the reagents into the array card, or you may blow the solution out of the port.



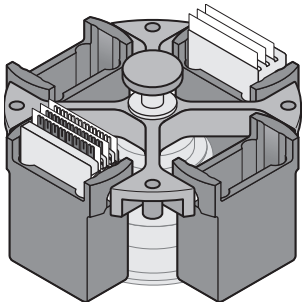
IMPORTANT! Do not allow the tip to contact and possibly damage the coated foil beneath the fill port.

4. Place the filled array card(s) into a centrifuge array card carrier clip and place empty array card(s) in the remaining slots. Make sure that the labels on the buckets and clips face the same way.



IMPORTANT! Make sure to balance the loads in opposite buckets in the centrifuge.

5. Place the filled carrier clips into the centrifuge buckets. Make sure that the array-card fill reservoirs and bucket and clip labels face outward when loaded into the centrifuge. Balance the loads in opposite buckets.



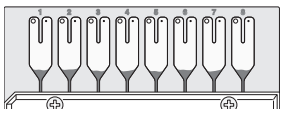
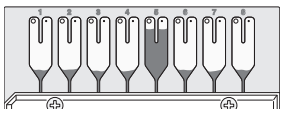
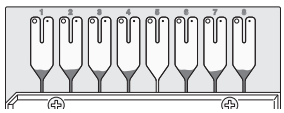
IMPORTANT! You must run the centrifuge with all four buckets in place and each of the two carriers filled with the array card. Place empty array cards (4-pack PN 4334812 and 1-pack PN 4351471) into unfilled slots.

6. Close the centrifuge cover, then spin the array card(s) for 1 minute at 1200 rpm.

7. When the run is finished, stop the centrifuge, then spin the array card(s) again for 1 minute at 1200 rpm.

IMPORTANT! Do not try to save time by doing one spin for 2 minutes. The two sets of ramps are important for a good fill into the array card.

8. When the second run is finished, open the centrifuge and check that the fluid levels in the reservoirs of each array card have decreased by the same amount. Also, check for the formation of bubbles in all wells and note possible problems.

Correct	Incorrect	
		

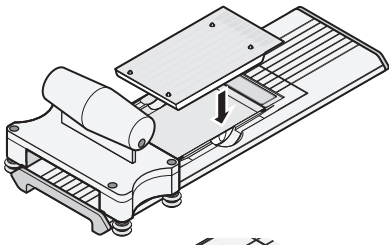
9. If necessary, centrifuge the array card(s) for an additional minute to fill any unfilled wells. Do not exceed three 1-minute runs or centrifuge the array card for longer than 1 minute at a time.

Note: Visit **http://www2.appliedbiosystems.com/lib/multimedia/taqman_tlda/tlda_1.cfm** to view an online video of loading an array card.

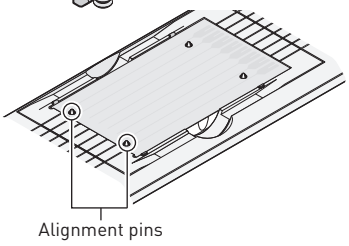
Seal the array card(s)

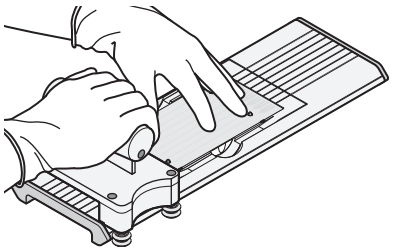
1. With the carriage (roller assembly) of the TaqMan® Array Micro Fluidic Card Sealer in the Start position, place a filled array card into the fixture with the foil side up so that the fill reservoirs are the farthest away from the carriage.

2. Press down on all four corners of the array card to ensure that it is fully seated within the fixture.

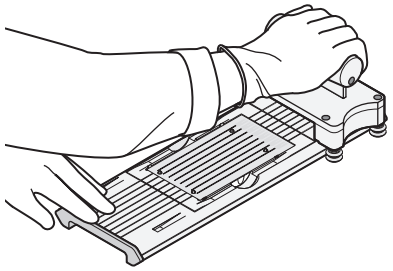


3. Use the two alignment pins in the fixture to position the array card correctly.

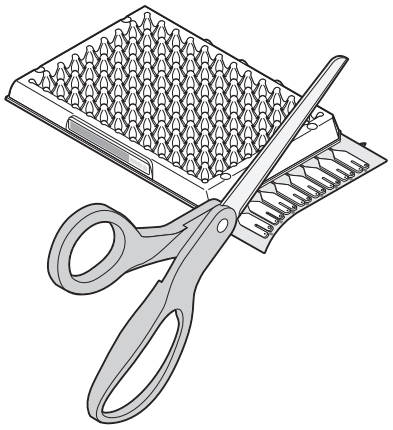




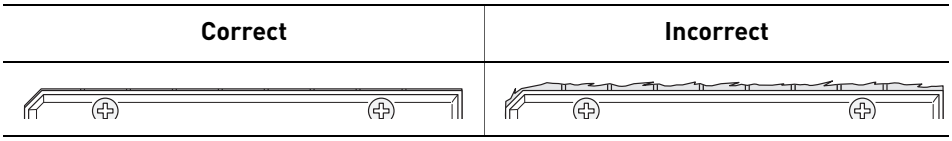
4. Seal the array card by running the carriage slowly over it, in one direction only. Do not apply downward force on the carriage as you move it forward over the card.



5. Remove the sealed array card from the fixture and trim the fill reservoirs from the array card assembly using scissors. Trim the foil array card so that the edge is even with the plastic carrier.



IMPORTANT! Completely remove the fill reservoirs from the array card so that the edge is free of residual plastic. The plastic from the fill reservoirs that extends beyond the edge of the card can prevent the card from seating properly on the sample block and affect amplification.



IMPORTANT! As you seal the remaining filled array cards, store them in a dark place until you are ready to use them. The fluorescent dyes in the array card are photosensitive. Prolonged exposure to light can diminish the fluorescence of the dye.

Capping and uncapping the 96-well reaction tubes and tube strips

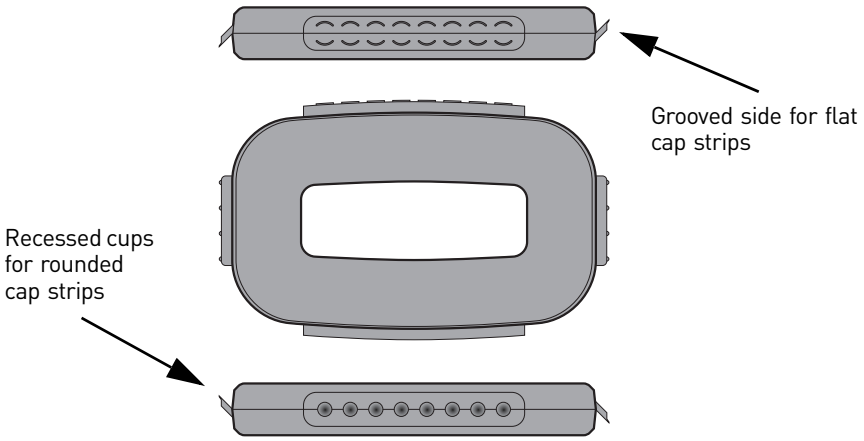
Note: Make sure that you secure the caps on the tubes and tube-strips tightly to avoid sample evaporation.

If you use the 96-well MicroAmp® Optical 8-Tube Strips or MicroAmp® Optical Tubes without Cap, use the MicroAmp® Cap Installing Tool (PN 4330015) and follow the instructions below for:

- Applying the MicroAmp® Optical 8-Cap Strip or MicroAmp® Optical Tubes without Cap to the tubes
- Removing a cap string from a plate

Required materials:

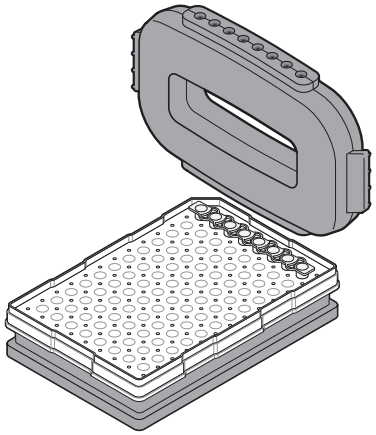
- MicroAmp® Cap Installing Tool
- MicroAmp® Optical 8-Tube Strips or MicroAmp® Optical Tubes without cap
- MicroAmp® Optical 8-Cap Strip



MicroAmp® Cap Installing Tool

Apply the MicroAmp® Optical 8-Cap Strip (flat)

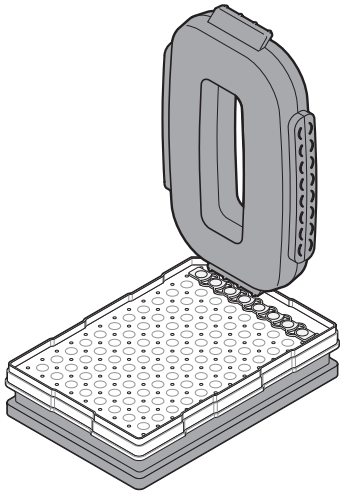
1. Grasp the Cap Installing Tool so that the grooved side is exposed.
2. Hold the strip of caps over the tube strip or the row of tubes.
3. Use the grooved side (shown) of the Cap Installing Tool to push and seat each cap firmly in place. Use a rocking motion to properly seat each cap.



Remove a cap string from a plate

The MicroAmp® Cap Installing Tool is also used for removing the MicroAmp® Optical 8-Cap Strip from the 96-well optical plates and tray/retainer assemblies. To remove the cap or cap strip:

1. Insert the small protrusions on the side of the Cap Installing Tool under the webbing between the caps on a cap strip.
2. Slowly pry the strip from the plate or Tray/ Retainer assembly.



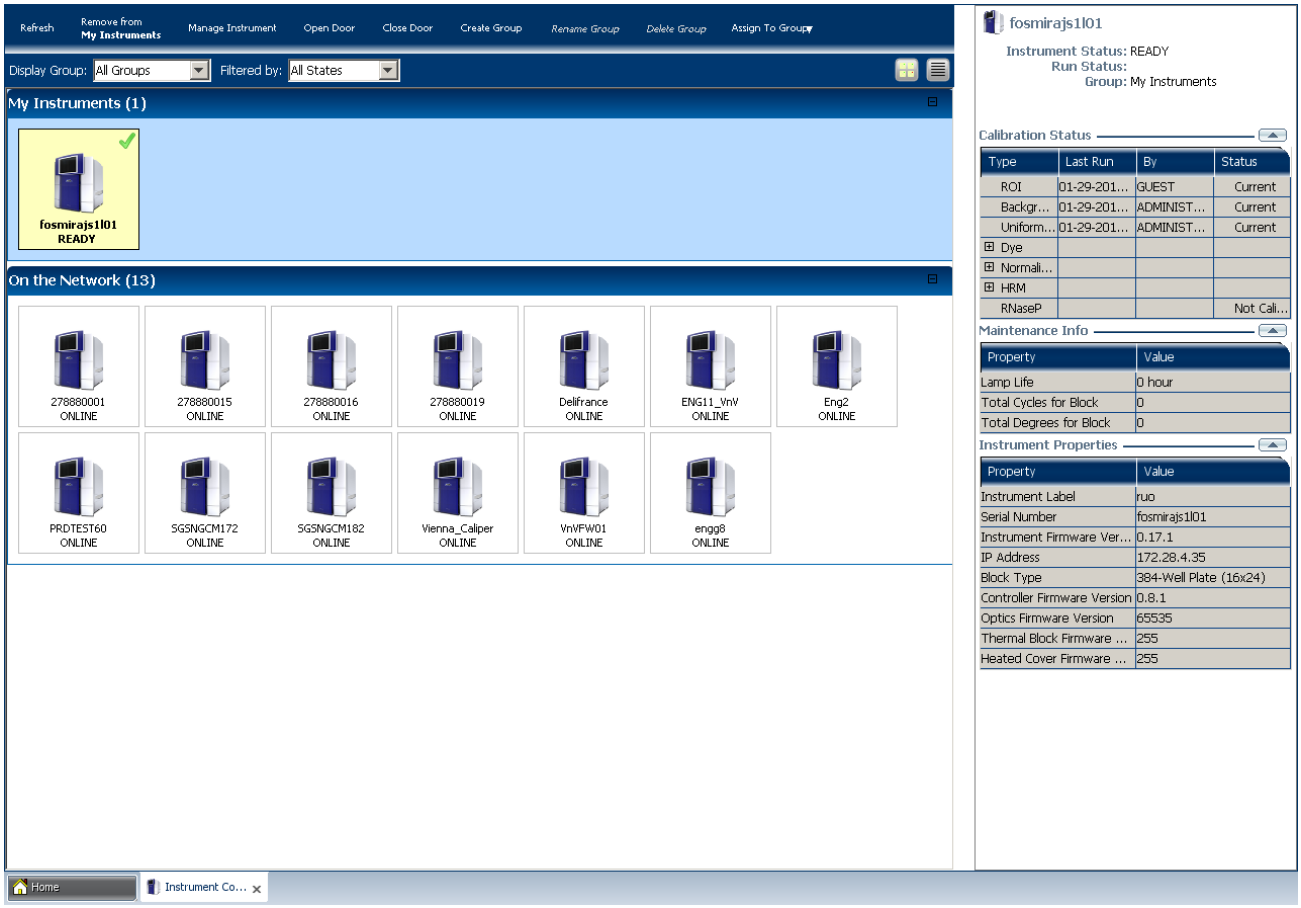
Start the experiment

To start an experiment:

1. Access the Instrument Console.
2. Load the reaction plate or array card into the instrument, as shown on page 41.
3. Run the experiment as shown on page 43.

Instrument Console

The Instrument Console displays all the ViiA™ 7 Instruments discovered on a network.



Left panel

The features on the left panel of the Instrument Console allow:

- Instrument access: Open and close the ViiA™ 7 Instrument door from the ViiA™ 7 Software user interface.
- Group management:
  - Create, rename, and delete groups and assign instruments to the groups.
  - Add and remove instruments to and from My Instruments.

    Note: To add instruments, select the icon of the ViiA™ 7 Instrument that you want to add to the My Instruments list. Then click **Add to My Instruments**. Similarly, click **Remove from My Instruments** to remove an instrument from the My Instruments list. You can also drag and drop the instrument icon into My Instruments or into the group created by you.

  - Display instrument groups from the Display Group drop-down menu. according to their activity. Select the status from the Filtered By drop-down menu. For more information on the status of an instrument, see "Monitor the experiment" on page 44.
- Instrument management:
  - Monitor experiments (check the run status or monitor a temperature plot or amplification plot during a run). For more information on monitoring experiments, see "Monitor the experiment" on page 44.
  - Maintain instruments (check the calibration status of instruments and perform different calibrations). For more information on Instrument maintenance, refer to *Applied Biosystems ViiA™ 7 Real-Time PCR System User Guide* (PN 4442661).
  - Manage files (upload setup files; download completed experiments; and create, rename, and delete experiment files and plate setup folders).

    Note: Completed experiments are downloaded into the default folder **Completed Experiments**.

    Note: To manage files, click **Manage Instrument**.
    Use the File Manager to create, rename or delete folders for holding setup files for starting a run or completed experiments for analysis.
    To move setup files from one folder to the other, click **Move** and select the setup folder you want to shift the setup file into.

IMPORTANT! To Manage and Monitor, you must move instruments from On the Network to My Instruments or a custom group. You can start a run and calibrate instruments present only in the My Instruments group or the custom group(s) that you created.

Right panel

The right panel of the Instrument Console displays:

- The name of the instrument whose instrument icon is selected.
- The run status of the selected instrument.
- The group the instrument belongs to.
- The calibration status, maintenance reminders and instrument properties of the selected instrument.

The calibration status is indicated by the icon. The icon appears in the Status column of the Calibration Status table after the last reminder date before the calibration expires.

Status icons

You can monitor the instrument status and view calibration and other information in the Instrument Console.

ViiA™ 7 Instrument status icon

The status of an instrument is represented by an icon in the top-right corner of the thumbnail representation of the instrument on the Instrument Console. An instrument displays the status when you place the instrument icon under My Instruments or under the Group(s) that you created.

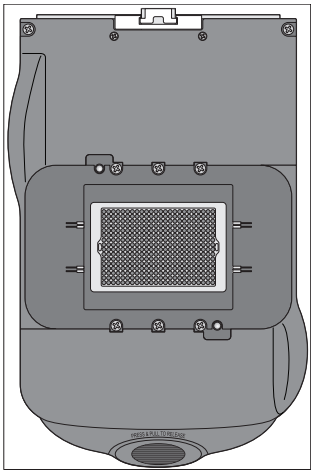
To monitor the instrument status:

1. On the Home tab ( ), select **Instrument Console**. If you do not see an instrument, click **Refresh** in the instrument console toolbar.
2. If needed, move the instrument from the On the Network group to a group which can be monitored:
   a. Click the instrument of interest, then click **Assign to Group** in the instrument console toolbar.
   b. Select the **My Instruments** or a personal group in the drop-down list. The instrument is now monitored.

Icon	Instrument status
	Ready
(no icon)	Available on the network but cannot be monitored because that instrument is not under My Instruments or a group you created.
	Run in process (The time remaining for the run is shown to the left of the icon.)
	Unavailable
	Incompatible firmware version
	No longer connected to the network
	Error occurred during run

Load the reaction plate or array card into the instrument

CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the temperature of the sample block(s) can exceed 100 °C. Keep your hands away until the sample block(s) reaches room temperature.



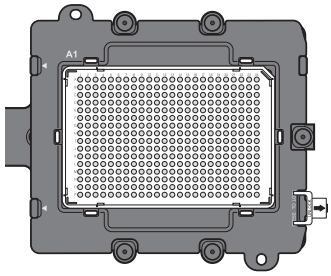
Sample block

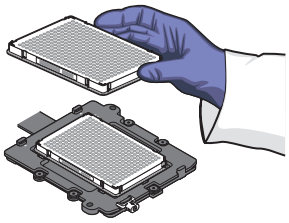
IMPORTANT! Wear powder-free gloves when you handle the **reaction plate or array card**.

IMPORTANT! Plates and array cards should be loaded and unloaded by operators who have been warned of the moving parts hazard and have been adequately trained.

1. Touch on the ViiA™ 7 Instrument touchscreen or click **Open Door** in the Instrument Console screen of the ViiA™ 7 Software to allow the plate adapter to come out from the instrument side.

2. Place the reaction plate or array card on the plate adapter. Ensure that the reaction plate or array card is properly aligned in the holder.
   - Make sure the well A1 is positioned at the top-left of the tray for any of the plate formats.
   - Make sure the barcode (for any of the plate formats) is facing the front of the instrument.





   - If using reaction tubes or tube strips, make sure you use adaptors. The adaptors are attached to the plate transport arm. The tray containing the tubes or tube strips must be placed on the adaptor and not into the sample block directly.

IMPORTANT! For optimal performance with partial loads, load at least 16 tubes and arrange them in:
Adjacent columns of 8 tubes, using rows A through H. For example, use wells in columns 6 and 7 (rows A through H).
Or
Adjacent rows of 8 tubes, using columns 3 through 10. For example, use wells in row D (columns 3 through 10) and row E (columns 3 through 10).

WARNING! Make sure that you use the flat caps for the 0.2µL tubes and 0.1µL tubes. Use of rounded caps damages the heated cover.

3. Touch on the ViiA™ 7 Instrument touchscreen or click **Close Door** in the Instrument Console screen of the ViiA™ 7 Software to retract the plate adapter back into the instrument.

Enable or change the Notification Settings

You can configure the ViiA™ 7 Software to alert you by email when the ViiA™ 7 Instrument begins and completes a run, or if an error occurs during a run.

Note: For details on using the Notification Settings feature, refer to the *Applied Biosystems ViiA™ 7 Real-Time PCR System User Guide* (PN 4442661).

Run the experiment

You can run the experiment in either of the following two ways:

- From the ViiA™ 7 Software
- From the ViiA™ 7 Instrument touchscreen

Note: The example experiments in each of the getting started guide booklets start a run from the ViiA™ 7 Software.

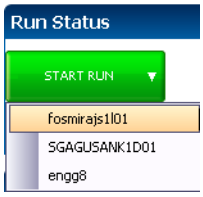
IMPORTANT! Make sure that instrument calibration is up-to-date. If a calibration has expired, you will get a warning when you start a run. For information on calibrating the ViiA™ 7 Instrument, refer to *Applied Biosystems ViiA™ 7 Real-Time PCR System User Guide* (PN 4442661).

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the ViiA™ 7 Instrument is in operation.

From the ViiA™ 7 Software

1. In the ViiA™ 7 Software, click **Run** in the navigation pane.
2. Click **START RUN**. Select the instrument for the run from the drop-down menu of the instruments placed under My Instruments.

   IMPORTANT! Make sure that the instrument to run the experiment on is in My Instruments or the custom group, and that it is ready to run an experiment. If the preferred instrument is not present under My Instruments or the custom group, or if it is unavailable, clicking START RUN does not display instrument names in the drop-down menu.



From the ViiA™ 7 Instrument touchscreen

1. Touch the ViiA™ 7 Instrument touchscreen to awaken it.

   Note: If the touchscreen is not at the Main Menu screen, touch .
2. In the Main Menu screen, touch **Browse Experiments**.
3. In the Browse screen, touch **Folders**, to display the folders containing the experiment setup files.
4. Touch any of the folder names to display the experiments in that folder.
5. In the Experiments screen, select the desired experiment, then touch **View/Edit** to view or edit the experiment before starting the run.

   Note: You can start a run immediately by clicking **Start Run**, then go to step 8.

6. *(Optional)* Modify the experiment parameters as needed. You can use the:
   - **Add** and **Delete** buttons to add and delete a stage or step to the thermal profile.
   - **Add Melt Curve button** to add a melt curve to the thermal profile.
   - **Save** button to save the experiment you modify.

7. In the Save Experiment screen, touch each field to edit the:
   - Experiment name
   - Folder to save the experiment
   - Reaction volume
   - Barcode Number
   - Notes

   When finished, touch **Save & Start Run** to start the experiment.

8. In the Start Run screen, touch each field as needed to modify the associated parameter, then touch **Start Run Now** to start the experiment.

   Note: When the run is complete, touch to unload the plate from the instrument. You can download the results of the experiment from a computer if the instrument is connected to a network, or copy the data to a USB device as explained in "Transfer experiment results" on page 50.

Monitor the experiment

Note: If there is loss of connection during an experiment, remove and then add the instrument to the My Instruments list. You may then resume monitoring the experiment.

You can monitor an experiment run in three ways:

- From the ViiA™ 7 Instrument touchscreen, in the same way that you run the experiment (see "From the ViiA™ 7 Instrument touchscreen" on page 43).
- From the Run screen of the ViiA™ 7 Software, while the experiment is in progress, as shown below.
- From the Instrument Console of the ViiA™ 7 Software (to monitor an experiment started from another computer or from the ViiA™ 7 Instrument touchscreen) as described in "From the ViiA™ 7 Software Instrument Console" on page 44.

From the ViiA™ 7 Software Run screen

1. Click **Amplification Plot** from the Run Experiment Menu to monitor the amplification plot of the experiment you are running.

   Note: For Melt Curve experiments, click **Melt Curve Plot** from the Run Experiment Menu.

2. Click **Temperature Plot** from the Run Experiment Menu to monitor the temperature plot of the experiment you are running.

From the ViiA™ 7 Software Instrument Console

1. In the Instrument Console screen, select the icon of the instrument that you are using to run the experiment.

2. Click **Manage Instrument**.

3. On the Instrument Manager screen, click **Monitor Running Instrument**.

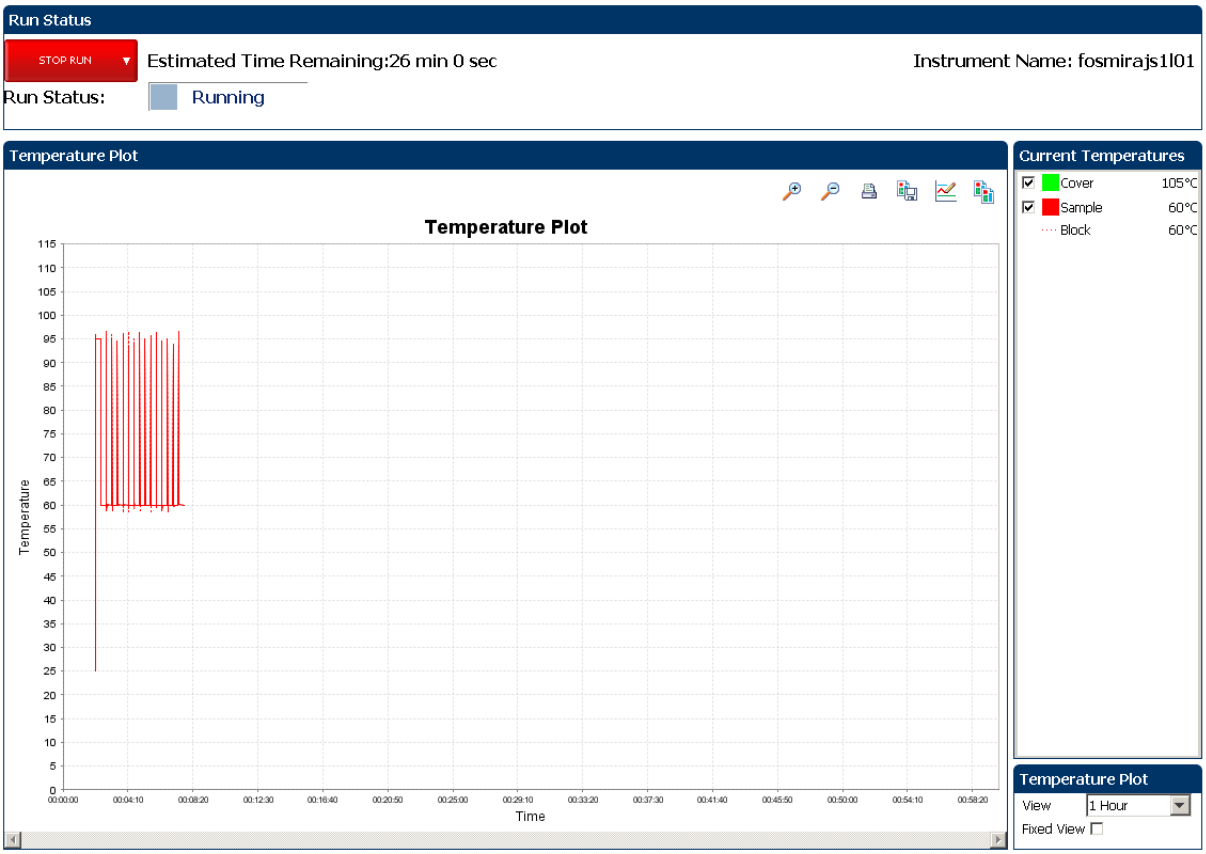
You can view the progress of the run in real time from the Run screen. During the run, periodically view the Amplification Plot, Temperature Plot and Run Method (see page 46) available from the ViiA™ 7 Software for potential problems.

To...	Action
Stop the run	• In the ViiA™ 7 Software, click **STOP RUN**. • In the Stop Run dialog, click one of the following: – **Stop Immediately** to stop the run immediately. – **Stop after Current Cycle/Hold** to stop the run after the current cycle or hold. – **Cancel** to continue the run.
View amplification data in real time	Select **Amplification Plot**. See "To monitor the Amplification Plot" on page 46.
View temperature data for the run in real time	Select **Temperature Plot**. See "To monitor the Temperature Plot" on page 47.
View progress of the run in the Run Method screen	Select **Run Method**. See "To monitor the Run Method" on page 47.
Enable/disable the Notification Settings	Select or deselect **Enable Notifications**. See "Enable or change the Notification Settings" on page 42.

Note: The individual experiment booklets provide illustrations of the different experiments in real time.

Note: For Melt Curve experiments, click **Derivative Melt Curve** from the Run Experiment Menu.

The Run screen for a Standard Curve experiment run looks like this:



To monitor the Amplification Plot

To view data in the Amplification Plot, click **Amplification Plot** from the Run Experiment Menu, select the Plate Layout tab, then select the wells to view.

The Amplification Plot screen allows you to view sample amplification as your instrument collects fluorescence data during a run. If a method is set up to collect real-time data, the Amplification Plot screen displays the data for the wells selected in the Plate Layout tab. The plot contrasts normalized dye fluorescence (ΔRn) and cycle number.

The Amplification Plot screen is useful for identifying and examining abnormal amplification, including:

- Increased fluorescence in negative control wells.
- Absence of detectable fluorescence at an expected cycle (determined from previous similar experiments run using the same reagents under the same conditions).

  Note: If you notice abnormal amplification or a complete absence of signal, troubleshoot the error as explained in the ViiA™ 7 Software Help (click or press **F1**).

To monitor the Temperature Plot

To view data in the Temperature Plot screen, click **Temperature Plot** from the Run Experiment Menu.

During a run, the Temperature Plot screen displays the temperatures of the sample block(s), the heated cover, and samples (calculated) in real-time.

To...	Action
Add or remove temperature plots	Select **Cover** or **Sample Block** to view the presence of the associated data in the plot.
Change the time to display in the plot	From the **View** drop-down menu, select the amount of time to display in the plot.
Display a fixed time window during the instrument run If the entire plot does not fit in the screen, the screen is not updated as the run progresses. For example, if you select 10 minutes from the View drop-down menu, the plot will show data for 10 minutes. If the Fixed View is: • Deselected, the plot updates as the run progresses even after 10 minutes. • Selected, the plot does not update as the run progresses even after 10 minutes.	Select **Fixed View**.

The Temperature Plot screen can be useful for identifying hardware failures. When monitoring the Temperature Plot screen, observe the Sample and Block plots for abnormal behavior.

- The Sample and Block plots should mirror each other approximately. A significant deviation of the plots may indicate a problem.
- The Cover plot should maintain the constant temperature specified in the method. A departure from the constant temperature may indicate a problem.

  Note: If you notice abnormal temperature plot, troubleshoot the error as explained in the ViiA™ 7 Software Help (click or press F1).

To monitor the Run Method

To view data in the Run Method screen, click **Run Method** from the Run Experiment Menu.

The Run Method screen displays the run method selected for the run in progress. The software updates the Run Status field throughout the run.

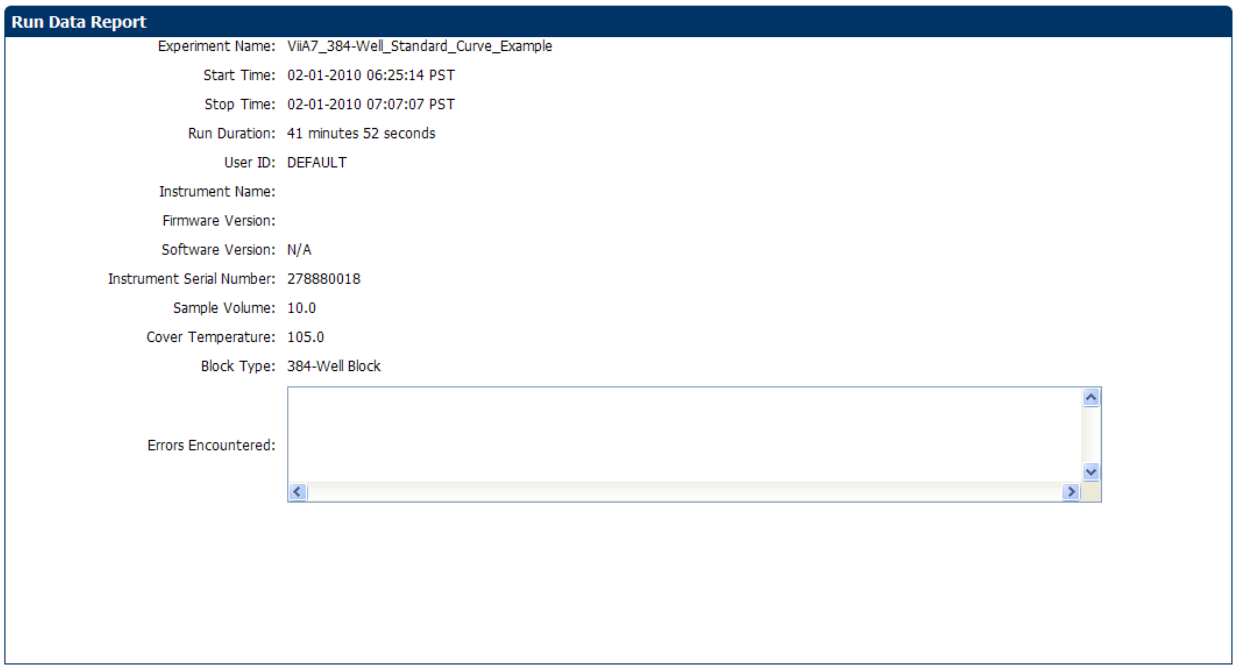
To...	Action
Change the number of cycles	In the **Adjust # of Cycles** field, enter the number of cycles to apply to the Cycling Stage.
Add a melt curve stage to the end of the run	Select **Add Melt Curve Stage to End**.

To...	Action
Add a Hold stage to the end of the run	Select **Add Holding Stage to End**.
Apply your changes	Click **Send to Instrument**.

If an alert appears, click the error for more information and troubleshoot the problem as explained in the ViiA™ 7 Software Help (click or press **F1**).

To view the run data

After a run is complete, you can view a run report by clicking **View Run Data**. The View Run Data screen displays information about the completed run, as in the following example from a Standard Curve experiment:



The run report data helps in:

- Comparing two experiments of the same type run on two different instruments.
- Troubleshooting. For example, after a firmware upgrade, you can compare an experiment run before and after the upgrade to determine if the upgrade affected performance.

From the ViiA™ 7 Instrument touchscreen

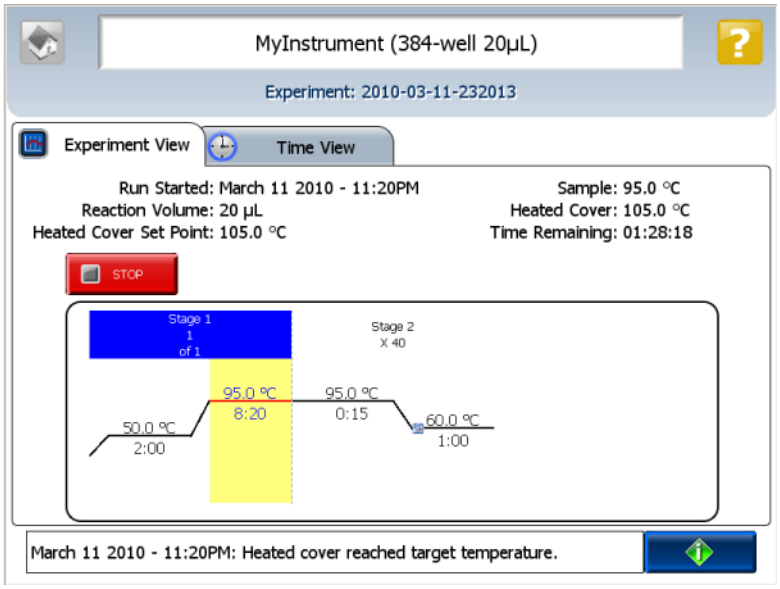
The touchscreen displays the method for the experiment, the date and time at which the run started, the time remaining in the run, and other information.

To...	Action
Display the time elapsed and the time remaining in the run	Touch the **Time View tab**, then touch **Experiment View** tab to return to the Run Method screen.
Stop the run	Touch STOP **STOP** to stop the protocol run immediately.

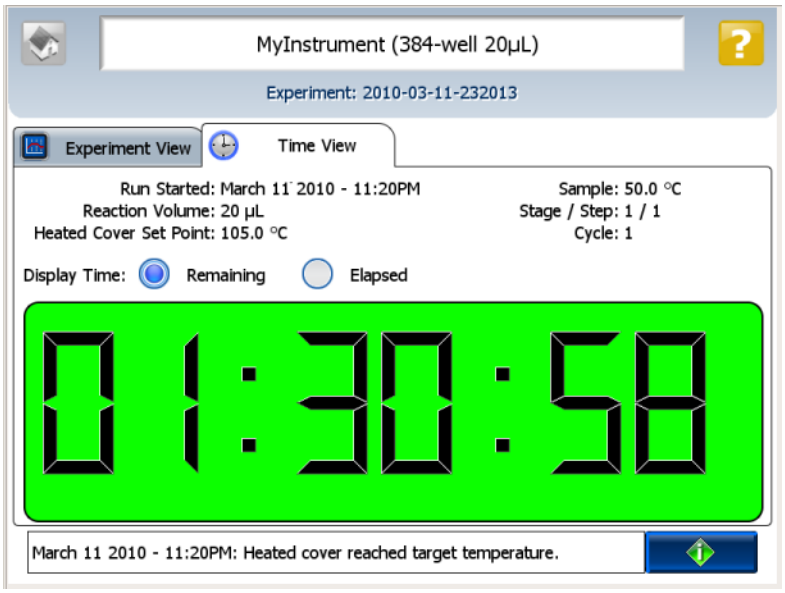
To...	Action
View the Events Log	Touch the status bar to display the events log.

The run method on the ViiA™ 7 Instrument touchscreen looks like this:

Experiment View



Time View



Unload the instrument

When your ViiA™ 7 Instrument displays the Main Menu screen, unload the reaction plate from the instrument and transfer the experiment data to the computer for analysis.

Unload the reaction plate or array card

CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the temperature of the sample block(s) can exceed 100 °C. Keep your hands away until the sample block(s) reaches room temperature.

1. Touch on the ViiA™ 7 Instrument touchscreen or click **Open Door** in the Instrument Console screen of the ViiA™ 7 Software.
2. Remove the reaction plate or array card from the plate adapter.
3. Touch or click **Close Door** to retract the plate adapter back into the instrument.

   If the ViiA™ 7 Instrument does not eject the plate, remove the plate as follows:

   a. Power off the ViiA™ 7 Instrument.
   b. Wait for 15 minutes, then power on the ViiA™ 7 Instrument and eject the plate.
   c. If the plate does not eject, power off and unplug the ViiA™ 7 Instrument, then open the access door.
   d. Wearing powder-free gloves, reach into the ViiA™ 7 Instrument and remove the plate from the heated cover, then close the access door.
   e. Perform a background calibration to confirm that the sample block has not been contaminated.

Transfer experiment results

You can transfer the experiment results in either of the following two ways:

Download the experiment from the ViiA™ 7 Instrument over the network

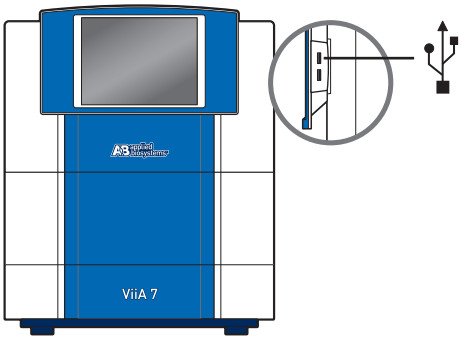
1. In the ViiA™ 7 Software, select **Instrument** ▸ **Instrument Console**.
2. Select the instrument icon of the **ViiA™ 7 Instrument** you just used to run the experiment from the My Instruments list.
3. Click **Manage Instrument** to open the Instrument Manager.
4. In the Instrument Manager, click **Manage Files**.
5. In the Experiments panel, select the experiment to download. Click **Download**.
6. In the Save dialog box, select the folder to hold the experiment results and click **Save**. The experiments folder is located at:

   <*drive*>:\Applied Biosystems\ViiA7 Software v1.1\experiments\

   where, <*drive*> is the computer hard drive on which the ViiA™ 7 Software is installed. The default installation drive for the software is the D: drive.

Transfer the experiment from the ViiA™ 7 Instrument to the computer via a USB drive:

1. If not already connected to the instrument, connect a USB drive to the USB port.



2. Touch the ViiA™ 7 Instrument touchscreen to awaken it.
3. If the touchscreen is not at the Main Menu screen, touch .
4. In the Main Menu, touch **Collect Results** to save the data to the USB drive.
5. Select one or multiple experiments (by touching them). Then touch **Copy to USB** to copy selected experiments to the USB drive.

   Note: If your instrument cannot find the USB drive, remove the USB drive, then try again. If the instrument still does not recognize the USB drive, try another USB drive.
6. Touch to return to the Main Menu.
7. Remove the USB drive from your instrument, then connect it to one of the USB ports on your computer.
8. In the computer desktop, use the Windows explorer to open the USB drive.
9. Copy the example experiment file to:
   <*drive*>:\Applied Biosystems\ViiA7 Software v1.1\experiments\
   where <*drive*> is the computer hard drive on which the ViiA™ 7 Software is installed. The default installation drive for the software is the D: drive.

Review experiment results

About analysis results

Immediately after a run, the ViiA™ 7 Software automatically analyzes the data using the default analysis settings, then displays the Amplification Plot screen.

Note: For auto-analysis of data, after a run, go to **Tools** ▸ **Preferences** ▸ **Experiment** and select the **Auto Analysis** check box.

Note: For Genotyping experiments, the ViiA™ 7 Software displays the Allelic Discrimination Plot screen.

To reanalyze the data, select all the wells in the plate layout, then click **Analyze**.

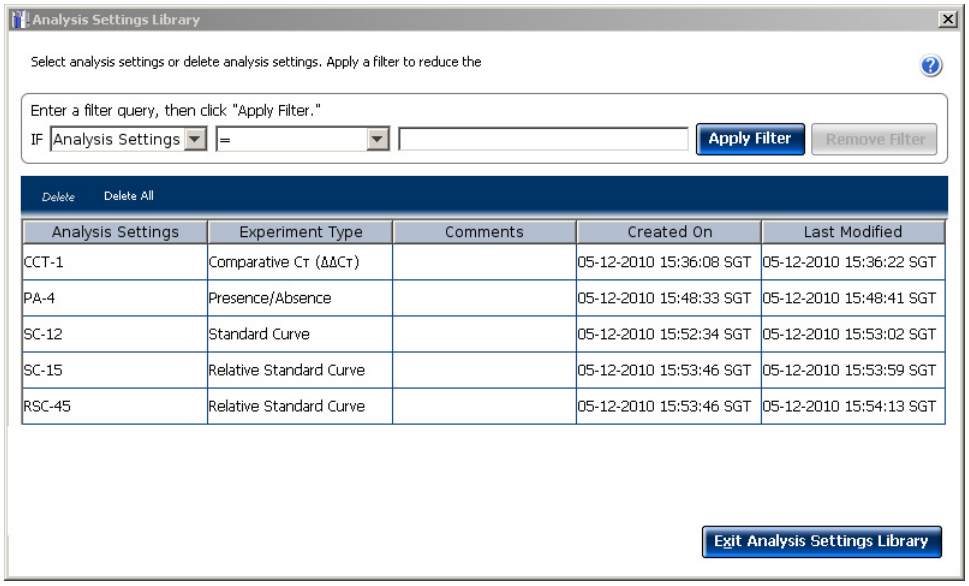
About the Analysis Settings Library

Analysis Settings are different for each experiment type. If the default analysis settings in the ViiA™ 7 Software are not suitable for your own experiment, you can change the settings in the Analysis Settings dialog box, then reanalyze your experiment.

You can save the changed analysis settings to the Analysis Settings Library so that you can use them in other experiments.

In the Analysis Settings Library dialog box you can apply a filter to reduce the number of settings protocols displayed.

You can access the Analysis Settings Library from the Tools menu. The Analysis Settings Library dialog box looks like this:



To change the analysis settings and to save them to the Analysis Settings Library:

1. From the Experiment Menu pane, select **Analysis**.

2. On the Analysis screen, click **Analysis Settings** to open the Analysis Settings dialog box.
3. Change the analysis settings as per your requirement.
4. Click **Save to Library** to save the changes you have made to the Analysis Settings Library.

You can import the analysis settings you have previously saved to the Analysis Settings Library, by clicking **Load from Library** in the Analysis Settings dialog box.

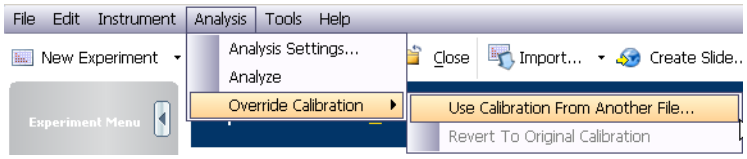
To override calibration

Each experiment file (.eds) stores the calibration data from the ViiA™ 7 Instrument it was run on. The calibration data can affect the analysis results of an experiment.

If you have run multiple experiments on different ViiA™ 7 Instruments and prefer the analysis results from a particular instrument, then you can choose to use the calibration data from another ViiA™ 7 Instrument.

To use the calibration data of another experiment

1. Open the experiment file (.eds), in which you want to import the calibration data from another ViiA™ 7 Instrument, in the ViiA™ 7 Software.
2. Go to **Analysis** ▸ **Override Calibration** ▸ **Use Calibration From Another File....**

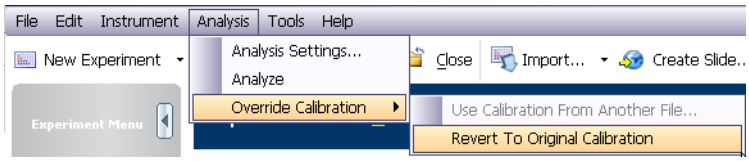


3. Browse to experiment file (.eds) from which you want to use the calibration data.

   Note: You can choose to override the calibration data in an experiment with the calibration data of only the same experiment type.
4. Click **Open**.

To revert to the original calibration data

1. Open the experiment file (.eds), in which you want to import the original calibration data, in the ViiA™ 7 Software.
2. Go to **Analysis** ▸ **Override Calibration** ▸ **Revert To Original Calibration.**



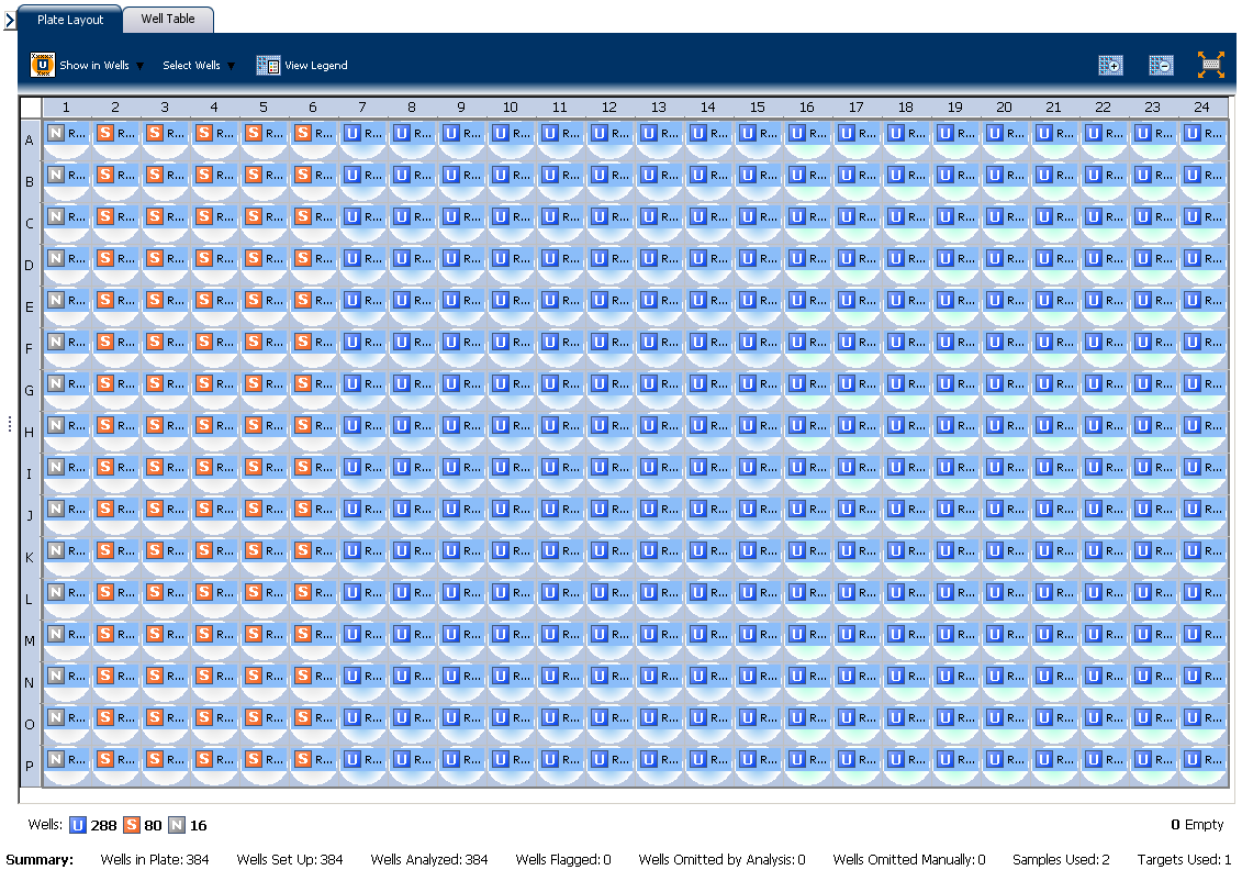
The experiment file will display analysis results as per the calibration data of the ViiA™ 7 Instrument that the experiment was run on.

To display wells

To display specific wells in the analysis plots, select the wells in the Plate Layout tab:

- To select wells of a specific type, use the Select Wells With drop-down menus: Select **Sample**, **Target**, or **Task**, then select the sample, target, or task name.
- To select a single well, click the well in the plate layout.
- To select multiple wells, click and drag over the desired wells, press **Ctrl-click**, or press **Shift-click** in the plate layout.
- To select all the wells, click the upper left corner of the plate layout.

The plate layout for a Standard Curve experiment looks like this:

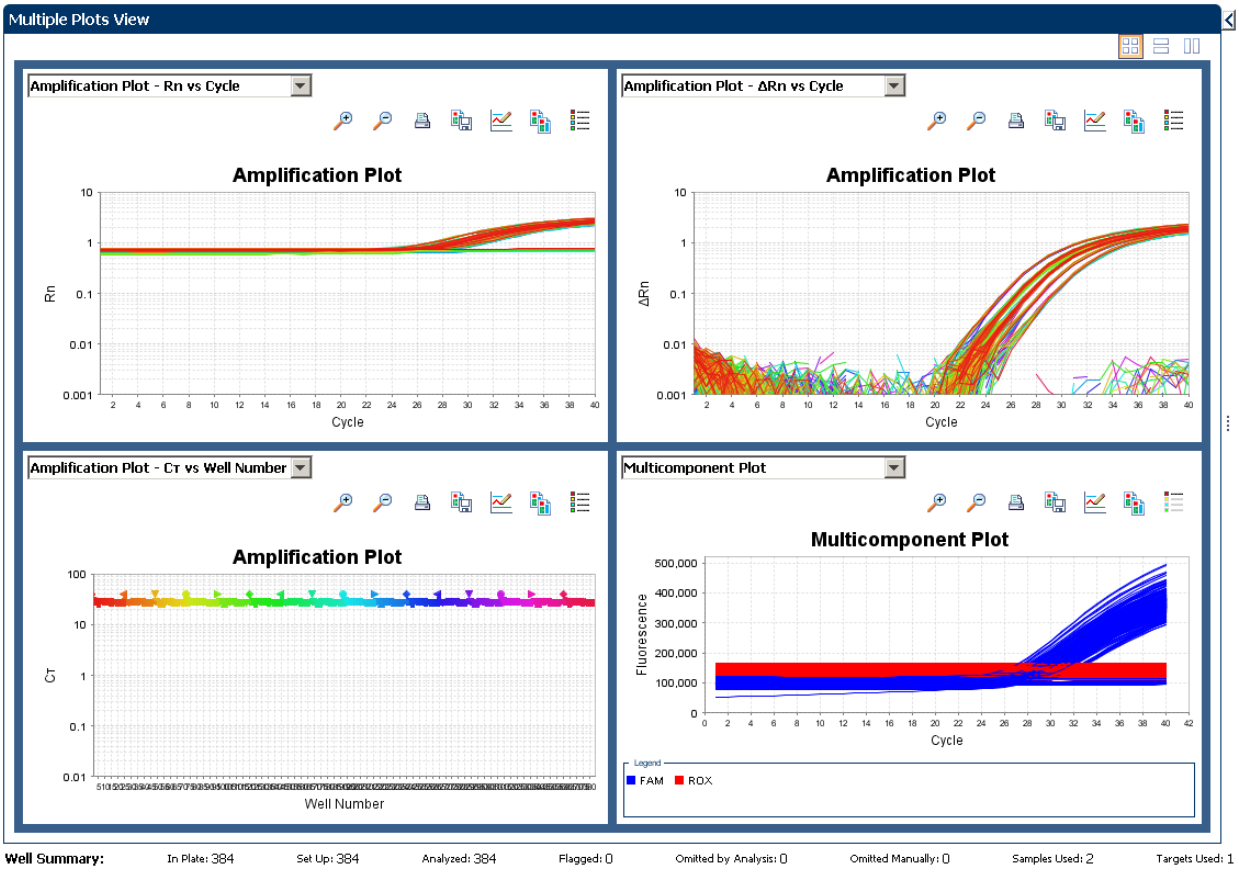


To display multiple plots

Use the Multiple Plots View screen to display up to four plots simultaneously. To navigate within the Multiple Plots View screen, from the Experiment Menu pane, select **Analysis ▸ Multiple Plots View**.

- To display four plots, click **Show plots in a 2 × 2 matrix**.
- Similarly, to display two plots in rows, click **Show plots in two rows**. and to display two plots vertically, click **Show plots in two columns**.
- To display a specific plot, select the plot from the drop-down menu above each plot display.

The Multiple Plots View screen for a Standard Curve example experiment looks like this:



To display an expanded view of a plot or wells

- Click to expand the view of a plot, displayed on the left-hand side of the screen.
- Click to expand the view of the Plate Layout or Well Table displayed on the right-hand side of the screen.

To edit plot properties

Use the Plot Properties dialog box on the Analysis screen to edit plot settings such as the font and color of the plot text, and the labels on the X axis and Y Axis.

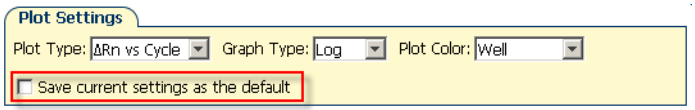
1. Click on the Analyze screen (the icon appears above the plot) to open the Plot Properties dialog box

2. Edit the settings under the General, X Axis, and Y Axis tab.
   - Click the General tab to edit the plot title text, font, or color. You can also select whether to show the plot title.
   - Click the X Axis tab to edit the x axis label text, font, or color; select the tick marks and tick mark labels to display; and select the range to display.
   - Click the Y Axis tab to edit the y axis label text, font, or color; select the tick marks and tick mark labels to display; and select the range to display.
3. Click **OK**.

To save current settings as default

You can change the Plot Settings for the different analysis plots, and save them as defaults.

Select the **Save current settings as the default** check box on the respective plot screens under the Analysis Experiment Menu.



To publish the analyzed data

To...	Click
Save a plot as an image file	
Print a plot	
Copy a plot to the clipboard	
Print a report	
Export data	

To...	Go to	Then
Print the plate layout	**File ▸ Print...**	Select the background color, and click **Print**
Create slides	**File ▸ Send to PowerPoint...**	Select the slides for your presentation, and click **Create Slides**
Print a report	**File ▸ Print Report...**	Select data for the report, and click **Print Report**

Export an experiment

About exporting an experiment

The Export feature of ViiA™ 7 Software allows you to export:

- Plate setup files for future experiments.

  Plate setup files contain setup information such as the well number, sample name, sample color, target name, dyes, and other reaction plate contents.
- Analyzed data in different formats for further analysis.

  The data can be exported in the ViiA™ 7 format, the 7900 SDS format, and the RDML format.

  The 7900 format is applicable only to Standard Curve, Relative Standard Curve, Genotyping, Presence/Absence, and Melt Curve experiments.

  The RDML export format is applicable only to Standard Curve, Relative Standard Curve, Comparative C_T, and Melt Curve experiments. The RDML format is available only in a single file format.

  For Standard Curve experiments, you can also export the analyzed data from the ViiA™ 7 Software to the external applications, TaqMan® Protein Expression Data Analysis Software and CopyCaller™ Software if they are installed on your computer before the ViiA™ 7 Software is installed. The applications appear in the Tools menu.
- Gene Expression studies to carry out a comparative analysis.

Export procedure

Note: If you choose the Auto Export option during experiment setup or before running an experiment, the data is automatically exported to the location you specified. If you did not set the Auto Export option, the analyzed data is not exported automatically.

1. Open the experiment file that contains the data to export, and from the Experiment Menu, click **Export.**
2. Select the format for exported data:
   - **ViiA™ 7 Format** (supports .txt, .xls, and .xlsx data).
   - **7900 Format** - Single experiments are exported in the SDS 2.4 detector centric export format of the 7900 Sequence Detecting System.
     Studies are exported in the SDS 2.3 RQ manager detector centric export format of the 7900 Sequence Detecting System
     The 7900 format supports only the .txt type of data.
   - **RDML Format** - Real Time Data Markup Language (supports only .xml type of data).

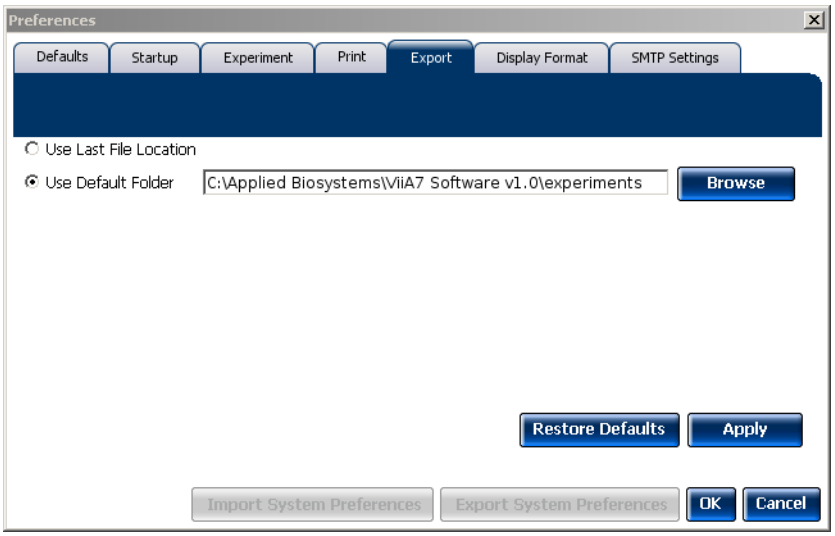
3. Select to export all data in one file or in separate files for each data type.
   - **One File** - All data types are exported in one file.
     - If you select the *.xls format, a worksheet is created for each data type.
     - If you select the *.txt format, the data are grouped by data type.
   - **Separate Files** - Each data type is exported in a separate file. For example, if you select three different data types Results, Amplification, and Multicomponent to export, three separate files (one each for Results, Amplification, and Multicomponent) are created. You can select the type of file (*.xls, *.xlsx or *.txt) to export from the **File Type** drop-down menu.

     Note: You cannot use an exported ***.xls** or an ***.xlsx** file when importing plate setup information.

4. *(Optional)* Select the **Open file(s) when export is complet**e check box to automatically open the file when export is complete.

5. Enter a file name and location.
   a. Enter a name for the export file in the **Export File Name** field.
   b. Enter the **Export File Location**. Click **Browse** if you do not want to save the export file in the default export folder.

      Note: To set up the Export File Location, go to **Tools** ▸ **Preferences**, and select the **Export** tab. You can select the **Use Last File Location** or **Use Default Folder** check box.



6. Select the data to export:

Select...	To export...
Sample setup	Well, sample name, sample color, and target name of samples in the plate
Raw data	Raw fluorescence data for each filter, for each cycle
Amplification data	Amplification results, such as C_T values, Rn, or ΔRn
Multicomponent data	Fluorescence data for each dye, for each cycle

Select...	To export...
Results	Results information, such as C_T values, Rn, or calls
Technical Replicate Results (Tech. Rep. Results)	Technical replicates information, such as Sample name, Target name, Task, or RQ
Biological Replicate Results (Bio. Rep. Results)	Biological replicates information, such as Biogroup name, Target name, Task, or RQ
Clipped Data	Information that is unique to the 7900 format. Data from the last three raw data points per step (clipped from the rest). The three data points are averaged to give you the final fluorescence data value for each step.

Note: Results data are not available for export until the run status is complete and the data are analyzed.

Note: The Technical Replicate Results, Biological Replicates Results, and Clipped Data are available only in Relative Standard Curve and Comparative C_T experiments.

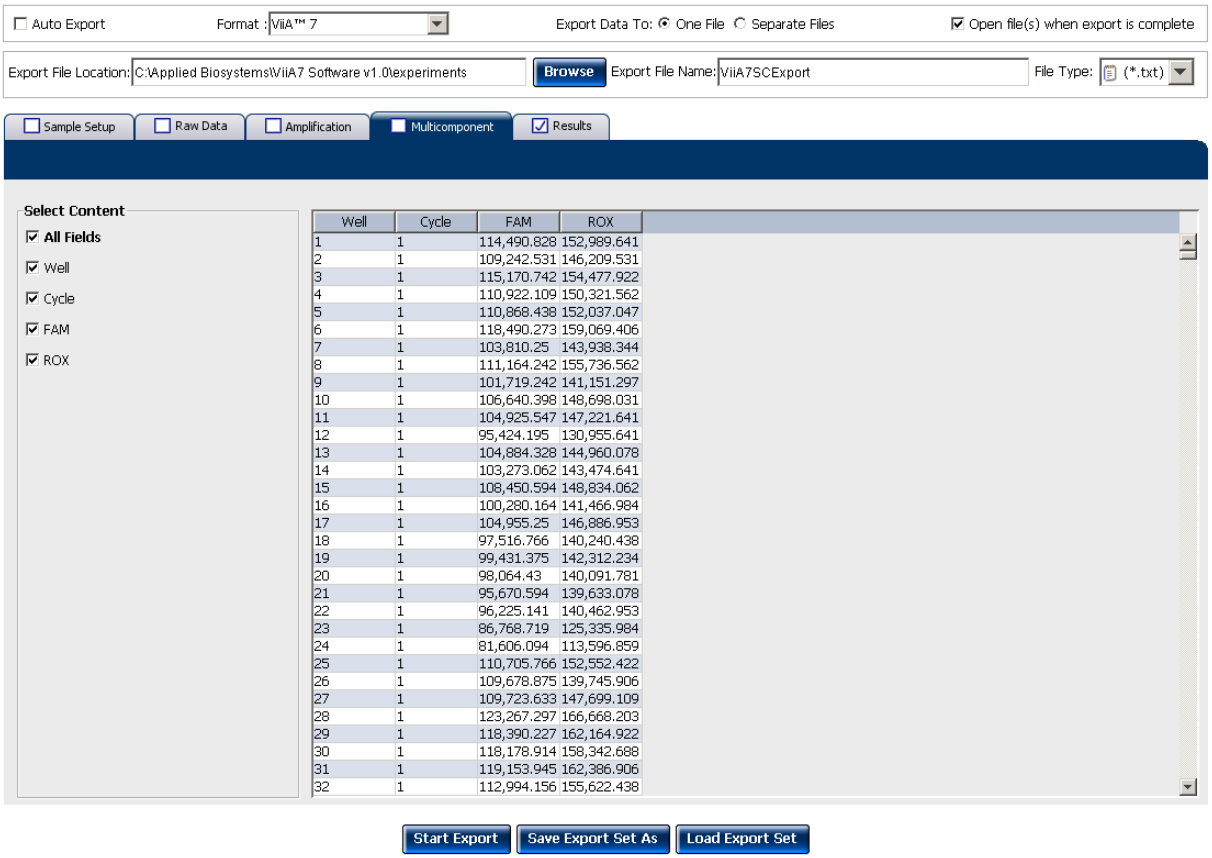
7. (*Optional*) For Standard Curve experiments, select the external application, **TaqMan® Protein Expression Data Analysis Software** or **CopyCaller™ Software** if either Software is installed on your computer.

8. *(Optional)* After you have defined the export properties or after moving the table headings order, you can save those export settings as an export set by clicking **Save Export Set As**. Later you can import the heading order into another file by clicking **Load Export Set**.

   Note: It is advisable to keep the default order of the table headings if you are using the external Applied Biosystems applications, **TaqMan® Protein Expression Data Analysis Software** or **CopyCaller™ Software** for further analysis.

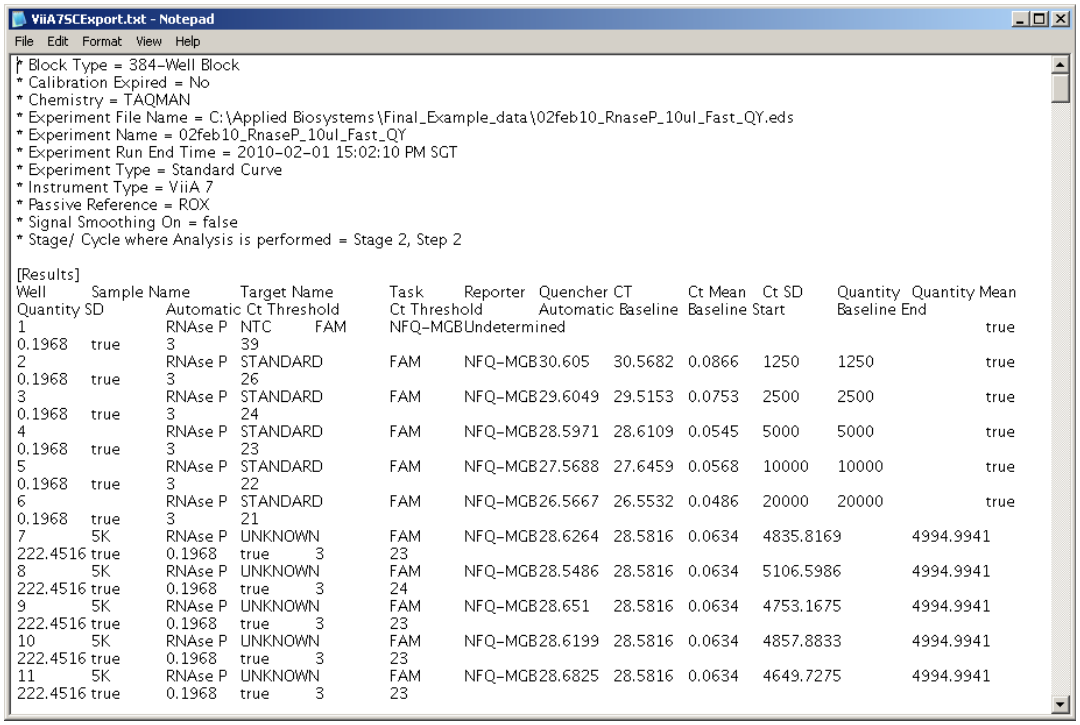
9. Click **Start Export**.

The Export screen for a Standard Curve experiment looks like this:



Well	Cycle	FAM	ROX
1	1	114,490.828	152,989.641
2	1	109,242.531	146,209.531
3	1	115,170.742	154,477.922
4	1	110,922.109	150,321.562
5	1	110,868.438	152,037.047
6	1	118,490.273	159,069.406
7	1	103,810.25	143,938.344
8	1	111,164.242	155,736.562
9	1	101,719.242	141,151.297
10	1	106,640.398	148,698.031
11	1	104,925.547	147,221.641
12	1	95,424.195	130,955.641
13	1	104,884.328	144,960.078
14	1	103,273.062	143,474.641
15	1	108,450.594	148,834.062
16	1	100,280.164	141,466.984
17	1	104,955.25	146,886.953
18	1	97,516.766	140,240.438
19	1	99,431.375	142,312.234
20	1	98,064.43	140,091.781
21	1	95,670.594	139,633.078
22	1	96,225.141	140,462.953
23	1	86,768.719	125,335.984
24	1	81,606.094	113,596.859
25	1	110,705.766	152,552.422
26	1	109,678.875	139,745.906
27	1	109,723.633	147,699.109
28	1	123,267.297	166,668.203
29	1	118,390.227	162,164.922
30	1	118,178.914	158,342.688
31	1	119,153.945	162,386.906
32	1	112,994.156	155,622.438

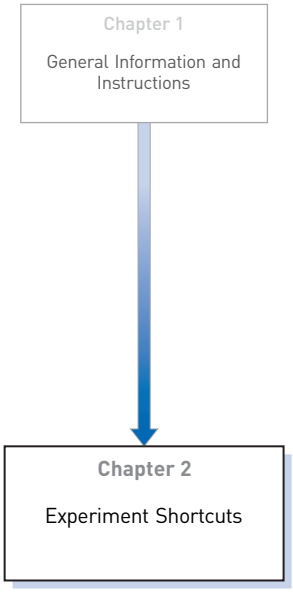
The exported file when opened in Notepad looks like this:




```
* Block Type = 384-Well Block
* Calibration Expired = No
* Chemistry = TAQMAN
* Experiment File Name = C:\Applied Biosystems\Final_Example_data\02feb10_RnaseP_10ul_Fast_QY.eds
* Experiment Name = 02feb10_RnaseP_10ul_Fast_QY
* Experiment Run End Time = 2010-02-01 15:02:10 PM SGT
* Experiment Type = Standard Curve
* Instrument Type = ViiA 7
* Passive Reference = ROX
* Signal Smoothing On = false
* Stage/ Cycle where Analysis is performed = Stage 2, Step 2

[Results]
Well    Sample Name    Target Name    Task    Reporter    Quencher    CT    Ct Mean    Ct SD    Quantity    Quantity Mean
Quantity SD    Automatic Ct Threshold    Ct Threshold    Automatic Baseline    Baseline Start    Baseline End
1    RNAse P    NTC    FAM    NFQ-MGB    Undetermined                    true
0.1968    true    3    39
2    RNAse P    STANDARD    FAM    NFQ-MGB    30.605    30.5682    0.0866    1250    1250    true
0.1968    true    3    26
3    RNAse P    STANDARD    FAM    NFQ-MGB    29.6049    29.5153    0.0753    2500    2500    true
0.1968    true    3    24
4    RNAse P    STANDARD    FAM    NFQ-MGB    28.5971    28.6109    0.0545    5000    5000    true
0.1968    true    3    23
5    RNAse P    STANDARD    FAM    NFQ-MGB    27.5688    27.6459    0.0568    10000    10000    true
0.1968    true    3    22
6    RNAse P    STANDARD    FAM    NFQ-MGB    26.5667    26.5532    0.0486    20000    20000    true
0.1968    true    3    21
7    5K    RNAse P    UNKNOWN    FAM    NFQ-MGB    28.6264    28.5816    0.0634    4835.8169    4994.9941
222.4516    true    0.1968    true    3    23
8    5K    RNAse P    UNKNOWN    FAM    NFQ-MGB    28.5486    28.5816    0.0634    5106.5986    4994.9941
222.4516    true    0.1968    true    3    24
9    5K    RNAse P    UNKNOWN    FAM    NFQ-MGB    28.651    28.5816    0.0634    4753.1675    4994.9941
222.4516    true    0.1968    true    3    23
10    5K    RNAse P    UNKNOWN    FAM    NFQ-MGB    28.6199    28.5816    0.0634    4857.8833    4994.9941
222.4516    true    0.1968    true    3    23
11    5K    RNAse P    UNKNOWN    FAM    NFQ-MGB    28.6825    28.5816    0.0634    4649.7275    4994.9941
222.4516    true    0.1968    true    3    23
```

2 Experiment Shortcuts



This chapter provides you with shortcuts to use in the ViiA™ 7 Software after you have learned experiment basics.

You can reuse experiment settings and plate setup information by: directly importing and editing a template, using the QuickStart feature with a template, importing experiment setup information, or importing a sample definition file; you can also prepare several experiments at once or create a new experiment using the ReadiApp feature.

The chapter covers:

- Create an experiment from a template 62
- Run an experiment with QuickStart 63
- Import plate setup for an experiment 65
- Import sample information 66
- Use a template to create a batch of experiments 69
- Create an experiment using ReadiApp 71

Create an experiment from a template

You can use a template to create a new experiment. Templates are useful when you want to create many experiments with the same experiment parameters.

You can create an experiment from a template from the ViiA™ 7 Software and from the ViiA™ 7 Instrument touchscreen.

Note: To access the ViiA™ 7 Software example templates, navigate to the templates folder located at <drive>:\Applied Biosystems\ViiA7 Software v1.1\config.

To create a template

1. Log in to the ViiA™ 7 Software and, from the Home screen, open an existing experiment, or create a new experiment.

   Note: To create a new experiment using the Experiment Setup, see "Set up an experiment" on page 13.

2. Select **File** ▸ **Save As Template**.

3. Enter a file name, select a location for the template, then click **Save** and **Close**.
   You can use that experiment as a template for similar experiments.

To create a new experiment using a template

1. From the Home screen, click **Template**.

2. Locate and select the template file, then click **Open**.
   A new experiment is created using the setup information from the template.

3. Edit the experiment properties, plate definitions, plate assignments, and run method before you prepare the reactions and run the experiment.

4. Proceed to preparing reactions, running the experiment, and analyzing the data.

To create an experiment using a template on the ViiA™ 7 Instrument touchscreen

You can run experiments using templates from the ViiA™ 7 Instrument touchscreen by importing the templates from the ViiA™ 7 Software instrument console or a USB drive. You can also modify the experiment parameters in the templates as per your requirement.

To run a pre-existing template

1. Touch **View Template** on the Home screen of the ViiA™ 7 Instrument touchscreen.

2. Select a pre-existing template from the templates list on the View Templates screen.

3. Touch **View** to see the run profile before you start a run.

4. After confirming the template setup is correct, touch to go back to View Template screen. Touch **Start Run**.

To edit a template before running the experiment

1. Touch **Create Experiment** on the View Templates screen to create a new experiment from the existing template.

   Note: Select a template before you touch **Create Experiment**.

2. Edit the experiment parameters in the Create New Experiment screen.

3. Touch **Save & Exit** to save and exit the experiment or touch **Save & Start Run** to save and start an experiment run.

Run an experiment with QuickStart

You can use a template to run an experiment with the ViiA™ 7 Software QuickStart feature:

QuickStart from the ViiA™ 7 Software

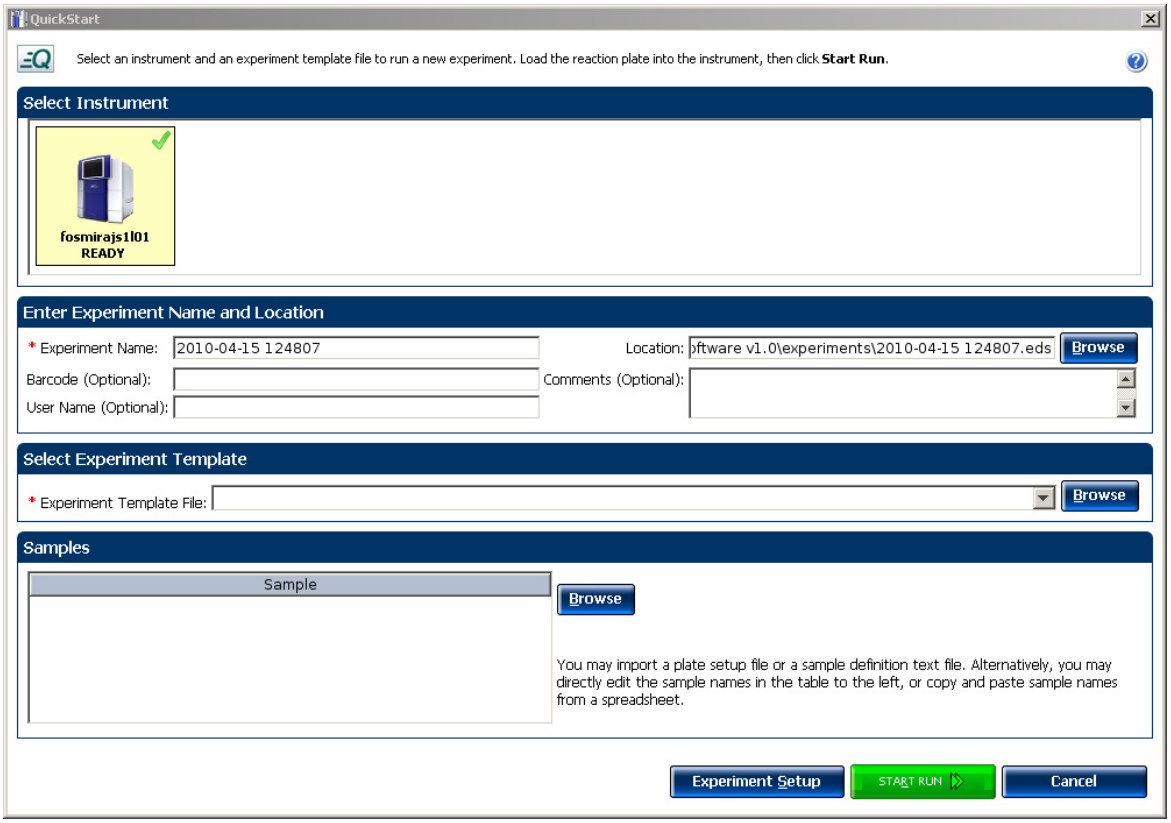
1. Prepare the reactions.

2. Log in to the ViiA™ 7 Software and, from the Home screen, click **QuickStart** to access the QuickStart dialog box.

3. In the QuickStart dialog box, enter or select the:
   a. Instrument icon of the instrument to perform the run on.
   b. Experiment name.
   c. Experiment template file.
   d. (*Optional*) Barcode and User Name for the experiment.

4. *(Optional)* To review the experiment or to make changes to any of the experiment parameters, click **Experiment Setup**.

The QuickStart dialog box looks like this:



5. Proceed to running the experiment and analyzing the data.

QuickStart from the ViiA™ 7 Instrument touchscreen

You can QuickStart an experiment from the ViiA™ 7 Instrument touchscreen in the following ways:

- Start an experiment using a pre-defined template.
- Start an experiment with a pre-defined short-cut button.

Start an experiment using a pre-defined template

You can use a pre-existing template from the default experiments folder or use a custom template from another folder to start a run.

Start an experiment with a pre-defined short-cut button

The ViiA™ 7 Instrument touchscreen displays up to 18 shortcut buttons to templates or folders that contain experiments to be run. The shortcut buttons are present under MY SHORTCUTS on the Home screen. To start a run, touch any of the pre-defined experiment or folder buttons.

To create a shortcut button for a preferred experiment or a folder that contains experiments:

1. Touch **Settings** to open the Settings Menu.

2. Touch **Set Up Shortcuts** to list the Shortcut Targets.

3. On the Shortcut Targets list screen, select an existing template Shortcut Target button or an unused button.

4. Touch **Set Shortcut**. If you selected an unused button, then touching Set Shortcut will list out the templates and folders to set the shortcut for.

5. Under the **From Templates** tab, select the templates for which you are creating the shortcut button.

6. *(Optional)* Create a shortcut button to show the templates or experiments in a particular folder for quick access, from those listed under the *From Folders* tab. You can touch *Edit* to create or edit shortcut buttons.

Import plate setup for an experiment

You can import the plate setup for a new experiment from an exported file with one of the following formats:

- *.txt - Text format
- *.xml - XML format
- *.csv - Comma separated values format
- *.sdt - Sequence detecting system (sds) template files format
- *.sds - 7900 v2.3 format

IMPORTANT! Make sure the file you select contains only plate setup data and that the experiment types match.

Note: For instructions on exporting an experiment, see "Export an experiment" on page 57.

To Import the plate setup data:

1. Create a new experiment or open an existing experiment.

2. In the Experiment Setup screen, select **File ▸ Import Plate Setup** or access the Import drop-down menu in the toolbar and select **Import Plate Setup**.

3. Click **Browse**, locate and select the file to import, then click **Select**.

4. Click **Start Import**. The setup data from the exported text file is imported into the open experiment.

   Note: If your experiment already contains plate setup information, the software asks if you want to replace the plate setup with the data from the import file. Click **Yes** to replace the plate setup.

5. After importing plate setup information, use Experiment Setup to set up your experiment, and then run the experiment.

Note: You can import plate setup information from a 96-well plate into a 384-well plate, provided that the file you are importing the information from is a .txt file.

Import sample information

You can import sample information from a sample definition file to include in the plate setup for your experiment. A sample definition file is a comma-delimited file (*.csv) or a tab-delimited text file (*.txt) that contains the following setup information: well number, sample name, and custom sample properties.

Note: Make sure that the sample definition file you select contains only sample information.

Create a sample definition file

1. Open a text editing program such as Notepad.
2. Enter the following column headers in the first row (press the Tab key between each entry if you are saving the file as *.txt or enter a comma between each entry if you are saving the file as *.csv):
   - Well
   - Sample Name
   - *(Optional)* Column header names for up to six user-defined custom fields (for example, **Custom 1**, **Custom 2**, etc.)
3. For each subsequent row, enter the well number, press the **Tab** key or enter a comma, then enter the sample name. Optionally, press the **Tab** key, then enter the custom properties for the sample.
4. Save the file with the .txt or .csv file extension.

An example sample definition, saved with the .csv extension, file looks like this:

	A	B	C	D	E	F	G	H
1	Well	Sample Name	ID	Age	Sex	Weight	HairColor	Smoker
2	1	Sample 1	1	22	Female	25	black	Yes
3	2	Sample 2	2	25	Male	26	brown	No
4	3	Sample 3	3	45	Female	50	blonde	Yes
5	4	Sample 4	4	31	Male	33	red	Yes
6	5	Sample 5	5	29	Female	46	grey	No
7	6	Sample 6	6	26	Male	35	black	No
8	7	Sample 7	7	31	Female	33	black	Yes
9	8	Sample 8	8	32	Male	67	black	No
10	9	Sample 9	9	32	Female	55	brown	Yes
11	10	Sample 10	10	33	Male	44	blonde	Yes
12	11	Sample 11	11	34	Female	25	red	No
13	12	Sample 12	12	34	Male	26	grey	No
14	13	Sample 13	13	35	Female	50	black	Yes
15	14	Sample 14	14	35	Male	33	black	No
16	15	Sample 15	15	36	Female	46	black	Yes
17	16	Sample 16	16	36	Male	35	brown	Yes
18	17	Sample 17	17	37	Female	33	blonde	No
19	18	Sample 18	18	37	Male	67	red	No
20	19	Sample 19	19	38	Female	55	grey	Yes
21	20	Sample 20	20	38	Male	44	black	No

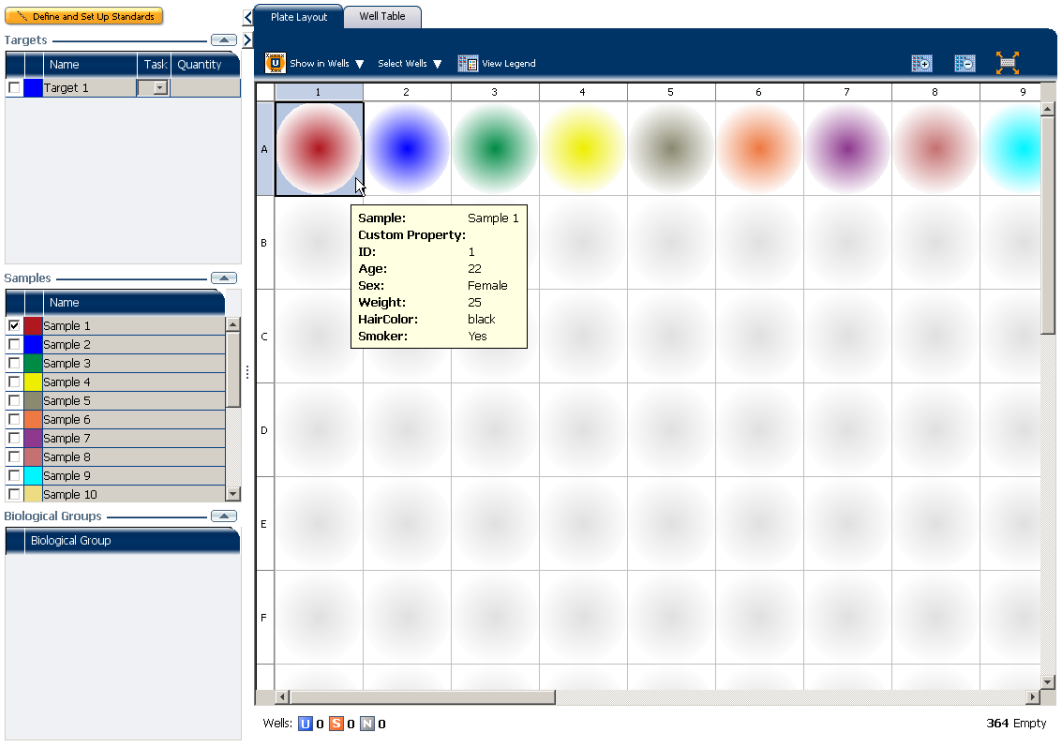
Import sample information from a sample definition file

1. Create a new experiment or open the experiment to receive the setup data (select **File ▸ Open**, select the file to open, then click **Open**).
2. From the open experiment, select **File ▸ Import Plate Setup.**
3. Click **Browse** to browse your computer for a sample definition text file (*.csv). After you locate the file and select it, click **Select**.
4. Click **Start Import**.
5. If your experiment already contains plate setup information, the software asks you if you want to replace the plate setup with the data from the file. Click **Yes** to replace the plate setup information.

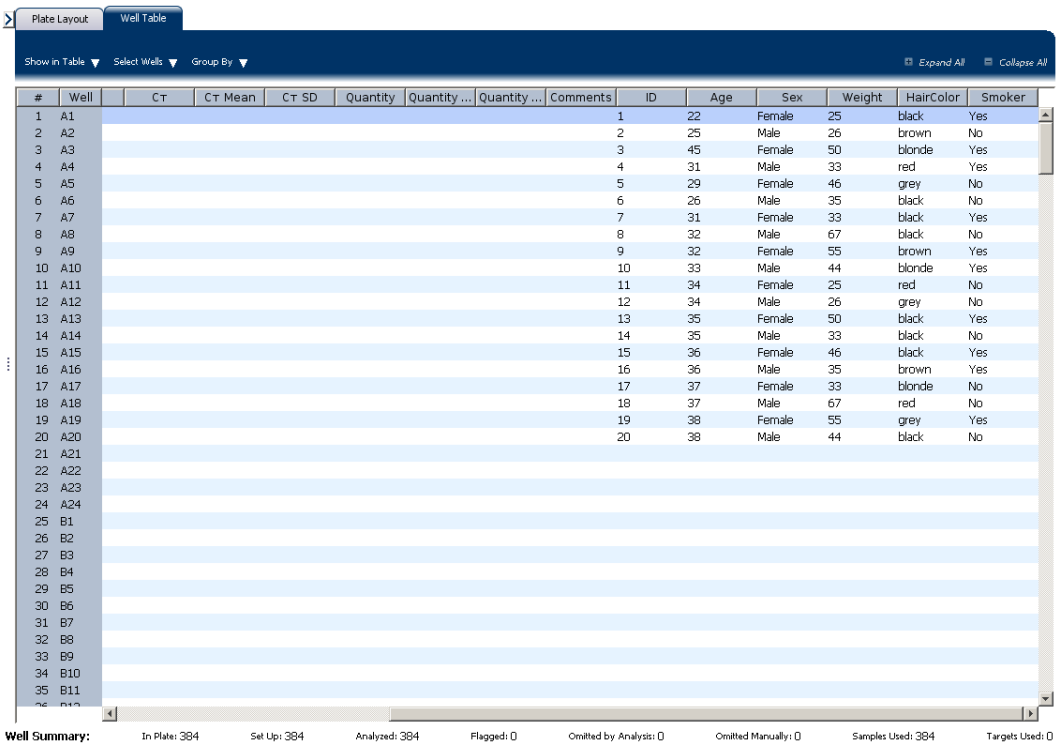
The samples appear in the Samples table for the experiment. All samples and well assignments in the experiment are replaced with those in the file. If defined, the custom sample properties also appear in the Well Table of the Analysis Section, and also in the Plate Layout tooltips in both the Setup and Analysis screens. The custom fields can be exported with the results data.

Note: You cannot edit the custom sample properties from within the Well Table. To modify this information, edit the custom fields in the sample definition file and import the file again. All of the sample information in the experiment is replaced with the information in the new file.

The Assign screen with information from the above sample definition file looks like this:



The Well Table in the Analysis section looks like this:

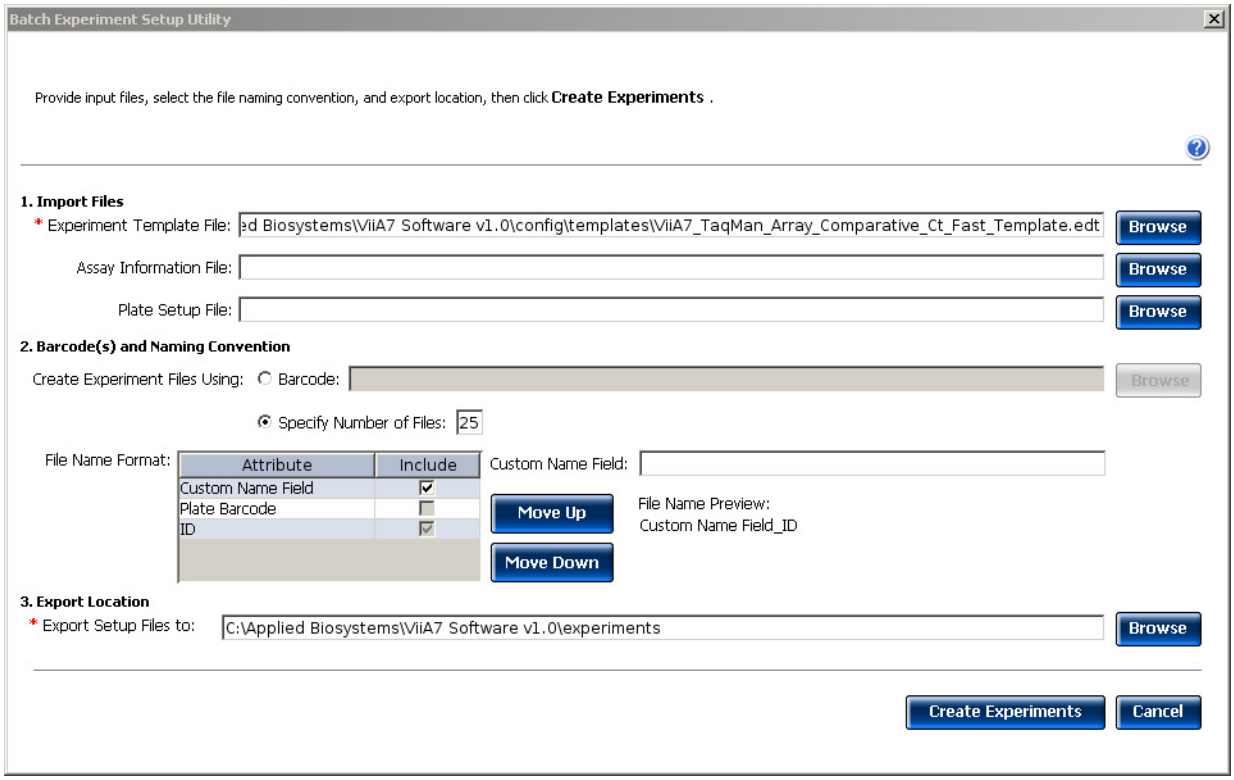


#	Well	CT	CT Mean	CT SD	Quantity	Quantity ...	Quantity ...	Comments	ID	Age	Sex	Weight	HairColor	Smoker
1	A1								1	22	Female	25	black	Yes
2	A2								2	25	Male	26	brown	No
3	A3								3	45	Female	50	blonde	Yes
4	A4								4	31	Male	33	red	Yes
5	A5								5	29	Female	46	grey	No
6	A6								6	26	Male	35	black	No
7	A7								7	31	Female	33	black	Yes
8	A8								8	32	Male	67	black	No
9	A9								9	32	Female	55	brown	Yes
10	A10								10	33	Male	44	blonde	Yes
11	A11								11	34	Female	25	red	No
12	A12								12	34	Male	26	grey	No
13	A13								13	35	Female	50	black	Yes
14	A14								14	35	Male	33	black	No
15	A15								15	36	Female	46	black	Yes
16	A16								16	36	Male	35	brown	Yes
17	A17								17	37	Female	33	blonde	No
18	A18								18	37	Male	67	red	No
19	A19								19	38	Female	55	grey	Yes
20	A20								20	38	Male	44	black	No
21	A21													
22	A22													
23	A23													
24	A24													
25	B1													
26	B2													
27	B3													
28	B4													
29	B5													
30	B6													
31	B7													
32	B8													
33	B9													
34	B10													
35	B11													

Use a template to create a batch of experiments

Use the batch experiment utility to create multiple experiment files from the same template without using Experiment Setup.

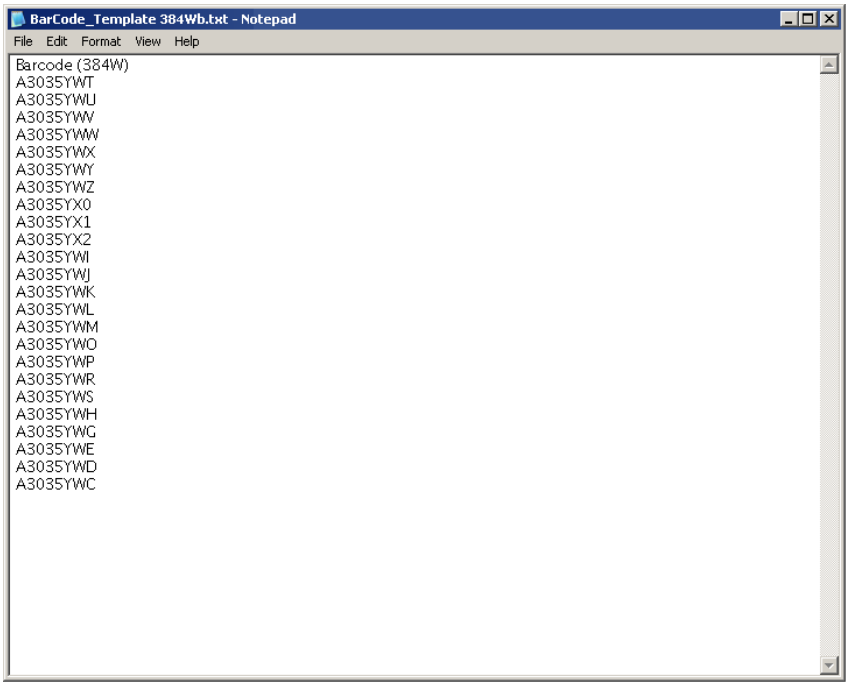
1. In the menu bar, select **Tools** ▸ **Batch Experiment Setup**.The Batch Experiment Setup Utility dialog box looks like this:



2. Select the file(s) to use to create the new experiments:
   a. Click **Browse** in the Experiment Template File field.
   b. Locate an *.edt file to import, then click **Select**.
   c. *(Optional)* Repeat **steps 2a** and **2b** for the remaining setup file types to import (Assay Information File (*.txt or *.xml), Plate Setup File (*.txt)).

3. Select the option to create experiment files. The selected option determines the number of experiment files created:
   - **Specify Number of Files** - Enter a number from 1 to 100.
   - **Barcode** - Click **Browse** and select a Barcode File (*.txt) to import. The software automatically adds the Plate Barcode attribute to the file name format. The number of experiments created equals the number of barcodes present in the barcode file.

     Note: A Barcode File contains one barcode per line. An example Barcode File looks like this:



4. *(Optional)* Edit the file name format. Use the File Name Preview to verify your settings.
   - Select the check box to include or exclude the **Custom Name Field** attribute from the file name. If included, click the Custom Name Field and enter up to 100 letters and/or numbers to identify the batch of experiments.

     Note: The file name can contain a total of 100 characters, including all file name attributes.

   - Click **Move Up** or **Move Down** to change the order of the selected file name attributes.

5. Select the location for the experiment files to be created:
   a. Click **Browse** in the Export Setup Files to: field.
   b. Review the location for the experiment files. Navigate to a new location if you do not want to export the experiment files to that folder, then click **Select**.

6. Click **Create Experiments**. A confirmation message appears when the batch of experiments has been created.

Create an experiment using ReadiApp

You can use the ReadiApp feature to set up an experiment in the ViiA™ 7 Software. The ReadiApp feature provides a shortcut to create experiments for the assays purchased from Applied Biosystems.

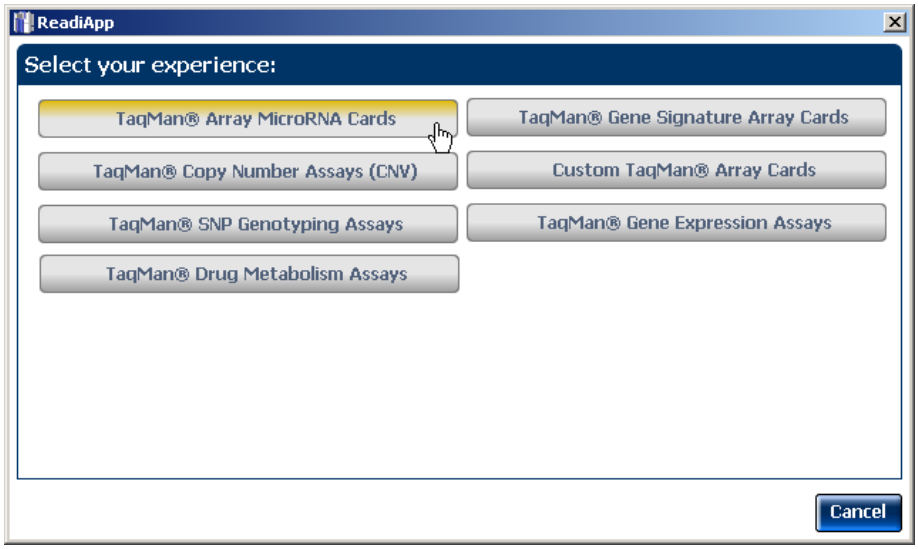
The default ReadiApp templates available in the ViiA™ 7 Software include:

- TaqMan® Gene Signature Array Cards
- Custom TaqMan® Array Cards
- TaqMan® Gene Expression Assays
- TaqMan® Drug Metabolism Assays
- TaqMan® array MicroRNA Cards
- TaqMan® Copy Number Assays (CNV)
- TaqMan® SNP Genotyping Assays

1. Log in to the ViiA™ 7 Software and, from the Set Up menu on the Home screen, click **ReadiApp**.

2. Click the assay to use to set up an experiment.

   Note: Click **Cancel** to exit the ReadiApp dialog box.



   A new experiment is created using the setup information from the template.

3. (*Optional*) Edit the experiment properties.

4. Proceed to preparing reactions, running the experiment, and analyzing the data.

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Part Number 4441434 Rev. C 12/2011

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BOOKLET 2

Running Standard Curve Experiments

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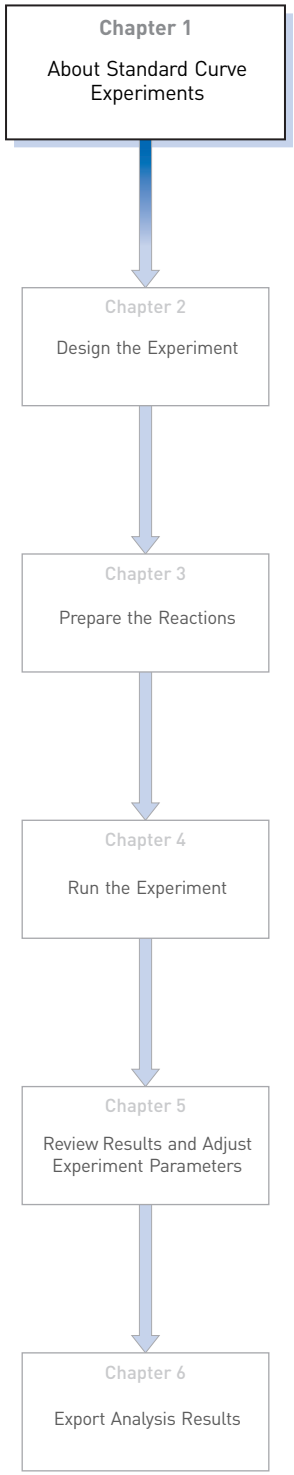
1 About Standard Curve Experiments



This chapter covers:

- Before you begin 6
- About the example experiment 7

IMPORTANT! First-time users of the ViiA™ 7 System please read Booklet 1, *Getting Started with ViiA™ 7 System Experiments* and Booklet 7, *ViiA™ 7 System Experiments - Appendixes* of this binder thoroughly. The booklet provides information and general instructions that are applicable to all the experiments described in this binder

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems ViiA™ 7 Software by pressing F1, clicking in the toolbar, or selecting **Help ▸ ViiA™ 7 Software Help**.

Before you begin

The Standard Curve method is used for determining absolute target quantity in samples. With the standard curve method, the software measures amplification of the target in samples and in a standard dilution series. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates the absolute quantity of target in the samples.

Assemble required components

- **Sample** – The tissue group that you are testing for a target gene.
- **Standard** – A sample that contains known quantities of the target; used in quantification experiments to generate standard curves.
- **Standard dilution series** – A set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards.
- **Replicates** – The total number of identical reactions containing identical samples, components, and volumes.
- **Negative controls** – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

PCR Options

When performing real-time PCR, choose between:

- Singleplex and multiplex PCR (below)

  and
- 1-step and 2-step RT-PCR (page 7)

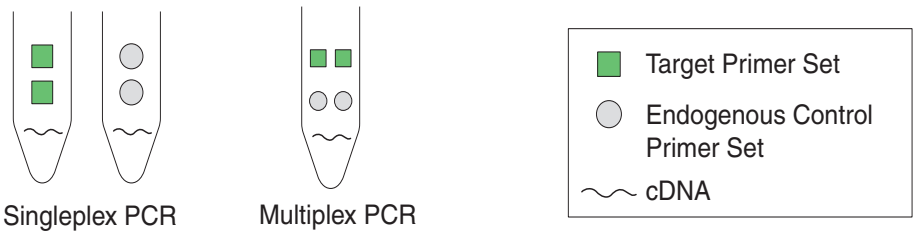
Singleplex and Multiplex PCR

You can perform a PCR reaction using either:

- **Singleplex PCR** – In singleplex PCR a single primer set is present in the reaction tube or well. Only one target or endogenous control can be amplified per reaction.

  or
- **Multiplex PCR** – In multiplex PCR, two or more primer sets are present in the reaction tube or well. Each set amplifies a specific target or endogenous control. Typically, a probe labeled with FAM™ dye detects the target and a probe labeled with VIC® dye detects the endogenous control.

IMPORTANT! SYBR® Green reagents cannot be used for multiplex PCR.



1- and 2-Step RT-PCR

You can perform reverse transcription (RT) and PCR in a single reaction (1-step) or in separate reactions (2-step). The reagent configuration you use depends on whether you are performing 1- or 2-step RT-PCR:

- **1-step RT-PCR**– In 1-step RT-PCR, RT and PCR take place in one buffer system. Using one buffer system provides the convenience of a single-tube preparation for RT and PCR amplification. However, you cannot use Fast PCR master mix or the carryover prevention enzyme, AmpErase® UNG (uracil-N-glycosylase), to perform 1-step RT-PCR.
- **2-step RT-PCR** – 2-step RT-PCR is performed in two separate reactions: First, total RNA is reverse-transcribed into cDNA, then the cDNA is amplified by PCR. This method is useful for detecting multiple transcripts from a single cDNA template or for storing cDNA aliquots for later use. The AmpErase® UNG enzyme can be used to prevent carryover contamination.

  Note: The Standard Curve example experiment is designed for singleplex PCR, where every well contains a primer/probe set for a single target; the reactions are set up for a 2-step RT-PCR.

About the example experiment

To illustrate how to perform Standard Curve experiments, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with the ViiA™ 7 System.

The objective of the Standard Curve example experiment is to determine the quantity of the RNase P gene in two populations.

In the standard curve example experiment:

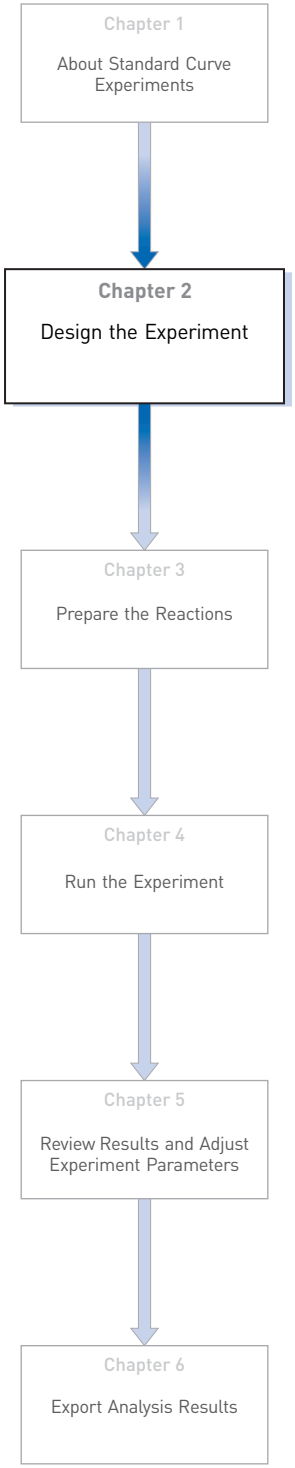
- The samples are genomic DNA isolated from two populations.
- The target is the RNase P gene.
- One standard curve is set up for the RNase P gene (target). The standard used for the standard dilution series contains known quantities of the RNase P gene. Because a single target is being studied, only one standard curve is required.

  Note: In experiments where multiple targets are being studied, a standard curve is required for each target.

- Three replicates of each sample and each dilution point in the standard curve are performed to ensure statistical significance.
- The experiment is designed for singleplex PCR, where every well contains a primer/probe set for a single target.
- Reactions are set up for 2-step RT-PCR.
- Primer/probe sets are from Applied Biosystems RNase P assay.

  Note: The human RNase P FAM™ dye-labeled MGB probe is not available as a TaqMan® Gene Expression Assay. It can be ordered as a Custom TaqMan® Gene Expression Assay (PN 4331348).

2 Design the Experiment



This chapter explains how to design the example experiment from the Experiment Setup menu.

This chapter covers:

- Define the experiment properties. 10
- Define targets, samples, and biological replicates 11
- Assign targets, samples, and biological groups 12
- Set up the run method 15
- Order materials for the experiment 16
- For more information. 16

Note: To automatically export the analyzed data to a specified location, select the Auto Export task from the Export screen, before running the experiment. For more information on Auto Export, refer to Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*.

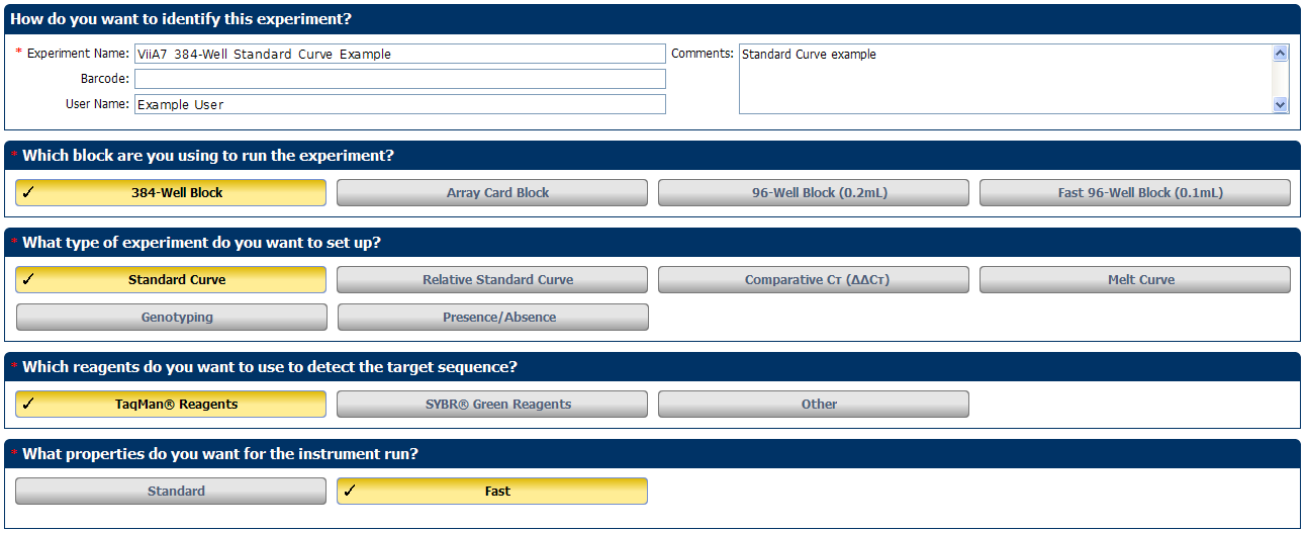
Define the experiment properties

Click **Experiment Setup** ▸ **Experiment Properties** to create a new experiment in the ViiA™ 7 Software. Enter:

Field or selection	Entry
Experiment Name	ViiA7_384-Well_Standard_Curve_Example
Barcode	Leave field empty
User Name	Example User
Comments	Standard Curve example
Block	384-Well Block
Experiment Type	Standard Curve
Reagents	TaqMan® Reagents
Ramp speed	Fast

Save the experiment.

Your Experiment Properties screen should look like this:



Define targets, samples, and biological replicates

Click **Define** to access the Define screen. Enter:

1. Targets

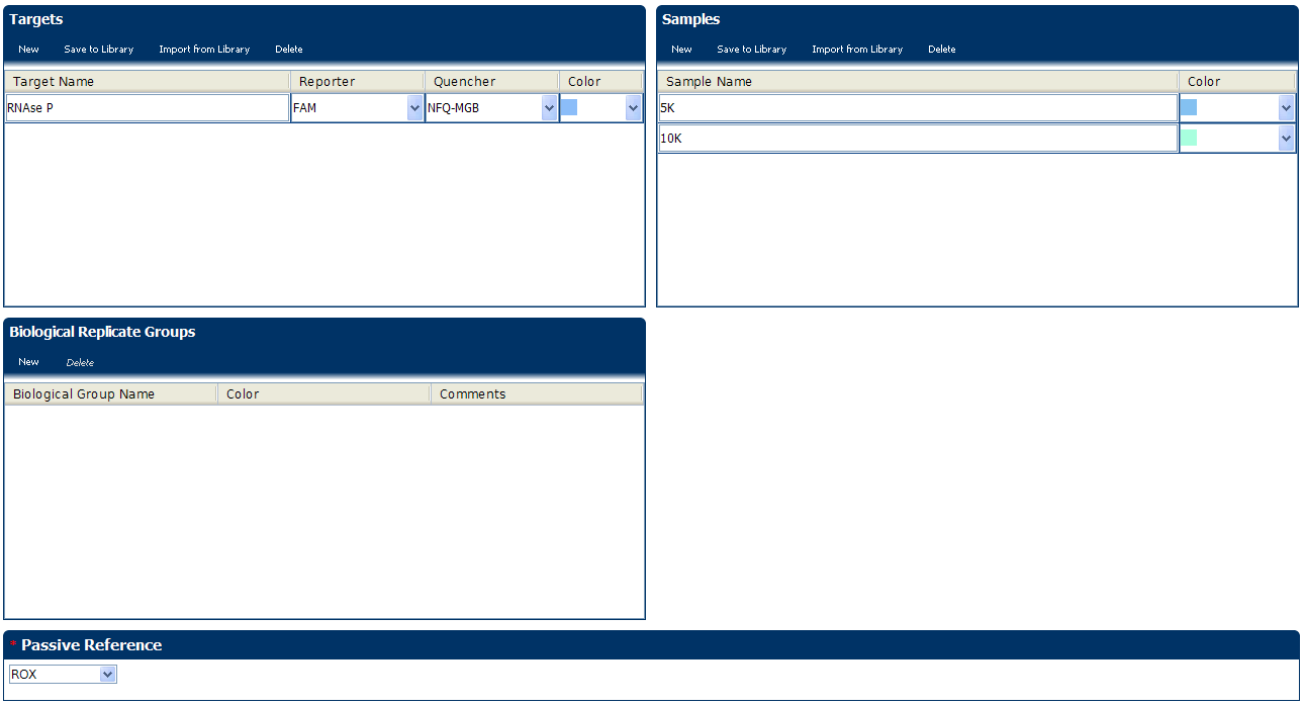
Target name	Reporter	Quencher	Color
RNaseP	FAM	NFQ-MGB	

2. Samples

Sample name	Color
5K	
10K	

3. Dye to be used as a Passive Reference
ROX

Your Define screen should look like this:



Note: This example experiment does not define biological replicate groups. Leave Biological Replicate Groups blank.

Assign targets, samples, and biological groups

Click **Assign** to access the Assign screen.

1. Define and set up standards.
   a. Click **Define and Set Up Standards** on the Assign screen.
   b. Select a target.

Field	Select
Select a target for this standard curve	RNaseP

   c. Define the standard curve.

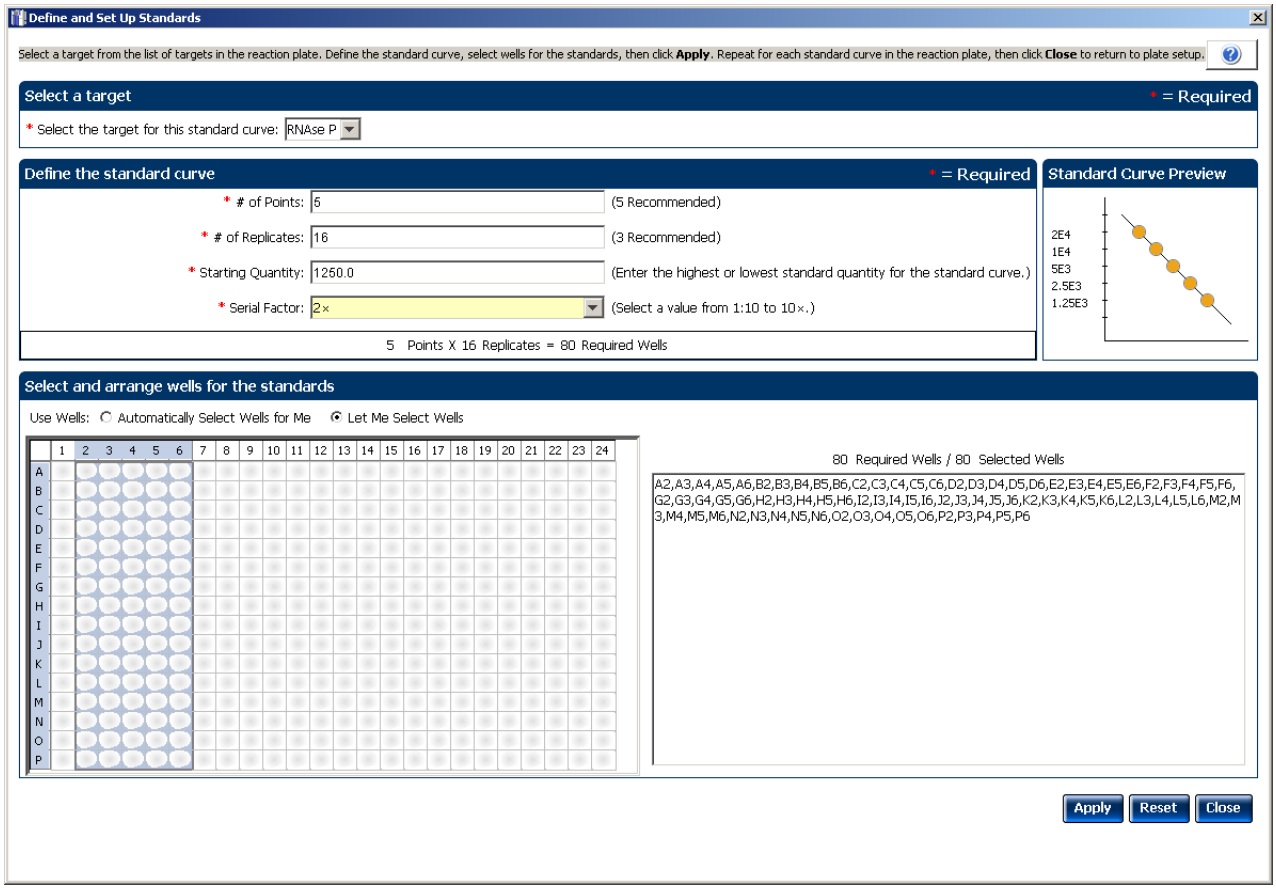
Field	Enter
# of Points	5
# of Replicates	16
Starting Quantity	1250.0
Serial Factor	2x

   d. Select and arrange wells for the standards.

Field	Select
Use Wells	Let Me Select Wells

e. Click **Apply**, and then **Close**.

Your Define and Set Up Standards dialog box should look like this:

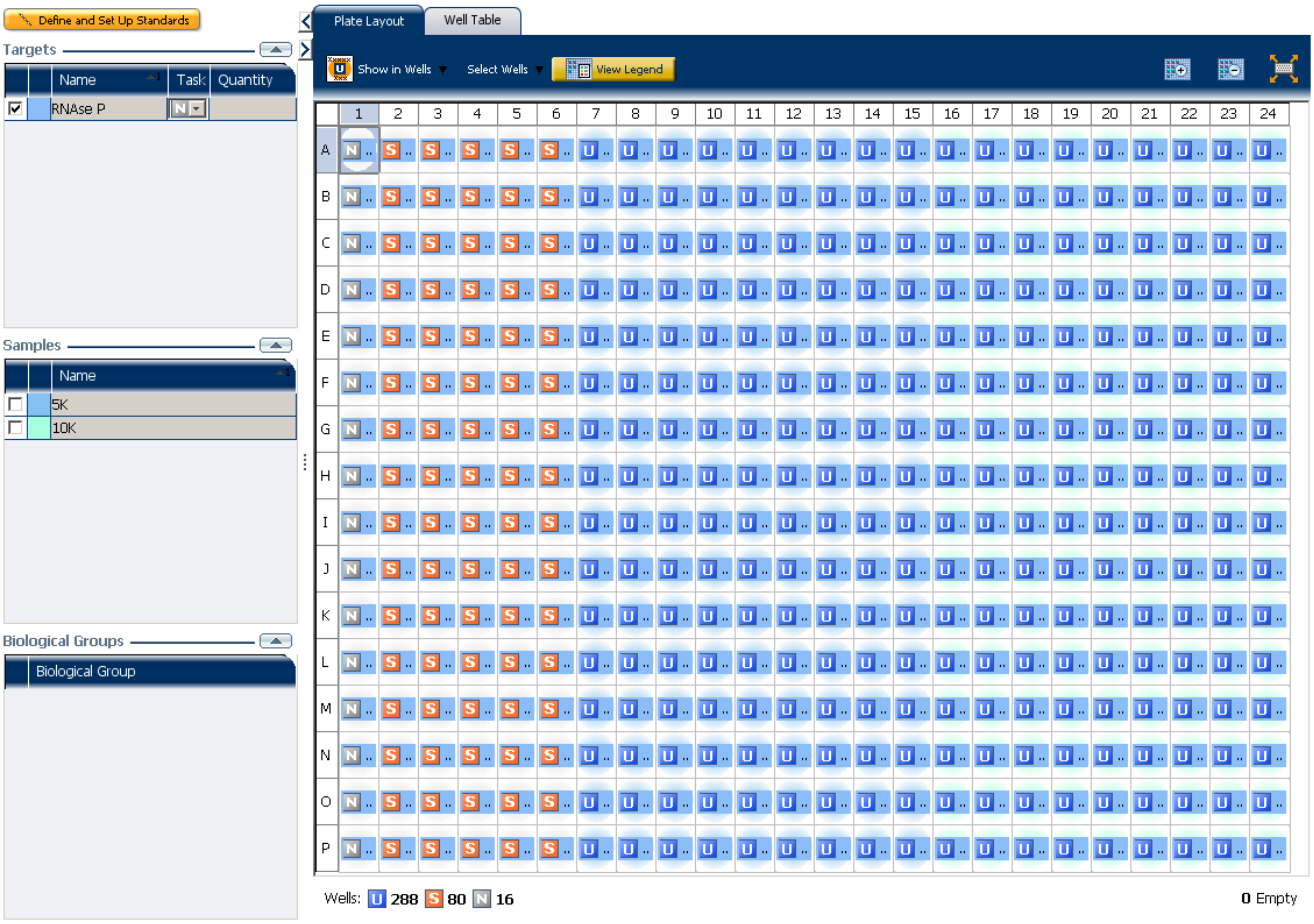


2. Assign targets and samples.

Target name	Well number	Task	Quantity	Sample name
RNaseP	A1 - P1 (column 1)	Negative	None	None
RNaseP	A2 - P2 (column 2)	Standard	1250	None
RNaseP	A3 - P3 (column 3)	Standard	2500	None
RNaseP	A4 - P4 (column 4)	Standard	5000	None
RNaseP	A5 - P5 (column 5)	Standard	10000	None
RNaseP	A6 - P6 (column 6)	Standard	20000	None

Target name	Well number	Task	Quantity	Sample name
RNaseP	A7 - P15 (columns 7 -15)	Unknown	Determined by run	5K
RNaseP	A16 - P24 (columns1 6 - 24)	Unknown	Determined by run	10K

Your Assign screen should look like this:



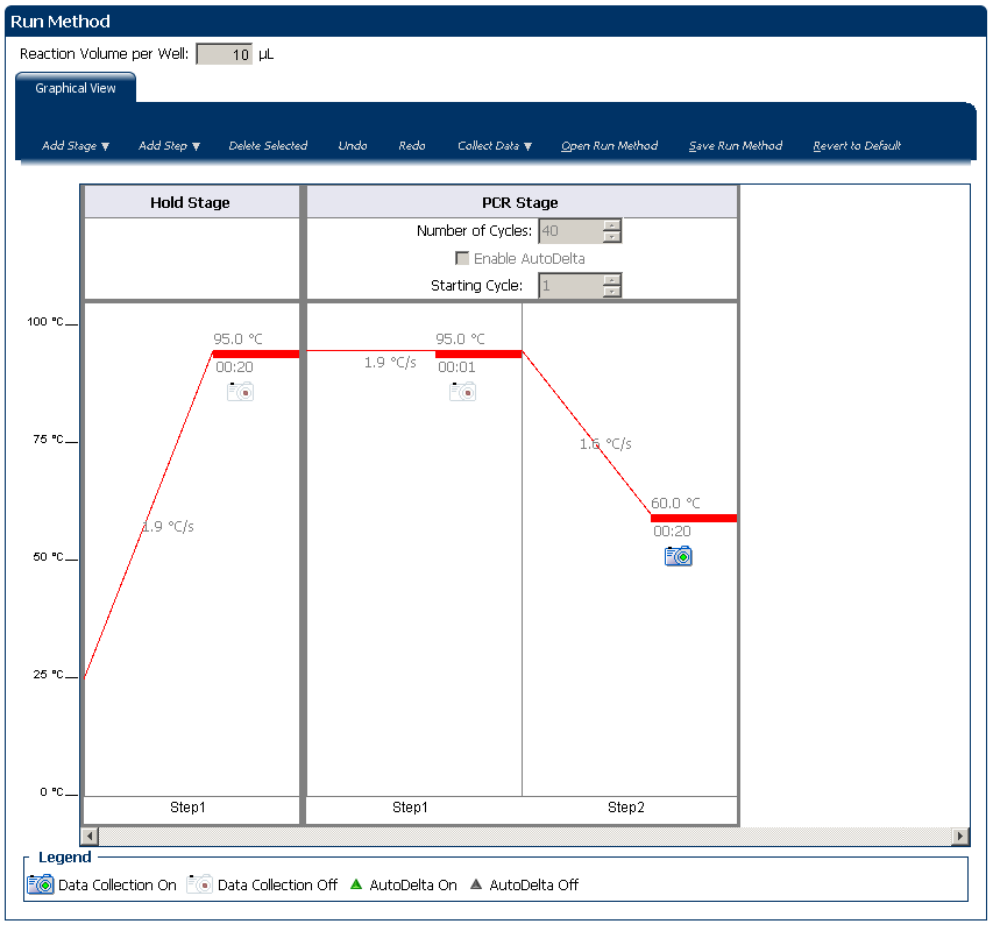
Set up the run method

Click **Run Method** to access the Run Method screen. Set the thermal profile under the Graphical View tab. Enter:

- Reaction Volume Per Well: 10µl
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Hold Stage	Step 1	1.9 °C/s	95ºC	20 seconds
PCR Stage Number of Cycles: 40 (default) Enable AutoDelta: Unchecked (default) Starting Cycle: Disabled when Enable AutoDelta is unchecked	Step 1	1.9 °C/s	95ºC	1 second
	Step 2	1.6 °C/s	60ºC	20 seconds

Your Run Method screen should look like this:



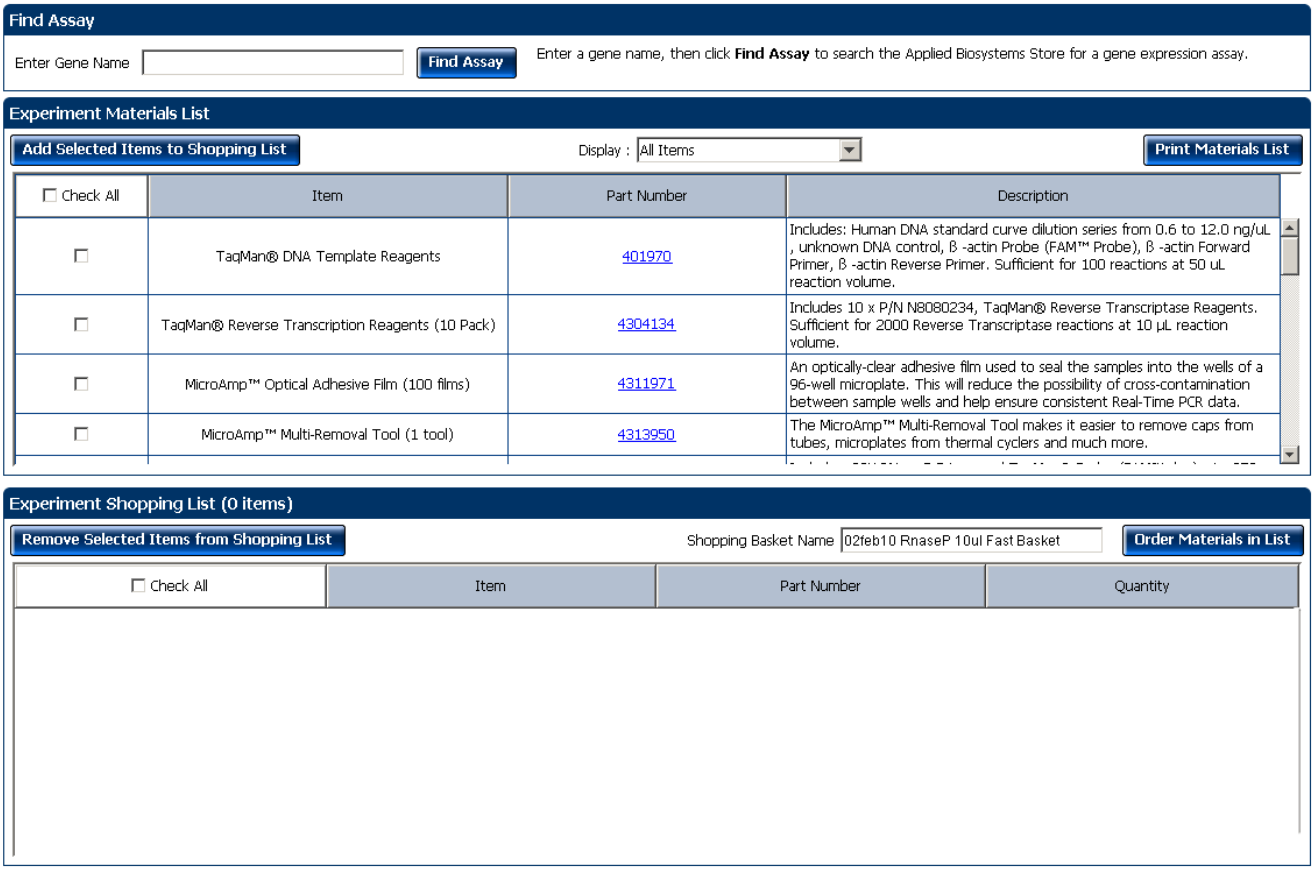
Order materials for the experiment

The recommended materials are:

- MicroAmp® Optical 384-Well Reaction Plate
- MicroAmp® Optical Adhesive Film
- TaqMan® Fast Universal PCR Master Mix
- Applied Biosystems RNase P assay (PN 4316831)

  Note: The human RNase P FAM™ dye-labeled MGB probe is not available as a TaqMan® Gene Expression Assay. It can be ordered as a Custom TaqMan® Gene Expression Assay (PN 4331348).

Your Materials list screen should look like this:

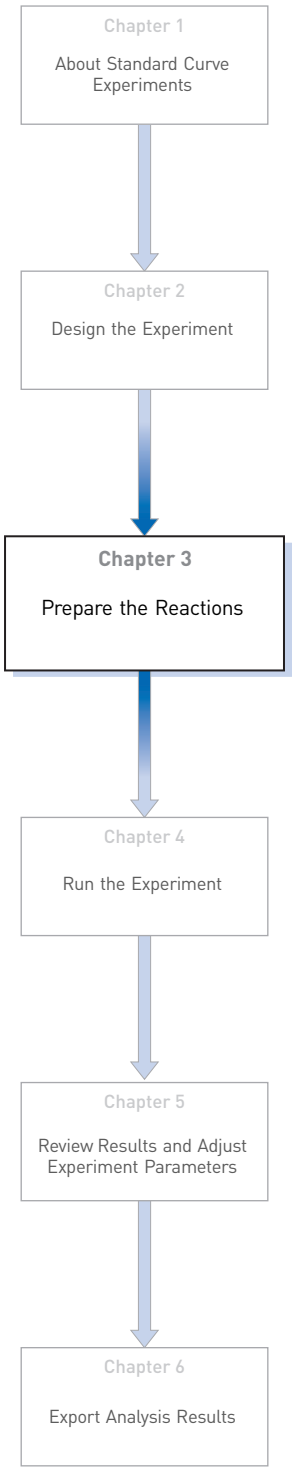


For more information

For more information on...	Refer to...	Part number
Consumables	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments* Appendix A in Booklet 7, *ViiA™ 7 System Experiments - Appendixes*	4441434

For more information on...	Refer to...	Part number
Using other quantification methods	Booklet 3, *Running Relative Standard Curve and Comparative C_T Experiments*.	4441434
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*	127AP05-03
Using Alternative Setup	Chapter 2 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

3 Prepare the Reactions



This chapter explains how to prepare the PCR reactions for the Standard Curve example experiment.

This chapter covers:

- Assemble required materials 20
- Prepare the sample dilutions 20
- Prepare the standard dilution series 21
- Prepare the reaction mix (“cocktail mix”). 21
- Prepare the reaction plate 22
- For more information. 25

Assemble required materials

- Items listed in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*
- Samples - Human Raji cell line-derived cDNA samples (100 ng/µL)
- Example experiment reaction mix components:
  - **TaqMan® Fast Universal PCR Master Mix**
  - RNase P Assay Mix (20X) (PN 4316831)

Prepare the sample dilutions

To determine the quantity of the RNase P gene in the example experiment, dilute the samples (as directed below) before adding the samples to the final reaction mix.

The stock concentration of each sample is 100 ng/µL. After dilution, the sample Pop1 has a concentration of 6.6 ng/µL and Pop 2 has a concentration of 3.3 ng/µL. Add 2µL to each reaction.

Use this table for sample dilution volumes for the example experiment.

Sample name	Stock concentration (ng/µL)	Sample volume (µL)	Diluent volume (µL)	Total volume of diluted sample (µL)
Pop1	100.0	25	355	380
Pop2	100.0	12.5	367.5	380

Note: For your own experiment, adjust the input amounts of the template depending on the template type and target abundance.

1. Label a separate microcentrifuge tube for each diluted sample:
   - **Pop 1**
   - **Pop 2**
2. Add the required volume of water (diluent) to each empty tube:

Tube	Sample name	Diluent volume (µL)
1	Pop 1	355
2	Pop 2	367.5

3. Add the required volume of sample stock to each tube:

Tube	Sample name	Sample volume (µL)
1	Pop 1	25

Tube	Sample name	Sample volume (µL)
2	Pop 2	12.5

4. Vortex each diluted sample for 3 to 5 seconds, then centrifuge the tubes briefly.
5. Place the diluted samples on ice until you prepare the reaction plate.

Prepare the standard dilution series

The standard concentration in stock is 20,000 copies/µL:

Standard name (labeled tube)	Dilution point	Source	Source volume (µL)	Diluent volume (µL)	Total volume (µL)	Standard concentration (copies/µL)
RNase P Std. 1	1 (20,000)	Stock	18	18	36	10,000
RNase P Std. 1	2 (10,000)	Dilution 1	18	18	36	5,000
RNase P Std. 1	3 (5,000)	Dilution 2	18	18	36	2,500
RNase P Std. 1	4 (2500)	Dilution 3	18	18	36	1250
RNase P Std. 1	5 (1250)	Dilution 4	18	18	36	625

1. Prepare five standard dilutions:

   Note: For dilution 1, first vortex the stock for 3 to 5 seconds, then centrifuge the RNase P Std. 1 tube briefly before pipetting stock into the tube.

   For each dilution:

   a. Use a new pipette tip to add 18 µL of source to the tube containing the standard.
   b. Vortex the tube for 3 to 5 seconds, then centrifuge the tube briefly.

2. Place the standards on ice until you prepare the reaction plate.

Prepare the reaction mix ("cocktail mix")

For the RNase P assay (Standard Curve example experiment), the following table lists the universal assay conditions (volume and final concentration) for using the TaqMan® Fast Universal PCR Master Mix.

Reaction component	Volume for 1 reaction (µL)	Volume for 384 reactions + 10% excess (µL)
TaqMan® Fast Universal PCR Master Mix Kit	5	2115
RNase P Assay (20X)	0.5	211.5
Water	3.5	1480.5
Total reaction mix volume	9	3807

1. Label an appropriately sized tube for the reaction mix: **RNase P Reaction Mix**.
2. Add the required volumes of each cocktail mix component to the tube.

   Note: Do not add the sample or standard at this time.
3. Mix the cocktail thoroughly by gently pipetting up and down several times, then cap the tube.
4. Centrifuge the tube briefly to remove air bubbles.
5. Place the cocktail mix on ice until you prepare the reaction plate.

Note: You can separately add the sample to the reaction plate, as opposed to preparing individual reaction mixes for each sample.

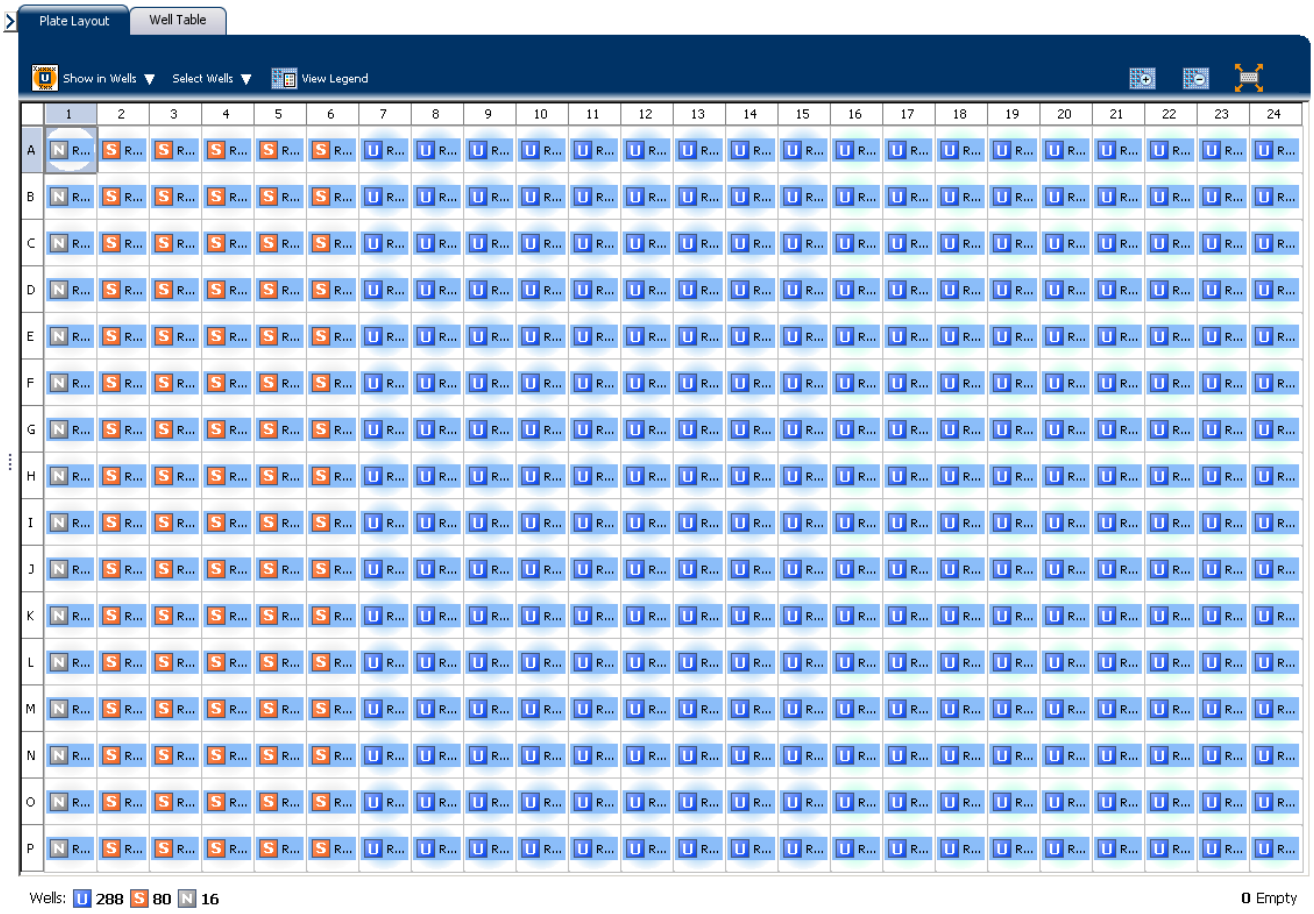
Prepare the reaction plate

Example experiment reaction plate components

The reaction plate for the Standard Curve example experiment contains:

- A MicroAmp® Optical 384-Well Reaction Plate
- Reaction volume: 10 µL/well
- 288 Unknown wells U
- 80 Standard wells S
- 16 Negative control wells N

The plate layout looks like this:



To prepare the reaction plate components

1. Prepare the negative control reactions for the target:
   a. To an appropriately sized tube, add the volumes of reaction mix and water listed below.

Tube	Reaction mix	Reaction mix volume (µL)	Water volume (µL)
1	RNase P reaction mix	157.5	17.5

   b. Mix the reaction by gently pipetting up and down, then cap the tube.
   c. Centrifuge the tube briefly to remove air bubbles.
   d. Add 10 µL of the negative control reaction to the appropriate wells in the reaction plate.

2. For each replicate group, prepare the standard reactions:
   a. To appropriately sized tubes, add the volumes of reaction mix and standard listed below.

Tube	Standard reaction	Reaction mix	Reaction mix volume (µL)	Standard	Standard volume (µL)
1	RNase P Std 1	RNase P reaction mix	157.5	RNase P Std 1	17.5
2	RNase P Std 2	RNase P reaction mix	157.5	RNase P Std 2	17.5
3	RNase P Std 3	RNase P reaction mix	157.5	RNase P Std 3	17.5
4	RNase P Std 4	RNase P reaction mix	157.5	RNase P Std 4	17.5
5	RNase P Std 5	RNase P reaction mix	157.5	RNase P Std 5	17.5

   b. Mix the reactions by gently pipetting up and down, then cap the tubes.
   c. Centrifuge the tubes briefly to remove air bubbles.
   d. Add 10 µL of the standard reaction to the appropriate wells in the reaction plate.

3. For each replicate group, prepare the reactions for the unknowns:
   a. To appropriately sized tubes, add the volumes of reaction mix and sample listed below.

Tube	Unknown reaction	Reaction mix	Reaction mix volume (µL)	Sample	Sample volume (µL)
1	RNase P pop1	RNase P reaction mix	1422	pop1	158
2	RNase P pop2	RNase P reaction mix	1422	pop2	158

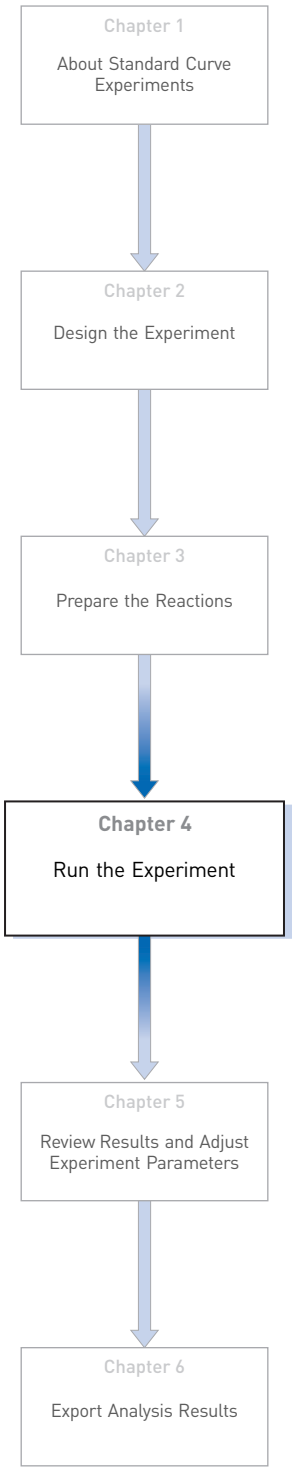
   b. Mix the reactions by gently pipetting up and down, then cap the tubes.
   c. Centrifuge the tubes briefly to remove air bubbles.
   d. Add 10 µL of the unknown (sample) reaction to the appropriate wells in the reaction plate.

4. Seal the reaction plate with optical adhesive film.
5. Centrifuge the reaction plate briefly to remove air bubbles.
6. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.
7. Until you are ready to perform the run, place the reaction plate at 4 °C, in the dark.

For more information

For more information on...	Refer to...	Part number
Assigning the reaction plate components	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

4 Run the Experiment



This chapter explains how to run the example experiment on the ViiA™ 7 Instrument.

This chapter covers:

- Start the run 28
- Monitor the run 28

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the ViiA™ 7 Instrument is in operation.

Start the run

1. Open the Standard Curve example file that you created using instructions in Chapter 2.
2. Load the reaction plate into the instrument.
3. Start the run.

Monitor the run

Monitor the example experiment run:

- From the ViiA™ 7 Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the ViiA™ 7 Software (to monitor an experiment started from another computer or from the ViiA™ 7 Instrument touchscreen).
- From the ViiA™ 7 Instrument touchscreen.

From the Instrument Console of the ViiA™ 7 Software

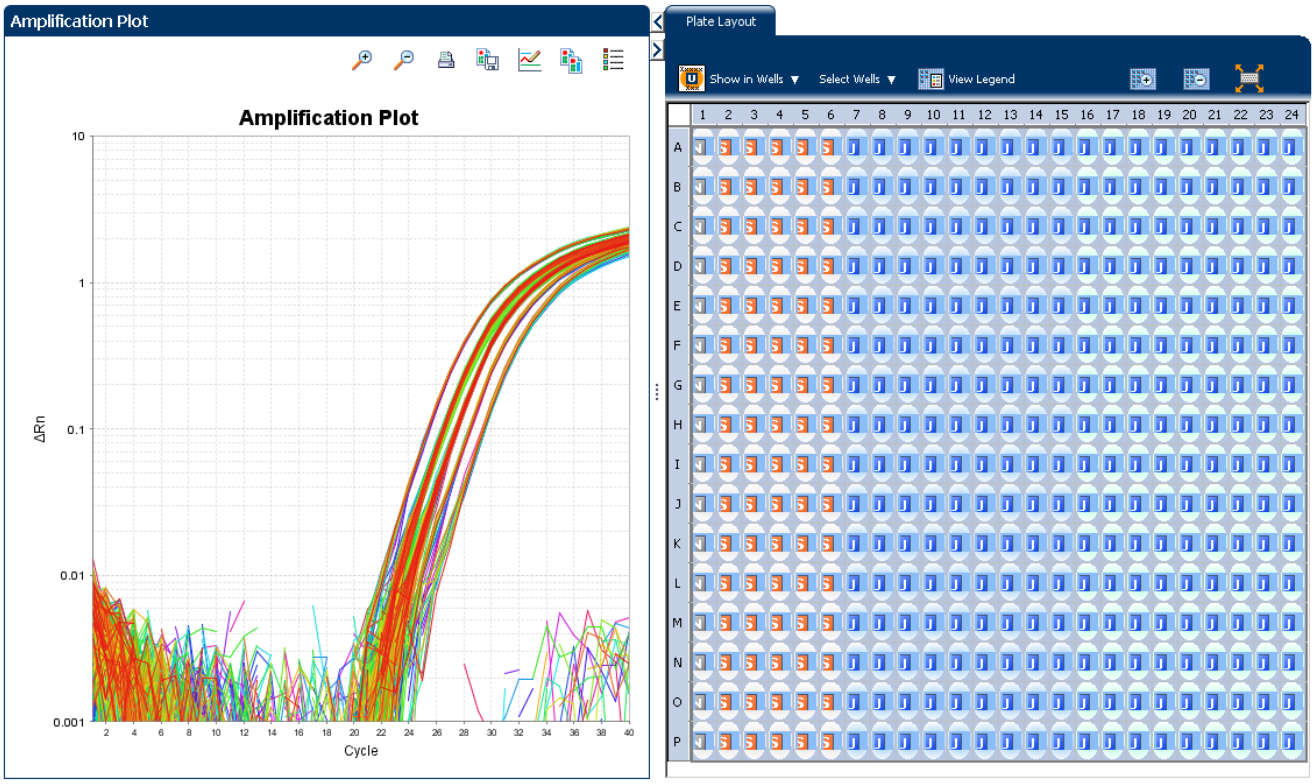
1. In the Instrument Console screen, select the instrument icon.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. On the Manage Instrument screen, click **Monitor Running Experiment** to access the Run screen.

View the Amplification Plot

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the ViiA™ 7 Software for potential problems.

Click **Amplification Plot** from the Run Experiment Menu, select the Plate Layout tab, then select the wells to view.

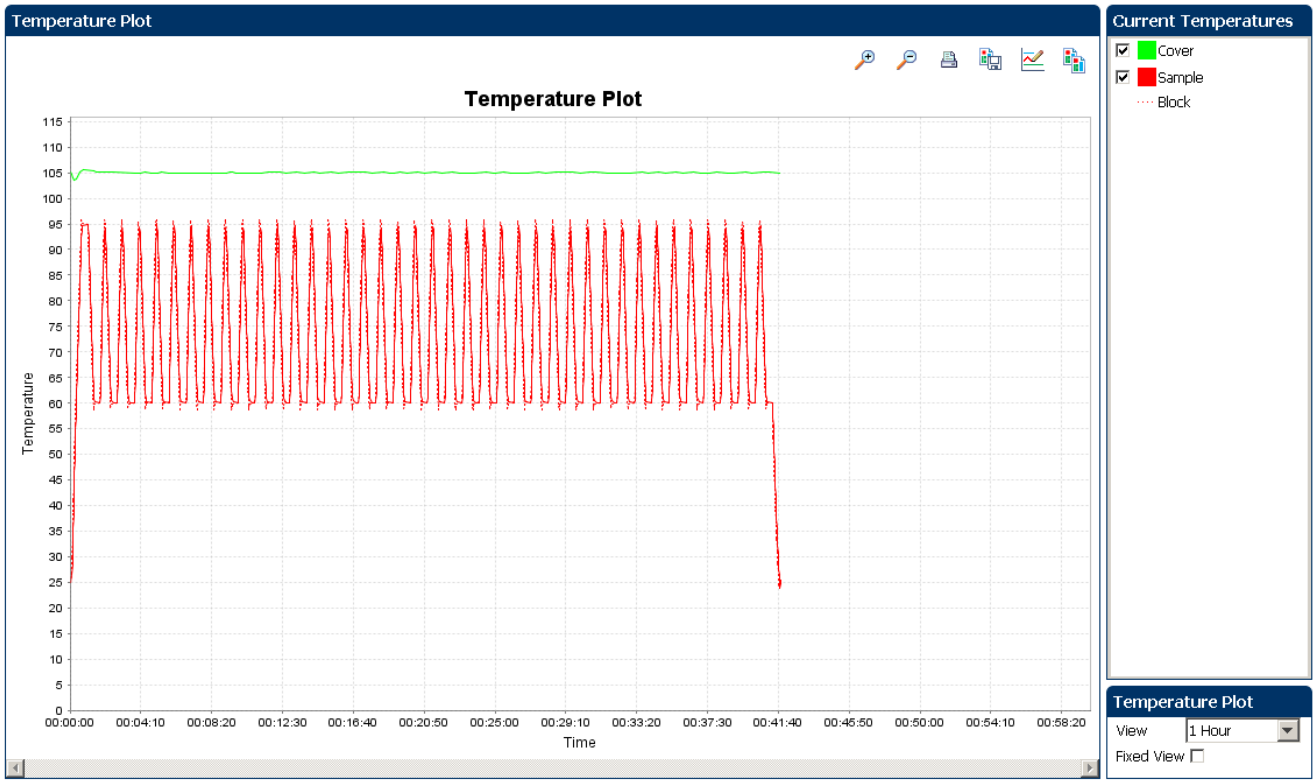
The figure below shows the Amplification Plot screen as it appears at the end of the example experiment.



View the Temperature Plot

Click **Temperature Plot** from the Run Experiment Menu.

The figure below shows the Temperature Plot screen as it appears during the example experiment.

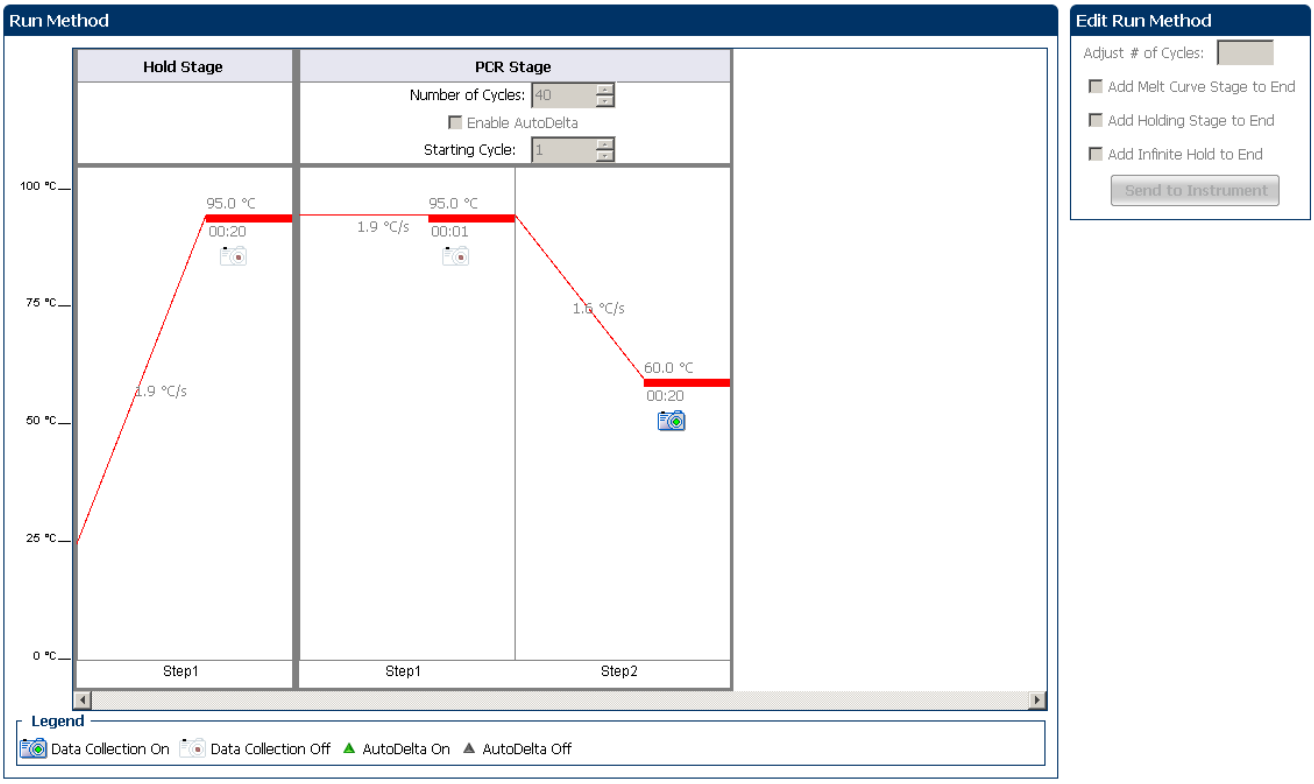


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

View the Run Method

Click **Run Method** from the Run Experiment Menu.

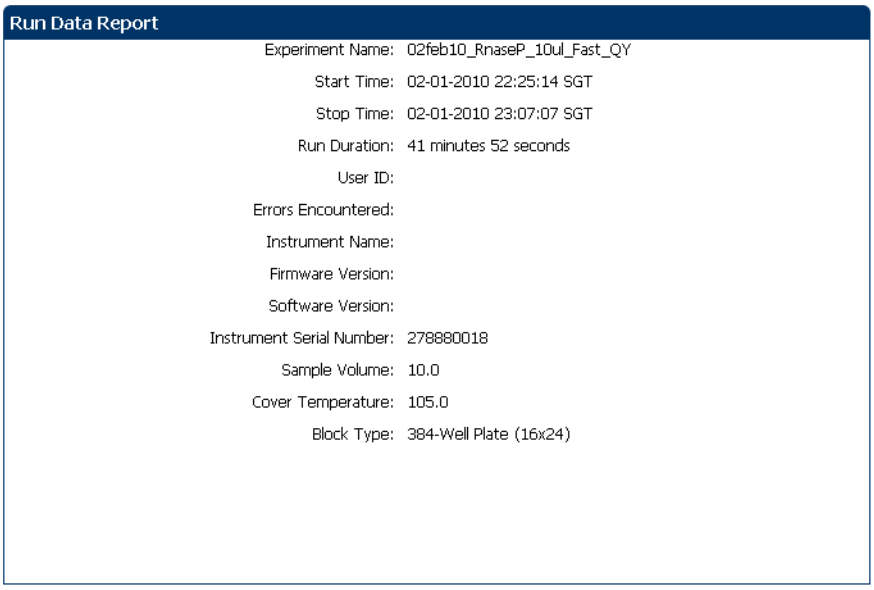
The figure below shows the Run Method screen as it appears in the example experiment.



View the run data

Click **View Run Data** from the Run Experiment Menu.

The figure below shows the View Run Data screen as it appears in the example experiment.

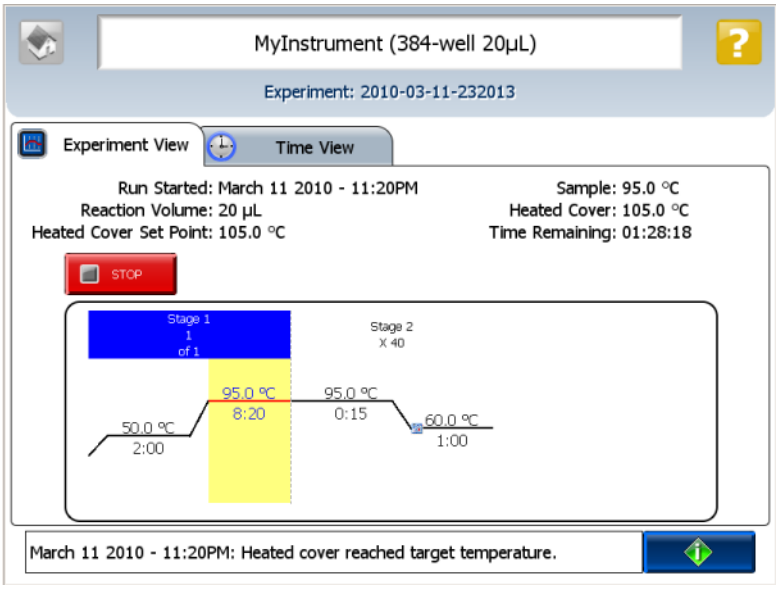


From the ViiA™ 7 Instrument touchscreen

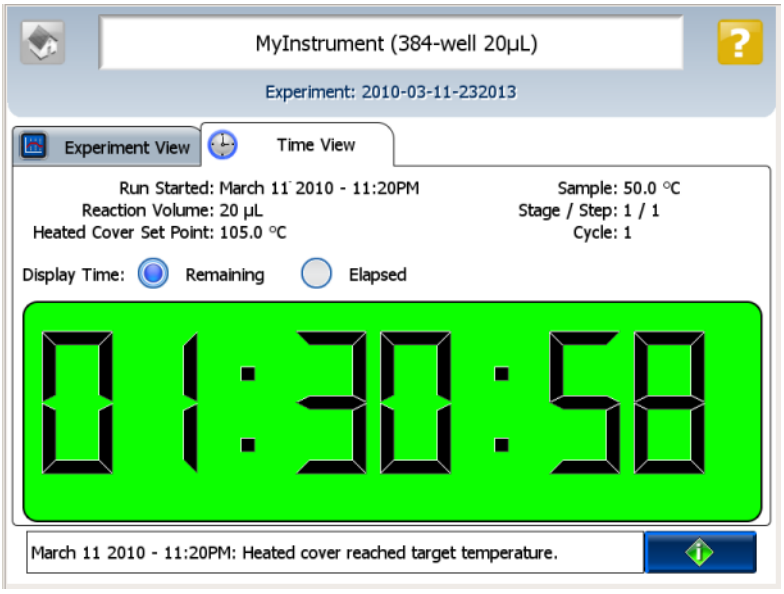
You can also view the progress of the run from the touchscreen of the ViiA™ 7 Instrument.

The Run Method screen on the ViiA™ 7 Instrument touchscreen looks like this:

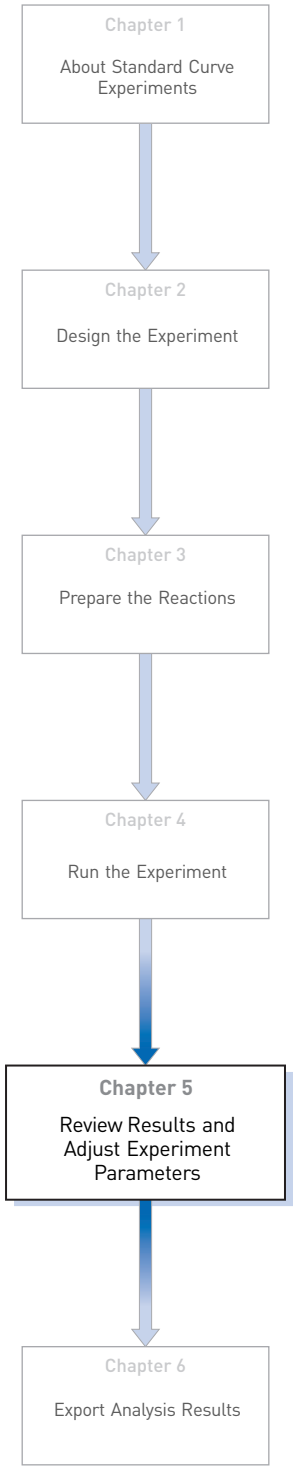
Experiment View



Time View



5 Review Results and Adjust Experiment Parameters



In Section 5.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 5.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

Section 5.1 Review Results 35

- Analyze the example experiment. 35
- View the Standard Curve Plot 35
- Assess amplification results using the Amplification Plot 38
- Identify well problems using the Well Table 45
- Confirm accurate dye signal using the Multicomponent Plot 49
- Determine signal accuracy using the Raw Data Plot 51
- Review the flags in the QC Summary 53
- For more information. 55

Section 5.2 Adjust parameters for re-analysis of your own experiments. 57

- Adjust analysis settings 57
- Improve C_T precision by omitting wells 61
- For more information. 62

5.1

Section 5.1 Review Results

Analyze the example experiment

1. Open the example experiment file that you ran in Chapter 4.
2. Click **Analyze**. The software analyzes the data using the default analysis settings.

   Note: You can also access the experiment to analyze from the Home screen.

View the Standard Curve Plot

The Standard Curve Plot screen displays the standard curve for samples designated as standards. The ViiA™ 7 Software calculates the quantity of an unknown target from the standard curve.

Purpose

The purpose of viewing the standard curve for the example experiment is to identify:

- Slope and amplification efficiency
- R^2 value (correlation coefficient)
- C_T values

To view and assess the Standard Curve Plot

1. From the Experiment Menu pane, select **Analysis ▸ Standard Curve.**

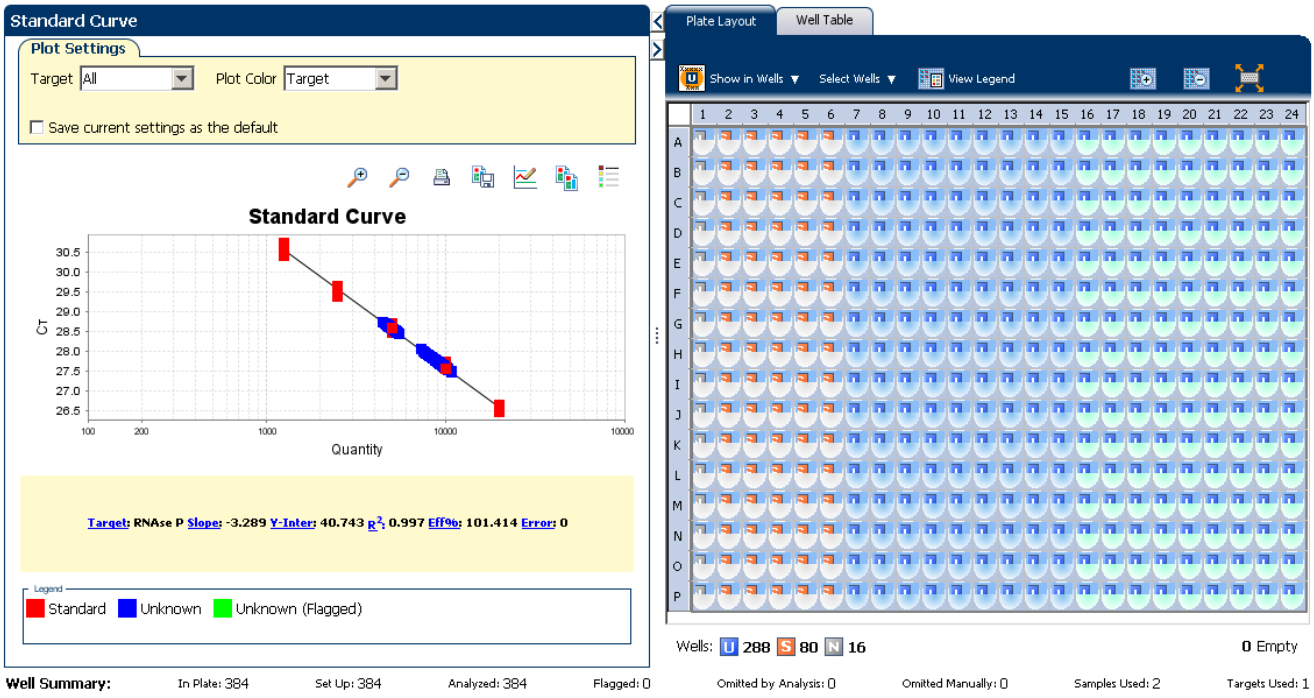
   Note: If no data are displayed, click **Analyze.**
2. Display all 384 wells in the Standard Curve Plot screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.
3. Enter the Plot Settings:

Menu	Selection
Target	All
Plot Color	Target
(This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.)	Check (default)

4. View the values displayed below the standard curve.

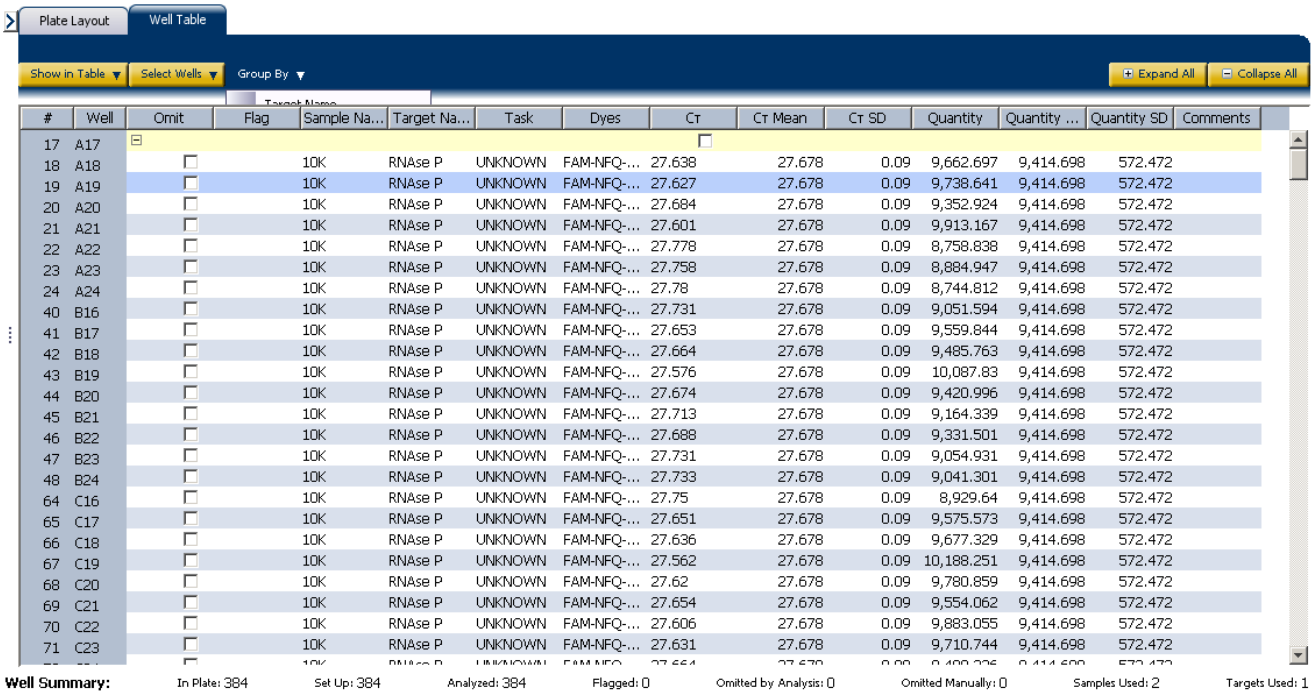
Menu	Selection
Slope	-3.289
R2	0.997
Amplification efficiency	101.414%
Error	0

5. Check that all samples are within the standard curve. In the example experiment, as shown below, all samples (blue dots) are within the standard curve (red dots).



6. Check the C_T values:
   a. Click the **Well Table** tab.
   b. From the Group By menu, select **Replicate**.

c. Look at the values in the C_T column. In the example experiment, the C_T values fall within the expected range (>8 and < 35).



#	Well	Omit	Flag	Sample Na...	Target Na...	Task	Dyes	CT	CT Mean	CT SD	Quantity	Quantity ...	Quantity SD	Comments
17	A17													
18	A18			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.638	27.678	0.09	9,662.697	9,414.698	572.472	
19	A19			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.627	27.678	0.09	9,738.641	9,414.698	572.472	
20	A20			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.684	27.678	0.09	9,352.924	9,414.698	572.472	
21	A21			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.601	27.678	0.09	9,913.167	9,414.698	572.472	
22	A22			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.778	27.678	0.09	8,758.838	9,414.698	572.472	
23	A23			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.758	27.678	0.09	8,884.947	9,414.698	572.472	
24	A24			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.78	27.678	0.09	8,744.812	9,414.698	572.472	
40	B16			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.731	27.678	0.09	9,051.594	9,414.698	572.472	
41	B17			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.653	27.678	0.09	9,559.844	9,414.698	572.472	
42	B18			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.664	27.678	0.09	9,485.763	9,414.698	572.472	
43	B19			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.576	27.678	0.09	10,087.83	9,414.698	572.472	
44	B20			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.674	27.678	0.09	9,420.996	9,414.698	572.472	
45	B21			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.713	27.678	0.09	9,164.339	9,414.698	572.472	
46	B22			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.688	27.678	0.09	9,331.501	9,414.698	572.472	
47	B23			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.731	27.678	0.09	9,054.931	9,414.698	572.472	
48	B24			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.733	27.678	0.09	9,041.301	9,414.698	572.472	
64	C16			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.75	27.678	0.09	8,929.64	9,414.698	572.472	
65	C17			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.651	27.678	0.09	9,575.573	9,414.698	572.472	
66	C18			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.636	27.678	0.09	9,677.329	9,414.698	572.472	
67	C19			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.562	27.678	0.09	10,188.251	9,414.698	572.472	
68	C20			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.62	27.678	0.09	9,780.859	9,414.698	572.472	
69	C21			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.654	27.678	0.09	9,554.062	9,414.698	572.472	
70	C22			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.606	27.678	0.09	9,883.055	9,414.698	572.472	
71	C23			10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.631	27.678	0.09	9,710.744	9,414.698	572.472	

Well Summary: In Plate: 384 Set Up: 384 Analyzed: 384 Flagged: 0 Omitted by Analysis: 0 Omitted Manually: 0 Samples Used: 2 Targets Used: 1

Tips for analyzing your own experiments

When you analyze your own standard curve experiment, look for:

- **Slope and amplification efficiency values** – The amplification efficiency is calculated using the slope of the regression line in the standard curve. A slope close to –3.3 indicates optimal, 100% PCR amplification efficiency. Factors that affect amplification efficiency:
  - Range of standard quantities – For accurate and precise efficiency measurements, use a broad range of standard quantities, 5 to 6 logs (10^5 to 10^6 fold).
  - Number of standard replicates – For accurate efficiency measurements, include replicates to decrease the effects of pipetting inaccuracies.
  - PCR inhibitors – PCR inhibitors in the reaction can reduce amplification efficiency.
- **R^2 values (correlation coefficient)** – The R^2 value is a measure of the closeness of fit between the regression line and the individual C_T data points of the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points. An R^2 value >0.99 is desirable.
- **C_T values** – The threshold cycle (C_T) is the PCR cycle number at which the fluorescence level meets the threshold.
  - A C_T value >8 and <35 is desirable.
  - A C_T value <8 indicates that there is too much template in the reaction.
  - A C_T value >35 indicates a low amount of target in the reaction; for C_T values >35, expect a higher standard deviation.

If your experiment does not meet the guidelines above, troubleshoot as follows:

- Omit wells (see "Improve C_T precision by omitting wells" on page 61).

  Or
- Rerun the experiment.

Assess amplification results using the Amplification Plot

Amplification plots available for viewing

The Amplification Plot screen displays amplification of all samples in the selected wells. There are three plots available:

- **ΔRn vs Cycle** – ΔRn is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. This plot displays ΔRn as a function of cycle number. Use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle** – Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays Rn as a function of cycle number. Use this plot to identify and examine irregular amplification.
- **C_T vs Well** – C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. Use this plot to locate outlying amplification (outliers).

Each plot can be viewed as a linear or log10 graph type.

Purpose

The purpose of viewing the amplification plot for the example experiment is to identify:

- Correct baseline and threshold values
- Outliers

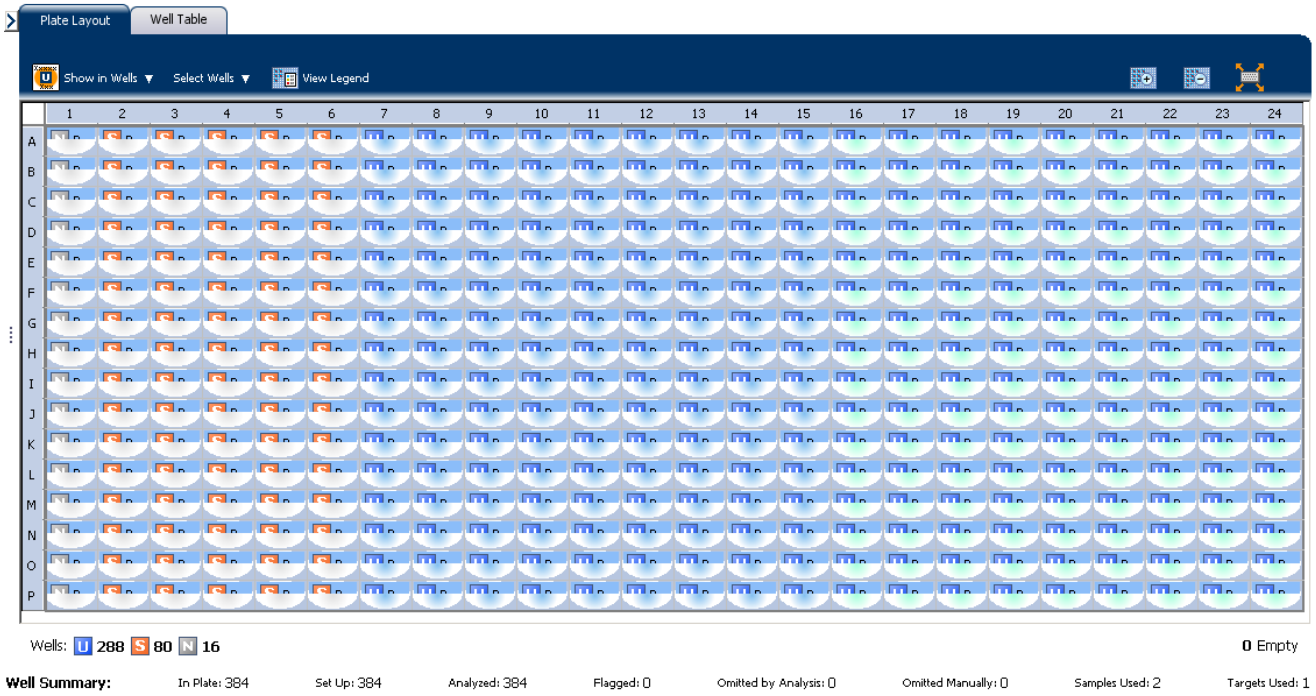
View the Amplification Plot

1. From the Experiment Menu pane, select **Analysis ▸ Amplification Plot.**

   Note: If no data are displayed, click **Analyze.**

2. Display the RNase P wells in the Amplification Plot screen. Click the **Plate Layout** tab. Enter the Plot Settings:

Menu	Selection
Select Wells With	**Target ▸ RNaseP**

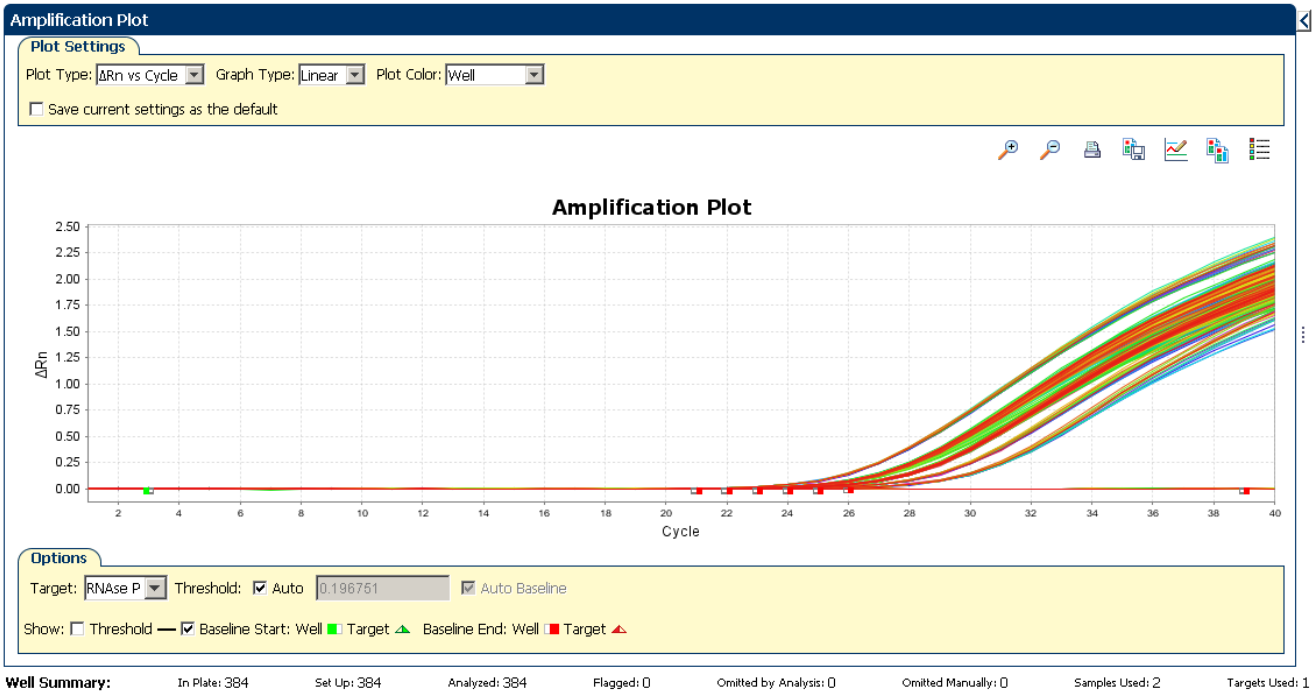


3. In the Amplification Plot screen, enter:

Menu	Select
Plot Type	**ΔRn vs Cycle**
Plot Color	**Well** (default)
(This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.)	Check (default)

4. View the baseline values.
   a. From the Graph Type drop-down menu, select **Linear**.
   b. Select the **Baseline** check box to show the start cycle and end cycle.

c. Verify that the baseline is set correctly: The end cycle should be set a few cycles before the cycle number where significant fluorescent signal is detected. In the example experiment, the baseline is set correctly.

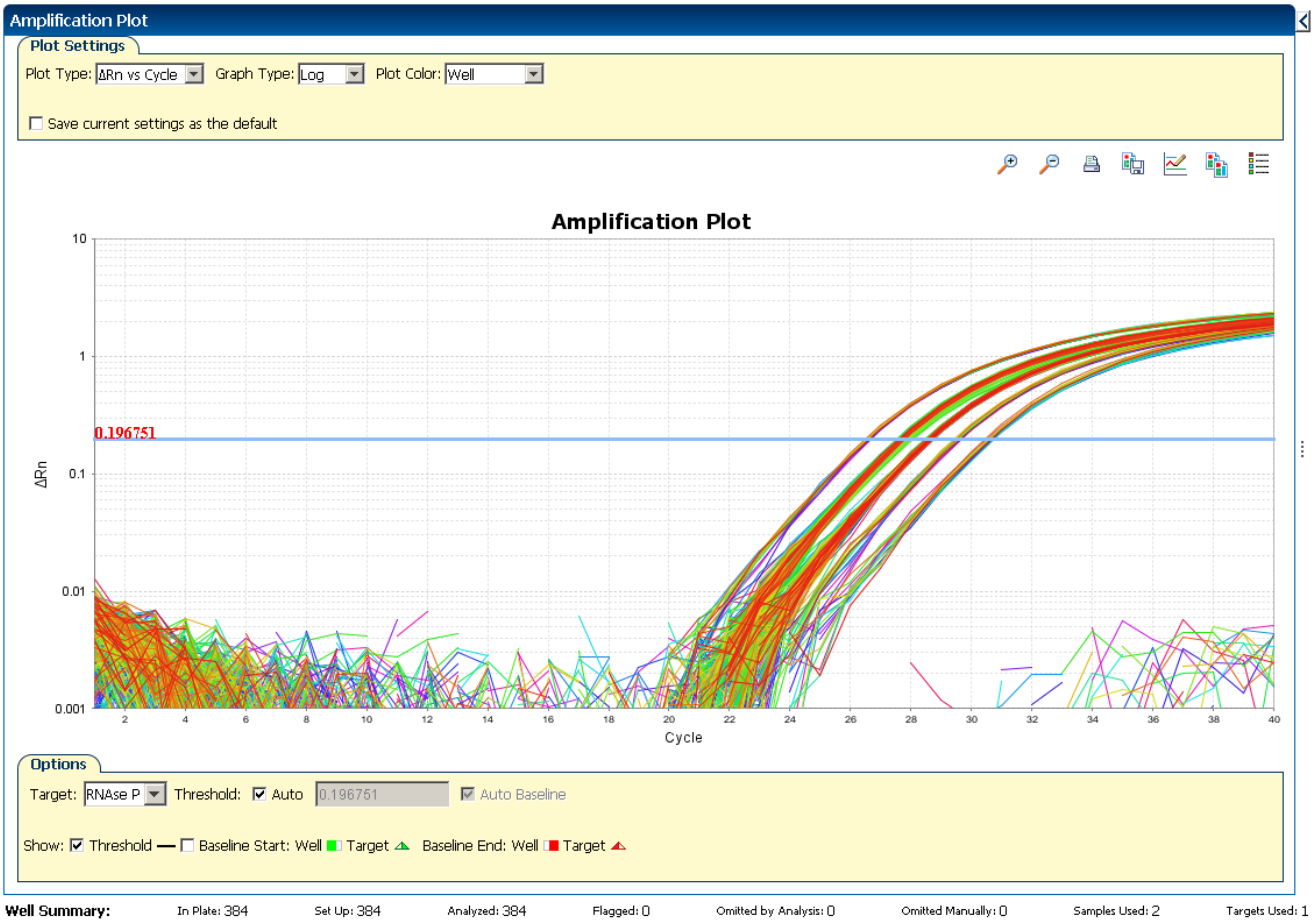


5. View the threshold values.

Menu	Select
Graph Type	Log
Target	RNaseP

a. Select the **Threshold** check box to show the threshold.

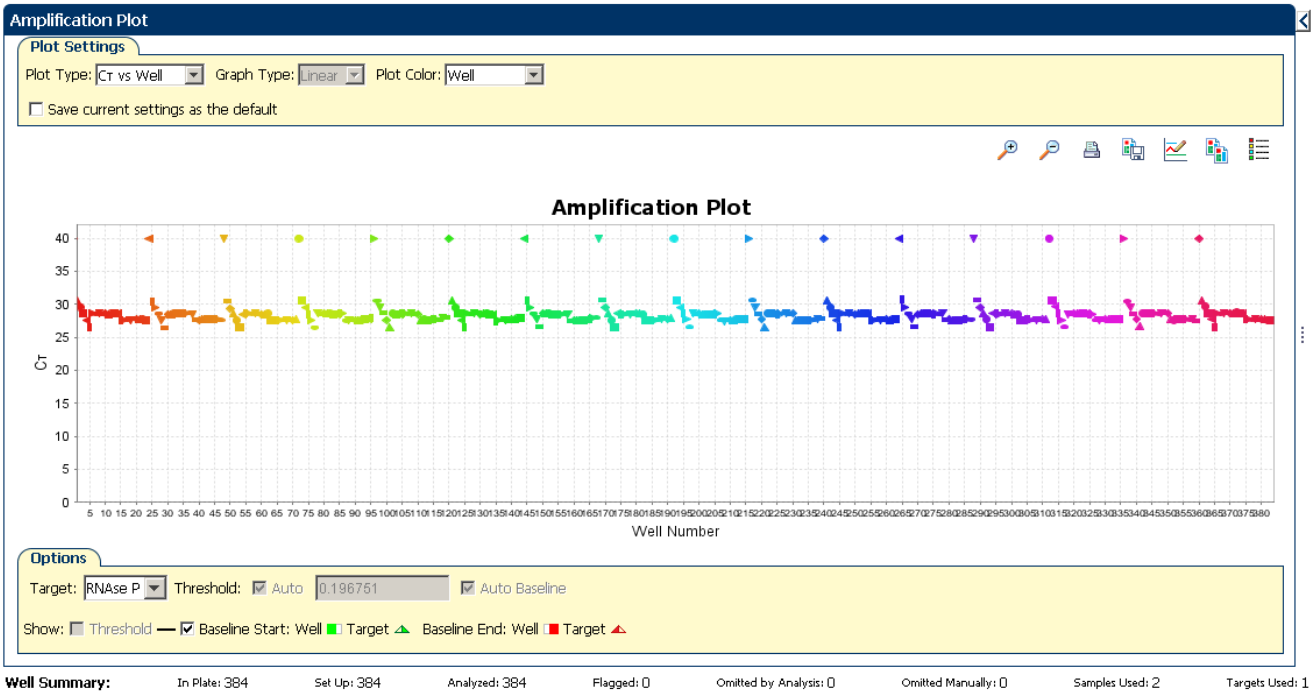
b. Verify that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.



6. Locate outliers:

   a. From the Plot Type drop-down menu, select **C_T vs Well**.

b. Look for outliers from the amplification plot. In the example experiment, there are no outliers for RNase P.



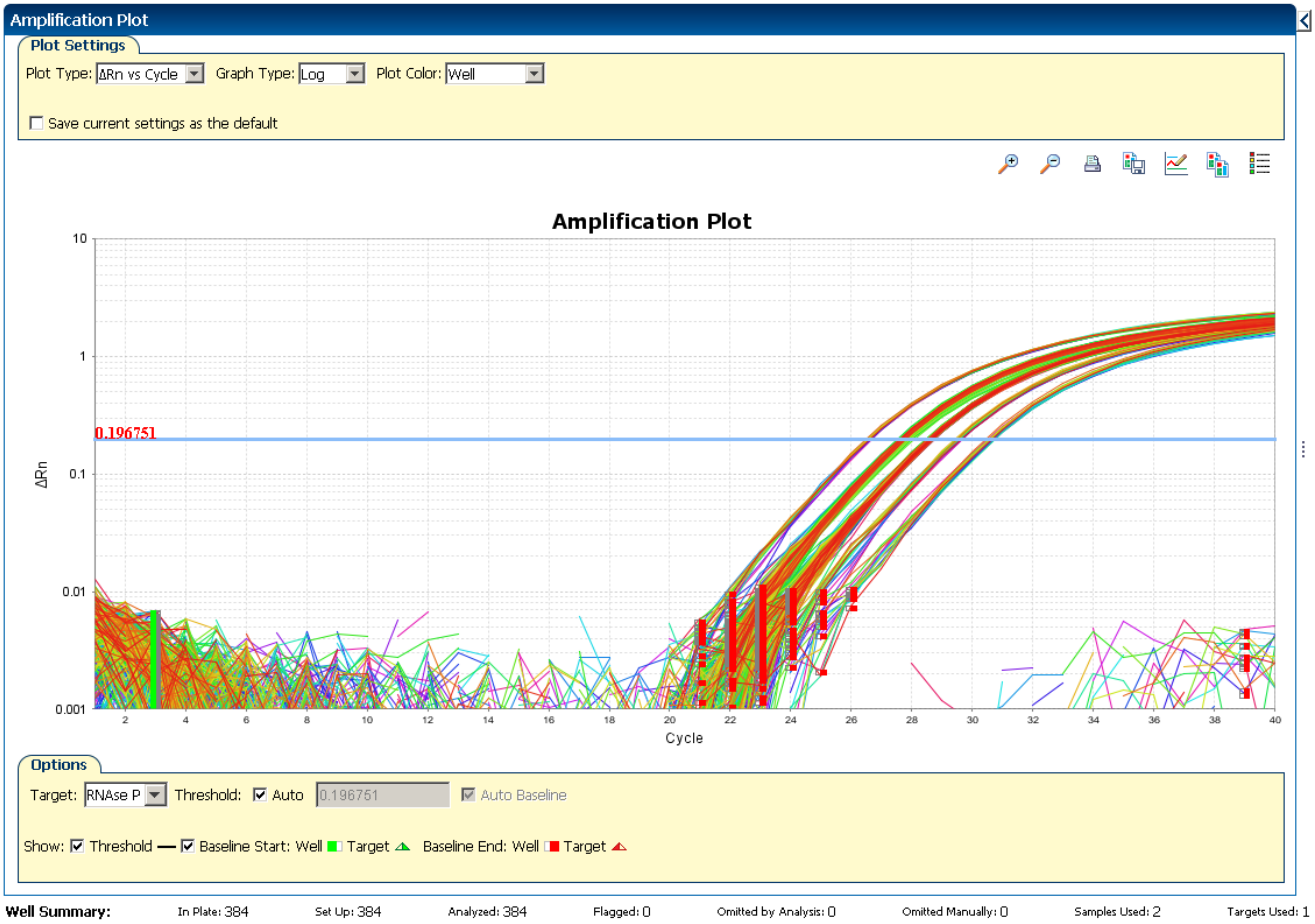
Tips for analyzing your own experiments

When you analyze your own standard curve experiment, look for:

Outliers

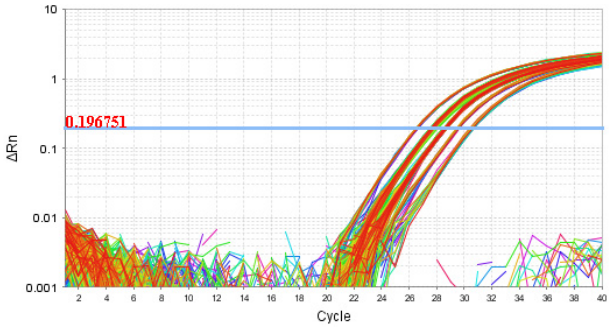
- **A typical amplification plot** – The ViiA™ 7 Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear phase
  - Exponential (geometric phase)
  - Baseline

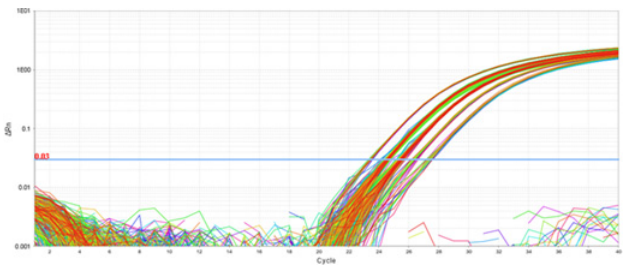
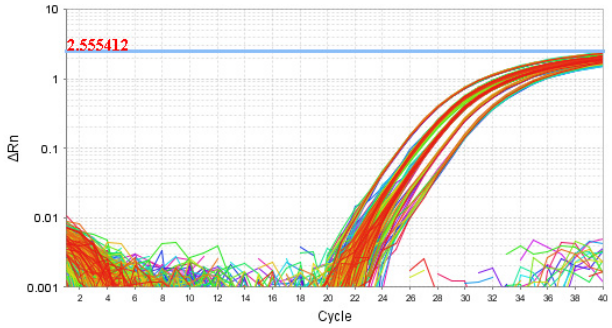
A typical amplification plot should look like this:



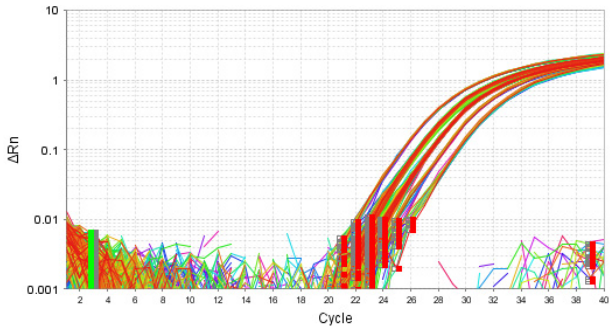
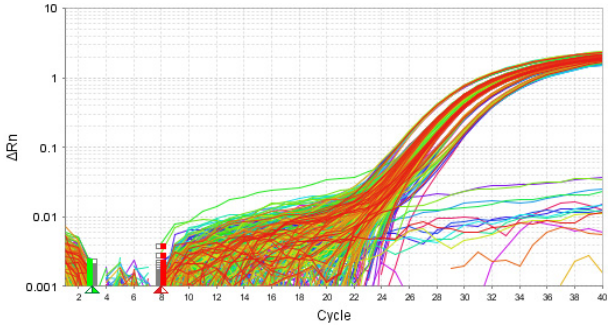
IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the ViiA™ 7 Software. Applied Biosystems recommends that you examine the Amplification Plot screen and review the assigned baseline and threshold values for each well after analysis.

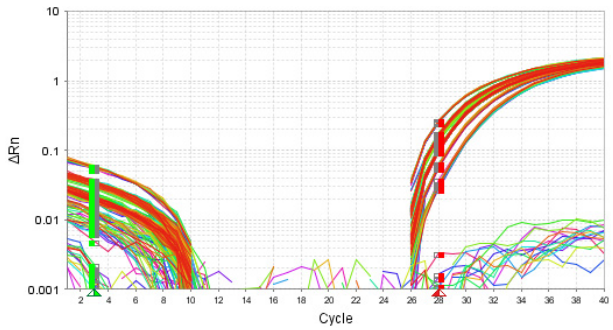
- **Correct threshold values:**

Threshold Set Correctly The threshold is set in the exponential phase of the amplification curve. Threshold settings above or below the optimum increase the standard deviation of the replicate groups.	

Threshold Set Too Low The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the exponential phase of the curve.	
Threshold Set Too High The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar down into the exponential phase of the curve.	

- **Correct baseline values**:

Baseline Set Correctly The amplification curve begins after the maximum baseline.	
Baseline Set Too Low The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.	

Baseline Set Too High The amplification curve begins before the maximum baseline. Decrease the End Cycle value.	

If your experiment does not meet the guidelines above, troubleshoot as follows:

- Omit wells (see "Improve C_T precision by omitting wells" on page 61).

  Or
- Manually adjust the baseline and/or threshold (see "Adjust analysis settings" on page 57).

Identify well problems using the Well Table

The Well Table displays data for each well in the reaction plate, including:

- The sample name, target name, task, and dyes
- The calculated threshold cycle (C_T), normalized fluorescence (Rn), and quantity values
- Comments
- Flags

Purpose

The purpose of viewing the well table is to identify:

- Quantity values
- Flags
- C_T values (including C_T standard deviation)

View the well table

1. From the Experiment Menu pane, select **Analysis**, then select the **Well Table** tab.

   Note: If no data are displayed, click **Analyze**.

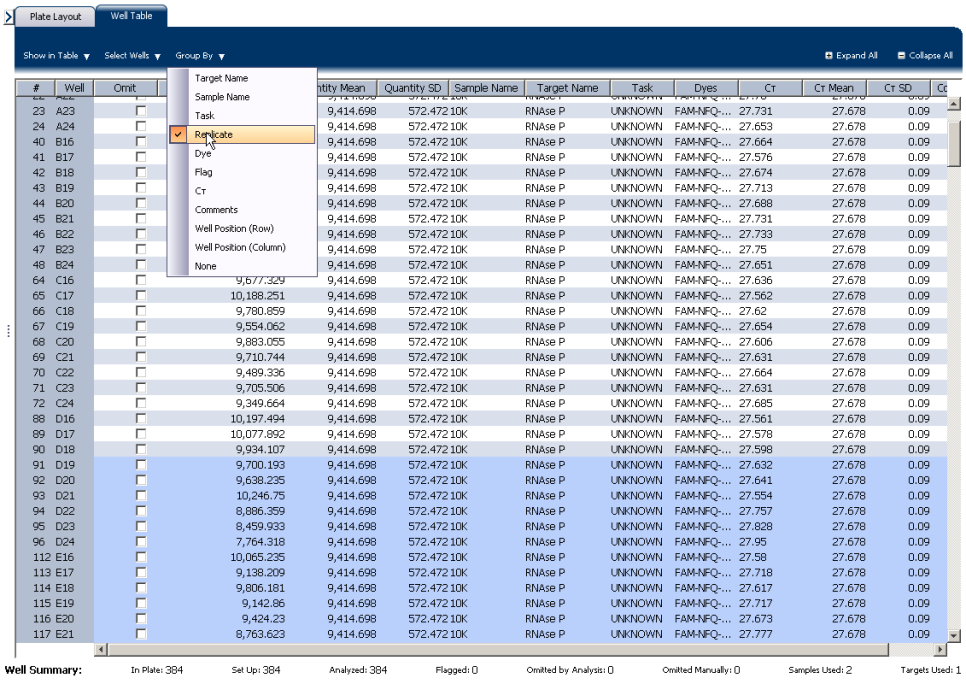
2. Use the Group By drop-down menu to group wells by a specific category. For the example experiment, group the wells by replicate, flag, or C_T value.

   Note: You can select only one category at a time.

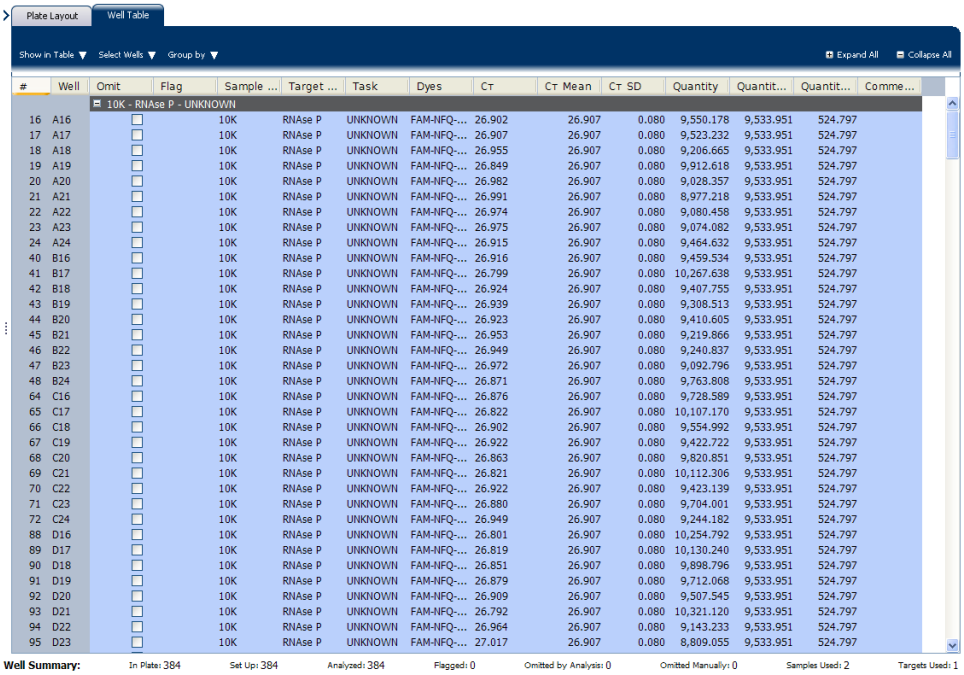
To group by replicate

From the Group By drop-down menu, select **Replicate**. The software groups the replicate wells: negative controls, standards, and samples. In the example experiment, note that the quantity values within each replicate group are similar.

Note: In the example experiment, the Quantity, Quantity Mean, and Quantity SD columns have been moved from their default locations to the beginning of the Well Table. To move a column, click and drag on the column heading.

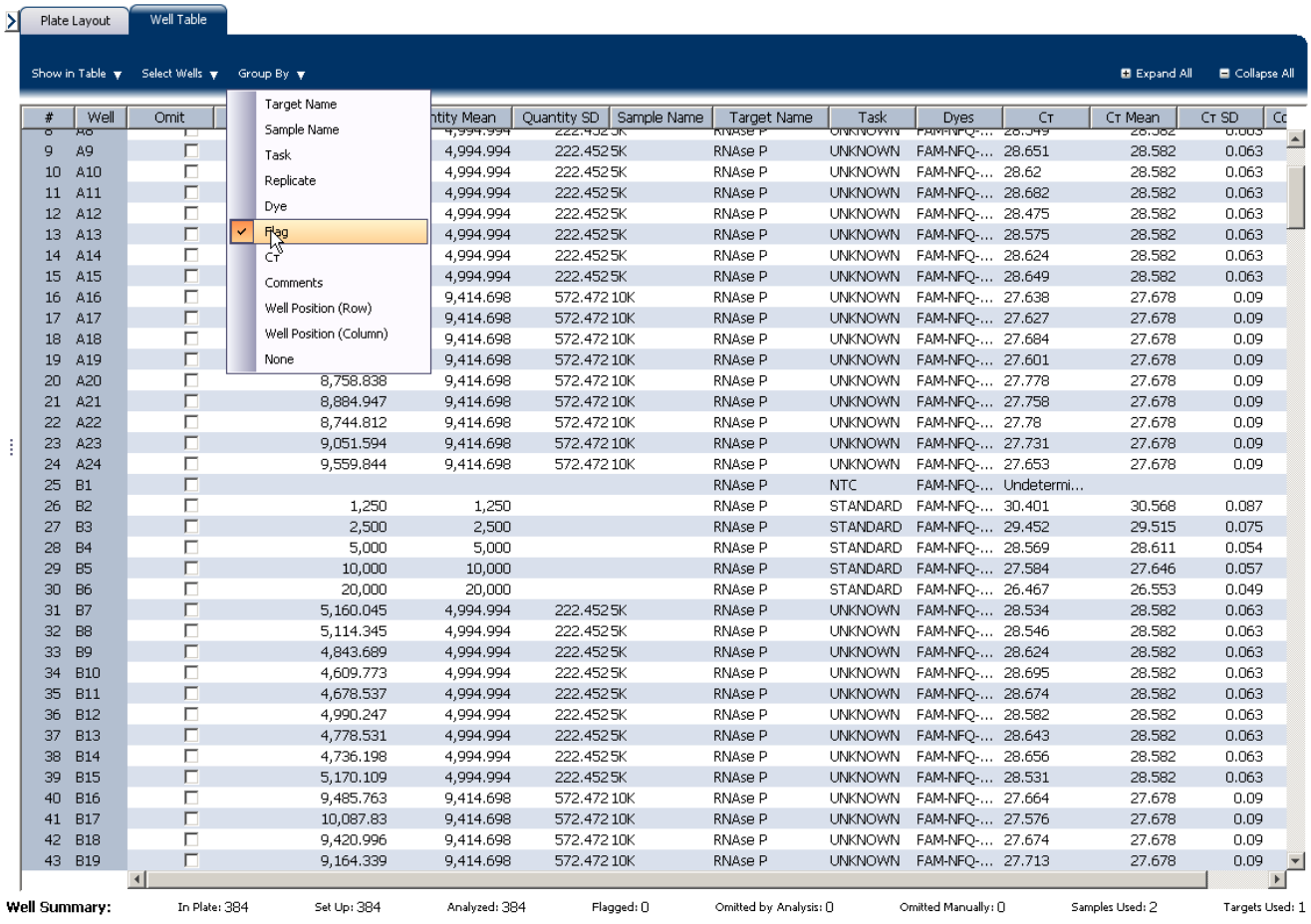


The well table looks like this:



To group by flag

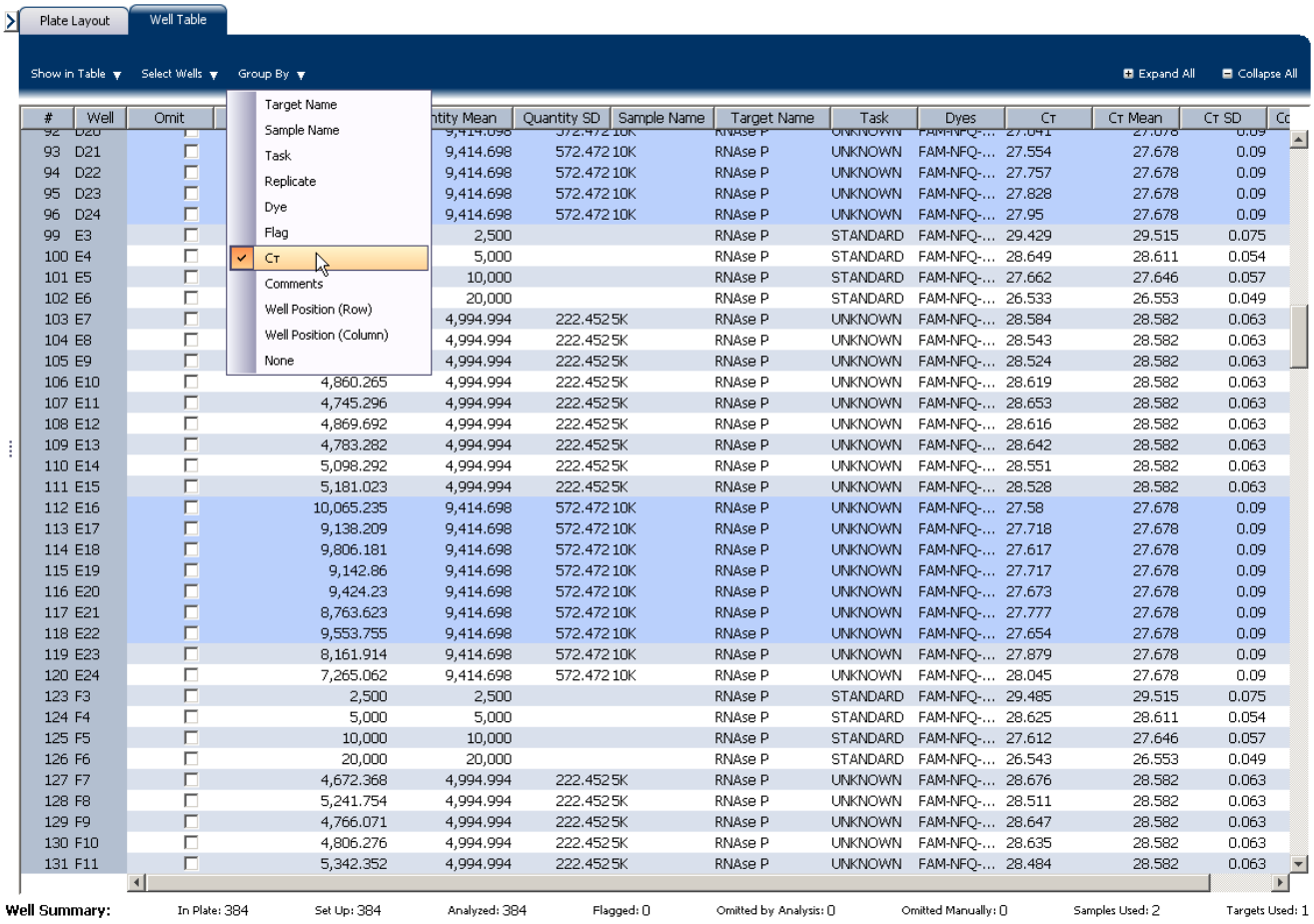
From the Group By drop-down menu, select **Flag**. The software groups the flagged and unflagged wells. In the example experiment, there are no flagged wells.



#	Well	Omit	Quantity Mean	Quantity SD	Sample Name	Target Name	Task	Dyes	Ct	Ct Mean	Ct SD
9	A9		4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.651	28.582	0.063
10	A10		4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.62	28.582	0.063
11	A11		4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.682	28.582	0.063
12	A12		4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.475	28.582	0.063
13	A13		4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.575	28.582	0.063
14	A14		4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.624	28.582	0.063
15	A15		4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.649	28.582	0.063
16	A16		9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.638	27.678	0.09
17	A17		9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.627	27.678	0.09
18	A18		9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.684	27.678	0.09
19	A19		9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.601	27.678	0.09
20	A20		8,758.838	9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.778	27.678	0.09
21	A21		8,884.947	9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.758	27.678	0.09
22	A22		8,744.812	9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.78	27.678	0.09
23	A23		9,051.594	9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.731	27.678	0.09
24	A24		9,559.844	9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.653	27.678	0.09
25	B1						RNAse P	NTC	FAM-NFQ-...	Undetermi...		
26	B2		1,250	1,250			RNAse P	STANDARD	FAM-NFQ-...	30.401	30.568	0.087
27	B3		2,500	2,500			RNAse P	STANDARD	FAM-NFQ-...	29.452	29.515	0.075
28	B4		5,000	5,000			RNAse P	STANDARD	FAM-NFQ-...	28.569	28.611	0.054
29	B5		10,000	10,000			RNAse P	STANDARD	FAM-NFQ-...	27.584	27.646	0.057
30	B6		20,000	20,000			RNAse P	STANDARD	FAM-NFQ-...	26.467	26.553	0.049
31	B7		5,160.045	4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.534	28.582	0.063
32	B8		5,114.345	4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.546	28.582	0.063
33	B9		4,843.689	4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.624	28.582	0.063
34	B10		4,609.773	4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.695	28.582	0.063
35	B11		4,678.537	4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.674	28.582	0.063
36	B12		4,990.247	4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.582	28.582	0.063
37	B13		4,778.531	4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.643	28.582	0.063
38	B14		4,736.198	4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.656	28.582	0.063
39	B15		5,170.109	4,994.994	222.452	5K	RNAse P	UNKNOWN	FAM-NFQ-...	28.531	28.582	0.063
40	B16		9,485.763	9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.664	27.678	0.09
41	B17		10,087.83	9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.576	27.678	0.09
42	B18		9,420.996	9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.674	27.678	0.09
43	B19		9,164.339	9,414.698	572.472	10K	RNAse P	UNKNOWN	FAM-NFQ-...	27.713	27.678	0.09

To group by C_T value

From the Group By drop-down menu, select **C_T**. The software groups the wells by C_T value: low, medium, high, and undetermined. In the example experiment, the C_T values are within the expected range (>8 and <35).



Tips for analyzing your own experiments

- **Replicate** – The software groups the wells by replicate: negative controls, standards, and samples. Look in the Quantity columns to make sure the quantity values for each replicate group are similar indicating tight C_T precision.
- **Flag** – The software groups the flagged and unflagged wells. A flag indicates that the software has found a potential error in the flagged well. For a description of the ViiA™ 7 Software flags, see "Flag Settings" on page 59.
- **C_T** – The threshold cycle (C_T) is the PCR cycle number at which the fluorescence level meets the threshold. A C_T value >8 and <35 is desirable. A C_T value <8 indicates that there is too much template in the reaction. A C_T value >35 indicates a low amount of target in the reaction; for C_T values >35, expect a higher standard deviation.

Confirm accurate dye signal using the Multicomponent Plot

The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the standard curve example experiment, you review the Multicomponent Plot screen for:

- ROX™ dye (passive reference)
- FAM™ dye (reporter)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

View the Multicomponent Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Multicomponent Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the unknown and standard wells one at a time in the Multicomponent Plot screen:

   a. Click the **Plate Layout** tab.

   b. Select one well in the plate layout; the well is shown in the Multicomponent Plot screen.

      Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

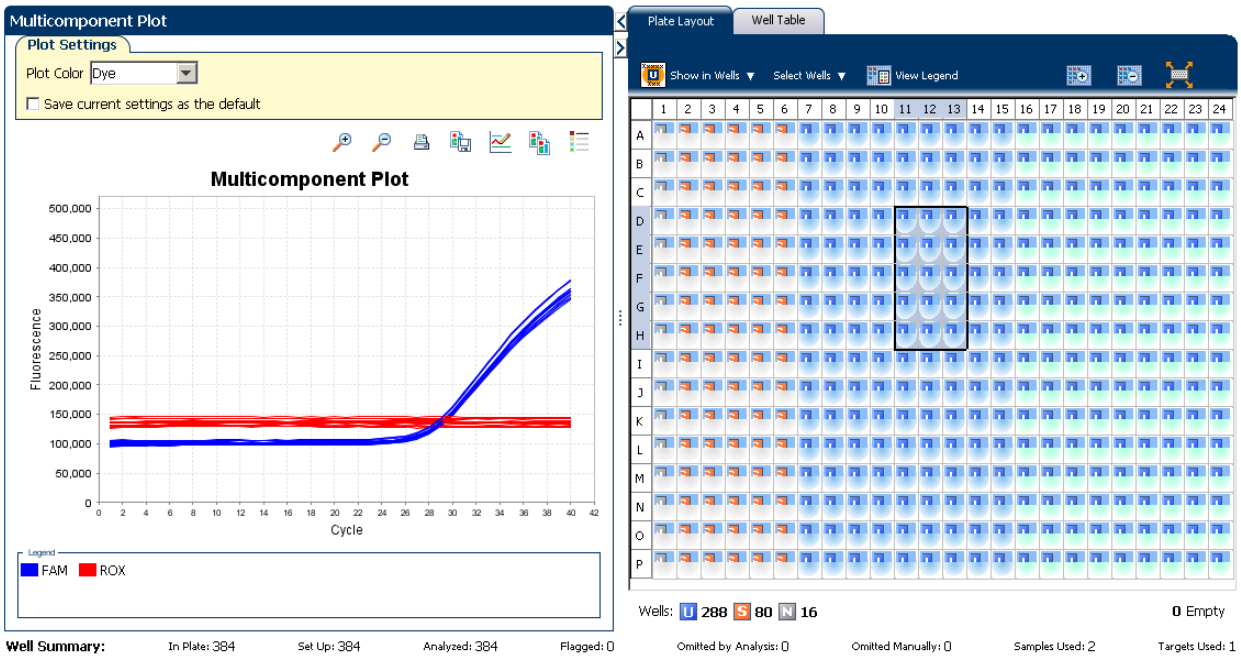
3. From the Plot Color drop-down menu, select **Dye**.

4. Click **Show a legend for the plot** (default).

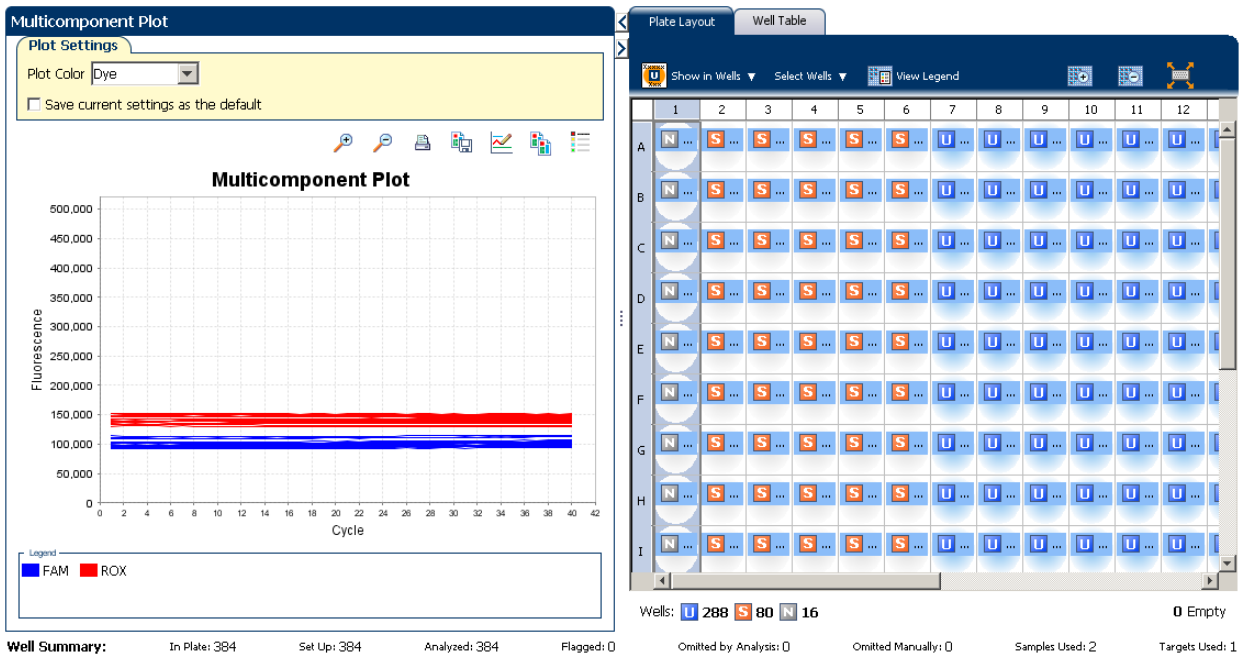
   Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

5. Check the ROX dye signal. In the example experiment, the ROX dye signal remains constant throughout the PCR process; a constant ROX dye signal indicates typical data.

6. Check the FAM dye signal. In the example experiment, the FAM dye signal increases throughout the PCR process; increase in FAM dye signal indicates normal amplification.



7. Select the negative control wells one at time and check for amplification. In the example experiment, there is no amplification in the negative control wells.



Tips for confirming dye accuracy in your own experiment

When you analyze your own standard curve experiment, look for:

- **Passive reference** – The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye** – The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- **Irregularities in the signal** – There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative control wells** – There should not be any amplification in the negative control wells.

Determine signal accuracy using the Raw Data Plot

The Raw Data Plot screen displays the raw fluorescence signal (not normalized) for each optical filter for the selected wells during each cycle of the real-time PCR.

Purpose

In the standard curve example experiment, you review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the Raw Data Plot

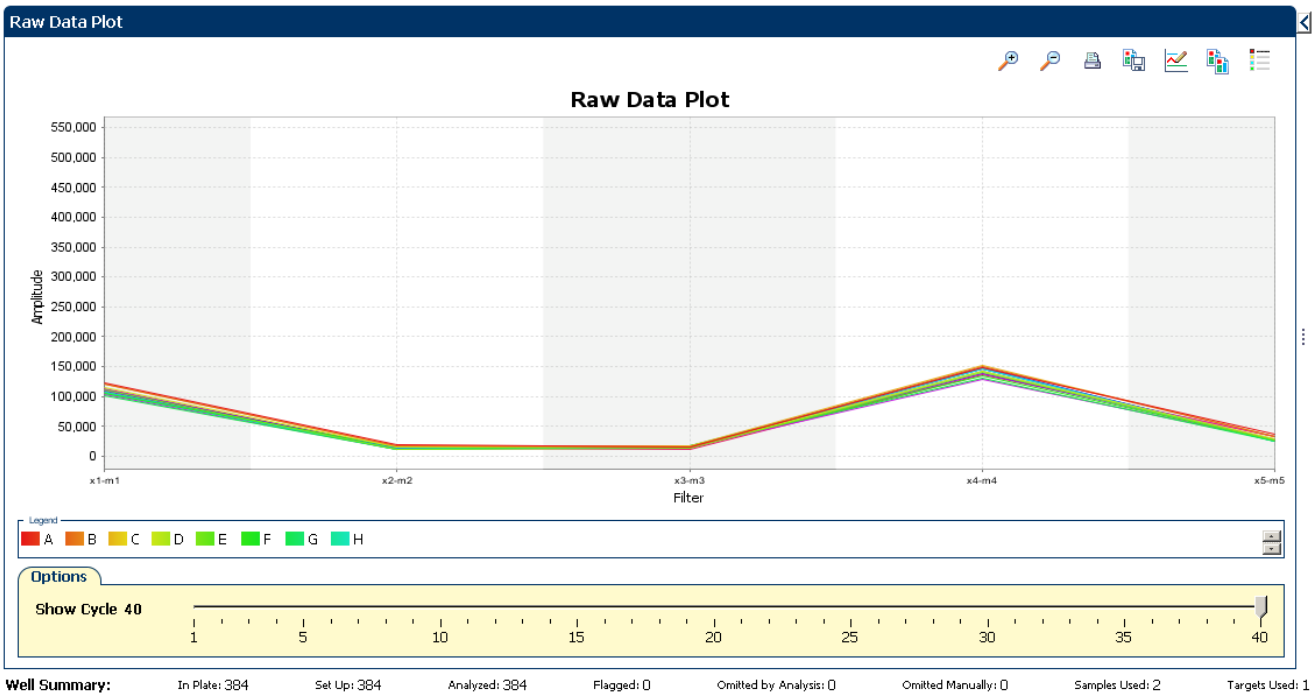
1. From the Experiment Menu pane, select **Analysis** ▸ **Raw Data Plot**.

   Note: If no data are displayed, click **Analyze**.

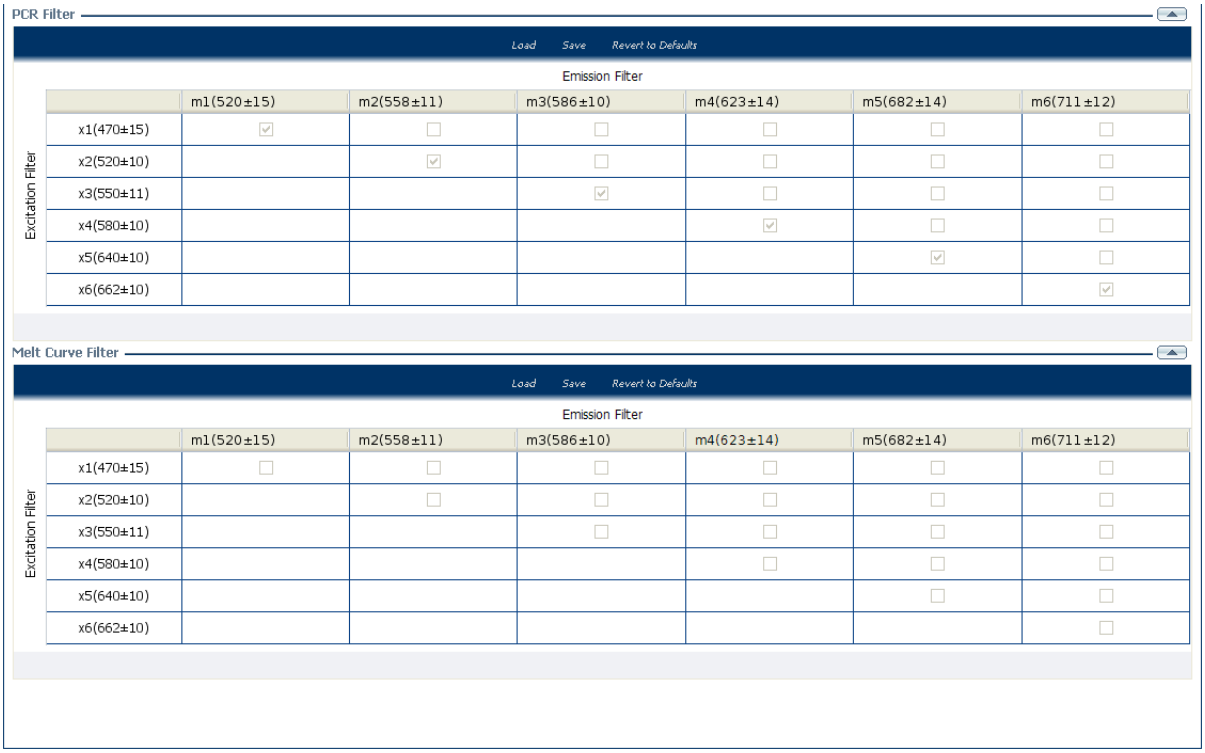
2. Display all 384 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the Plate Layout tab.

3. Click **Show a legend for the plot** (default). The legend displays the color code for each row of the reaction plate (see the legend in the Raw Data Plot shown below).

4. Click and drag the Show Cycle pointer from cycle 1 to cycle 40. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.



The filters used for the example experiment are:



Tips for determining signal accuracy in your own experiments

When you analyze your own standard curve experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

Review the flags in the QC Summary

The QC Summary screen displays a list of the ViiA™ 7 Software flags, including the flag frequency and location for the open experiment.

View the QC Summary

1. From the Experiment Menu pane, select **Analysis ▸ QC Summary**.

   Note: If no data are displayed, click **Analyze**.

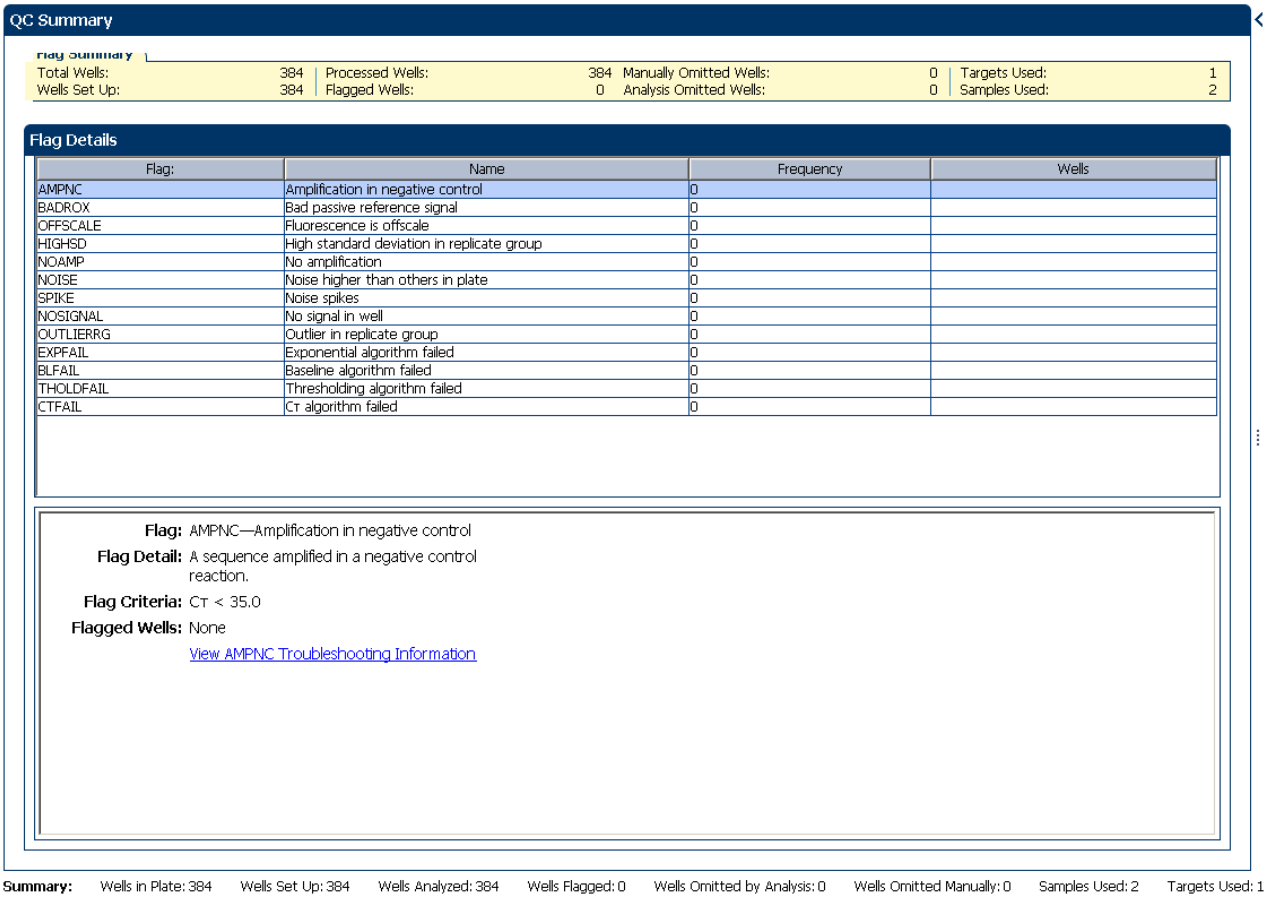
2. Review the Flags Summary.

   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment. If the frequency is >0, the flag appears somewhere in the experiment; the well position is listed in the Wells column.

   In the example experiment, there are no flagged wells.

3. In the Flag Details table, click each flag with a frequency >0 to display detailed information about the flag. In the example experiment, the Frequency column displays 0 for all flags.

4. *(Optional)* For those flags with frequency >0, click the troubleshooting link to view information on correcting the flag.



Possible flags

The flags listed below may be triggered by the experiment data.

Note: To change the flag settings, refer to "Flag Settings" on page 59.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed

Flag	Description
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
Secondary analysis flags	
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the Frequency column for each flag).

For more information

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

5.2 Section 5.2 Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

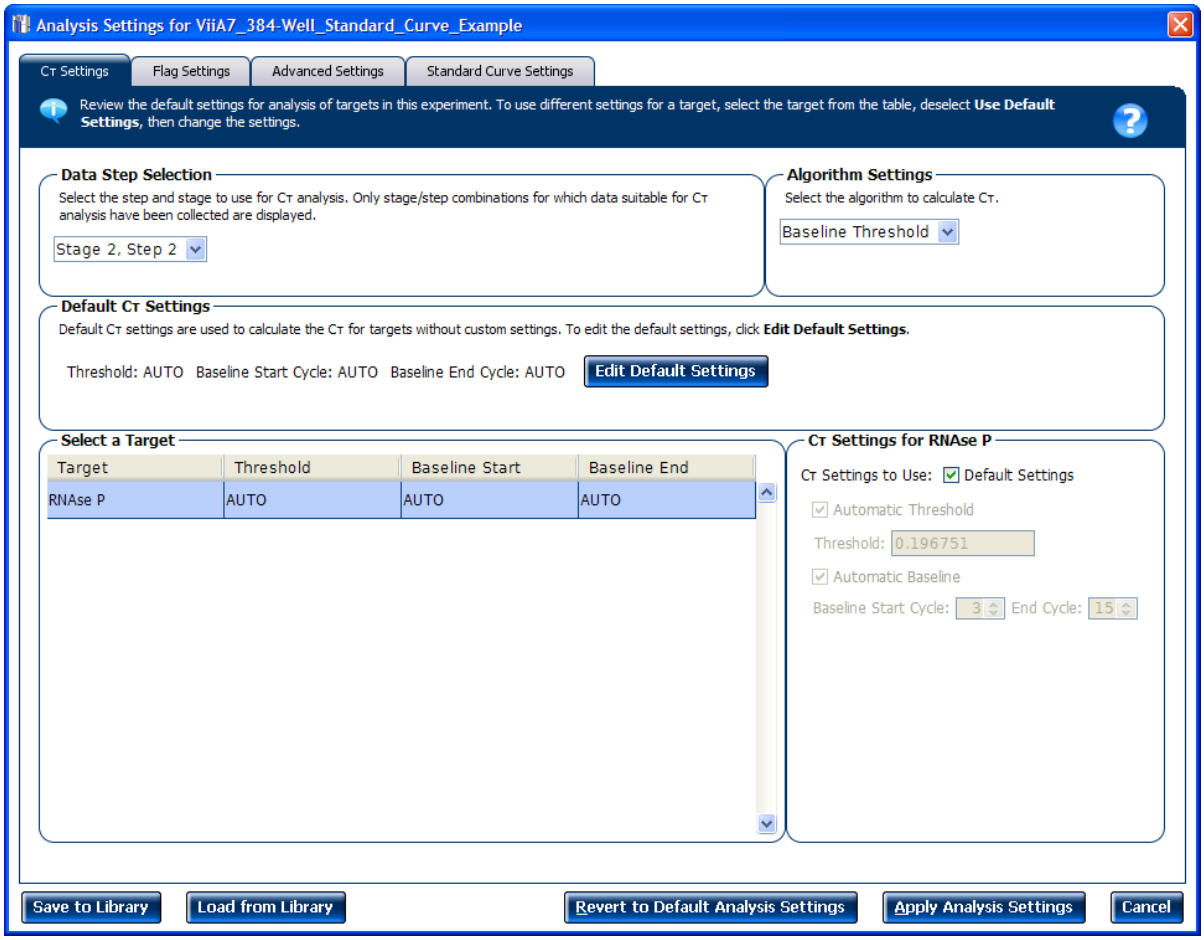
The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, and advanced options.

If the default analysis settings in the ViiA™ 7 Software are not suitable for your own experiment, you can change the settings in the Analysis Settings dialog box, then reanalyze your experiment.

View the analysis settings

1. From the Experiment Menu pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the Analysis Settings dialog box.
   In the example experiment, the default analysis settings are used for each tab:
   - C_T Settings
   - Flag Settings
   - Advanced Settings
   - Standard Curve Settings

The Analysis Settings dialog box for a Standard Curve experiment looks like this:



3. View and, if necessary, change the analysis settings (see "Adjust analysis settings" below).

   Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see About the Analysis Settings Library in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*.

4. Click **Apply Analysis Settings** to apply the current analysis settings.

   Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

C_T Settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method.

- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for gene quantification.

The Relative Threshold algorithm lets you compare the data on a per-well or per-target basis. This setting is ideal for analyzing a single gene across samples or a single sample across genes with no dependence on targets, thereby reducing variability. Any settings for baseline or threshold do not affect the analysis when you use the Relative Threshold algorithm.

- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the targets that do not have custom settings. To edit the default settings, click **Edit Default Settings**.

- **C_T Settings for Target**

  When you manually set the threshold and baseline, Applied Biosystems recommends:

Setting	Recommendation
Threshold	Enter a value for the threshold so that the threshold is: • Above the background. • Below the plateau and linear regions of the amplification curve. • Within the exponential phase of the amplification curve.
Baseline	Select the Start Cycle and End Cycle values so that the baseline ends before significant fluorescent signal is detected.

Note: This setting is applicable only to the Baseline Threshold algorithm.

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag Settings

Use the Flag Settings tab to:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the ViiA™ 7 Software.

To adjust the flag settings:

1. In the Use column, select the check boxes for flags to apply during analysis.
2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

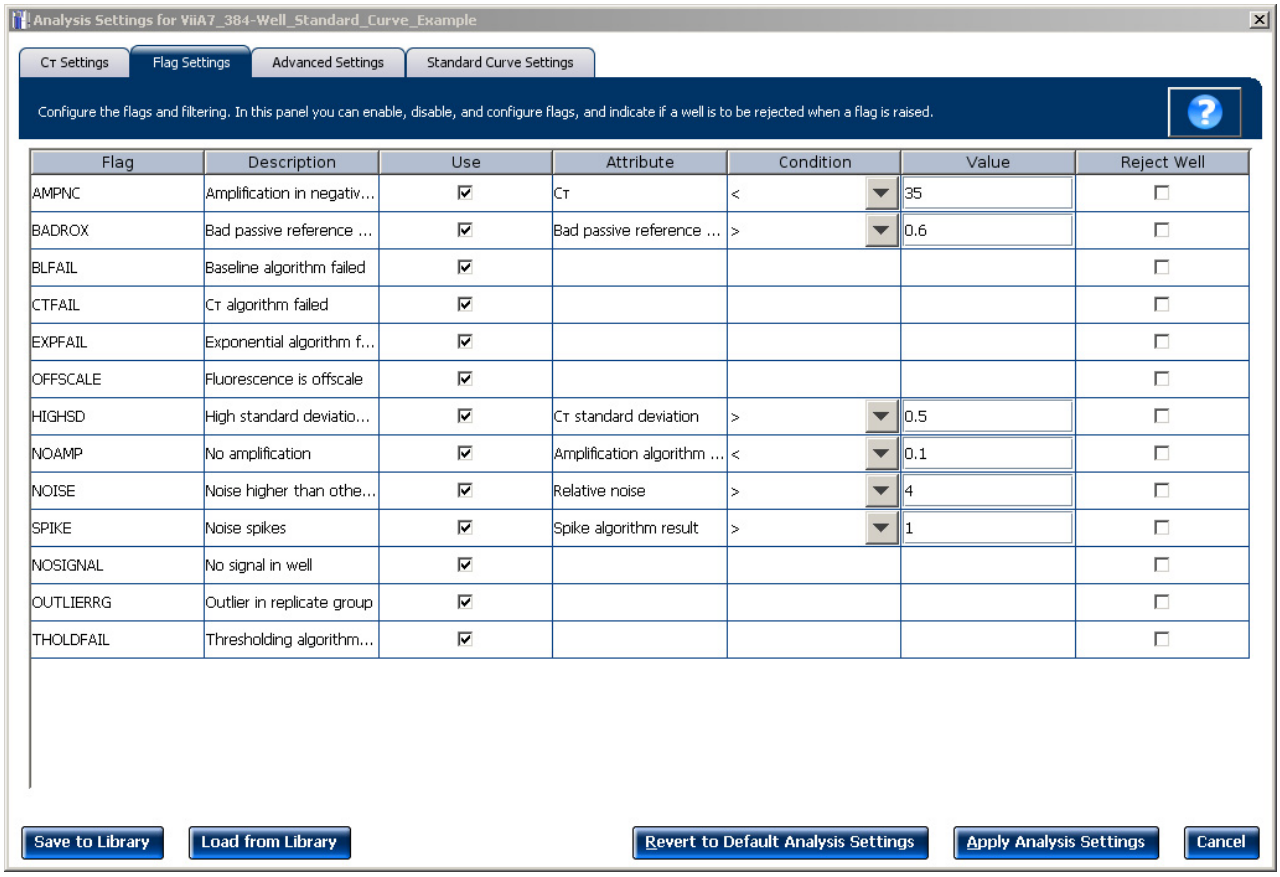
   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations may change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.

4. Click **Apply Analysis Settings** in the Analysis Settings dialog box. If the run status is complete, the data are reanalyzed.

   The Flag Settings tab looks like this:



Advanced Settings

Use the Advanced Settings tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold algorithm.

To use custom baseline settings for a well-target combination:

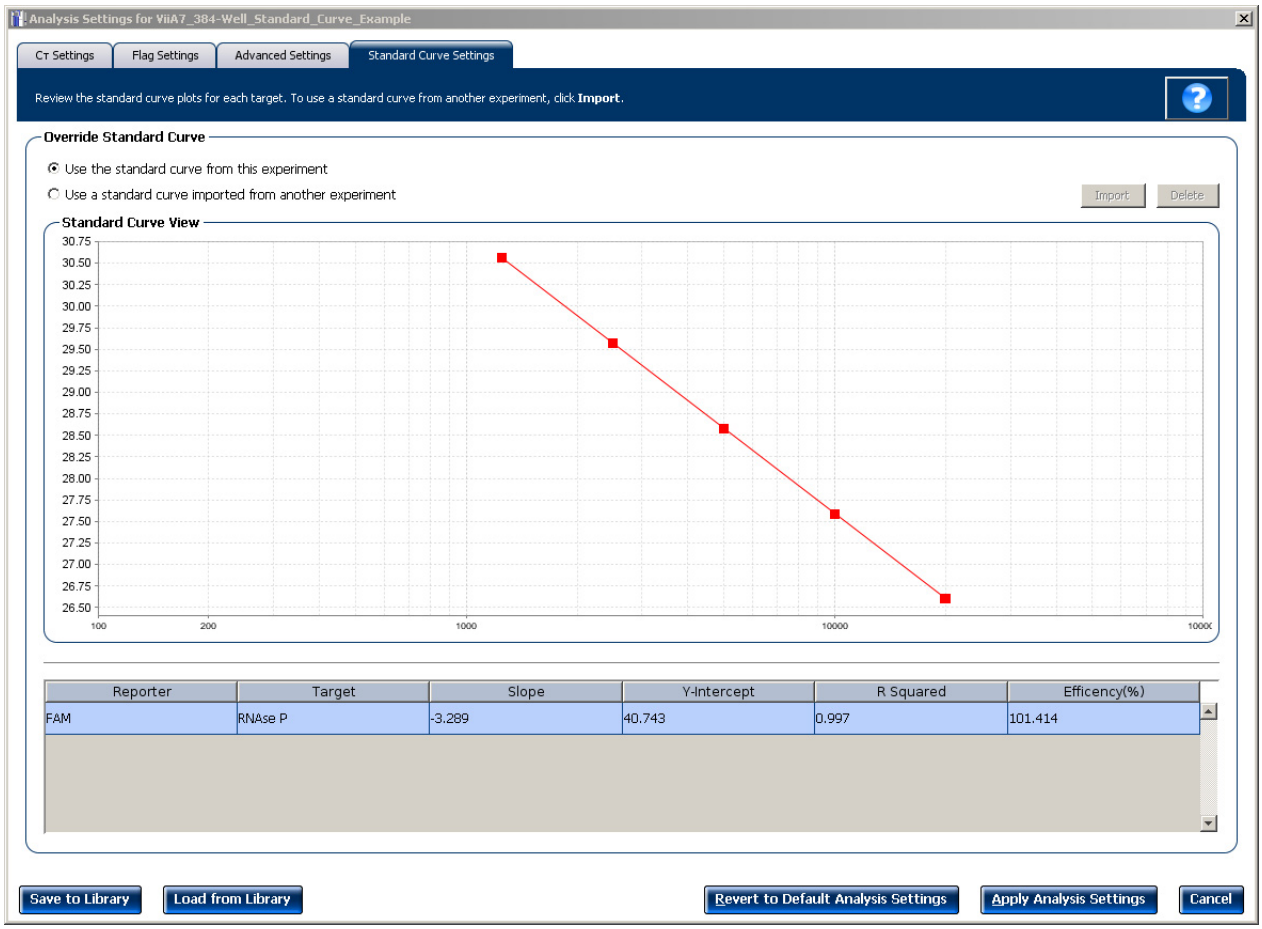
1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** check box.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, then enter the baseline start cycle and baseline end cycle.

Standard Curve Settings

Use this tab to review the settings of the current standard curve experiment or to import the standard curve from an external experiment (with the same samples and targets) and apply it to the current experiment.

Note: The run method must be the same. Applied Biosystems recommends using the standard curve from the current experiment.

For the example experiment, the settings from the current experiment have been used.



Improve C_T precision by omitting wells

Experimental error may cause some wells to be amplified insufficiently or not at all. These wells typically produce C_T values that differ significantly from the average for the associated replicate wells. If included in the calculations, these outliers can result in erroneous measurements; to ensure C_T precision, omit the outliers from the analysis.

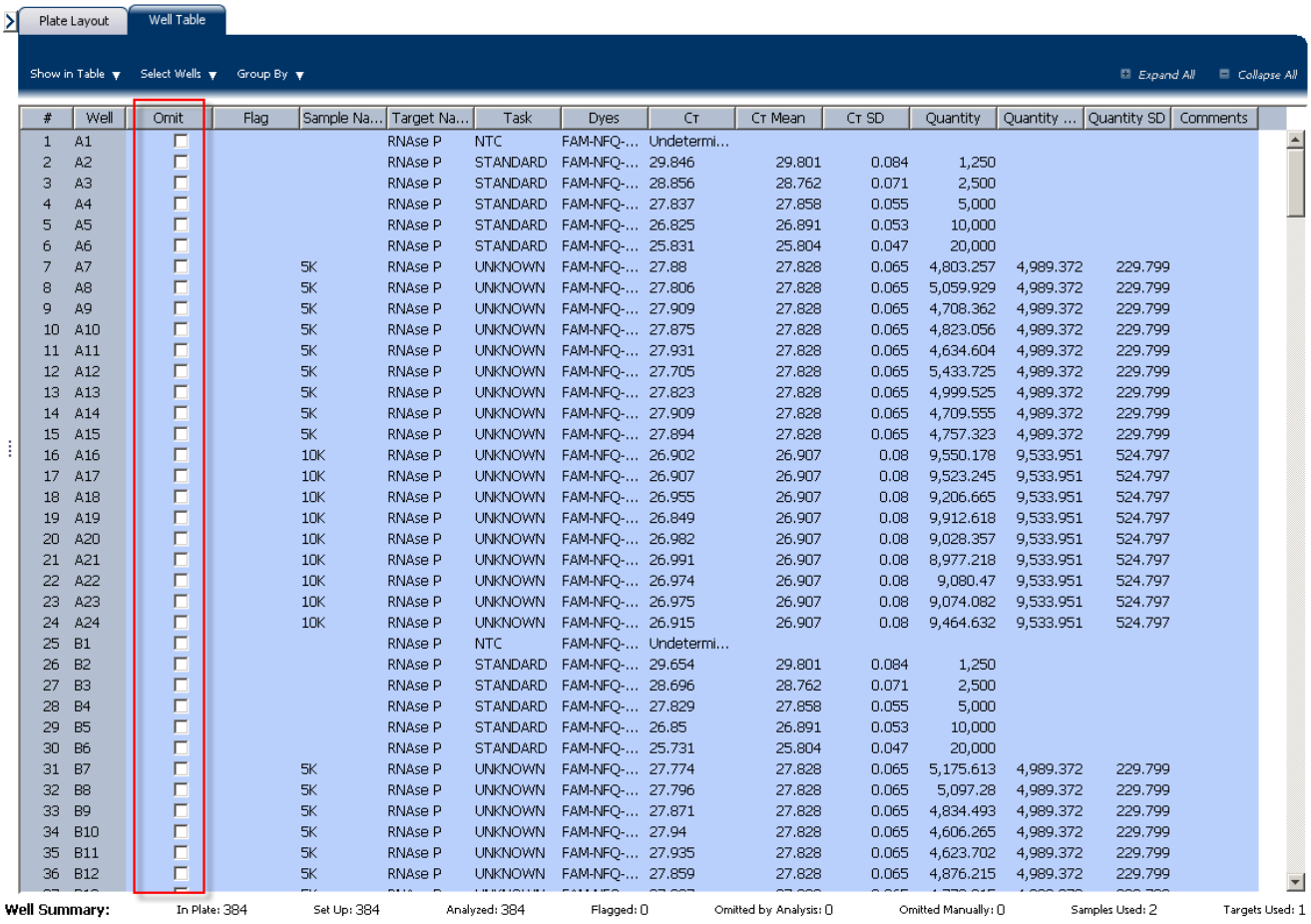
1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.

2. In the Amplification Plot screen, select **C_T vs Well** from the Plot Type drop-down menu.

3. Select the **Well Table** tab.

4. In the Well Table, view outliers:
   a. From the Group By drop-down menu, select **Replicate**.
   b. Look for any outliers in the replicate group (make sure they are flagged).
   c. Select the **Omit** check box next to outlying well(s), as shown below.



#	Well	Omit	Flag	Sample Na...	Target Na...	Task	Dyes	Ct	Ct Mean	Ct SD	Quantity	Quantity ...	Quantity SD	Comments
1	A1	☐			RNAse P	NTC	FAM-NFQ-...	Undetermi...						
2	A2	☐			RNAse P	STANDARD	FAM-NFQ-...	29.846	29.801	0.084	1,250			
3	A3	☐			RNAse P	STANDARD	FAM-NFQ-...	28.856	28.762	0.071	2,500			
4	A4	☐			RNAse P	STANDARD	FAM-NFQ-...	27.837	27.858	0.055	5,000			
5	A5	☐			RNAse P	STANDARD	FAM-NFQ-...	26.825	26.891	0.053	10,000			
6	A6	☐			RNAse P	STANDARD	FAM-NFQ-...	25.831	25.804	0.047	20,000			
7	A7	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.88	27.828	0.065	4,803.257	4,989.372	229.799	
8	A8	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.806	27.828	0.065	5,059.929	4,989.372	229.799	
9	A9	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.909	27.828	0.065	4,708.362	4,989.372	229.799	
10	A10	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.875	27.828	0.065	4,823.056	4,989.372	229.799	
11	A11	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.931	27.828	0.065	4,634.604	4,989.372	229.799	
12	A12	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.705	27.828	0.065	5,433.725	4,989.372	229.799	
13	A13	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.823	27.828	0.065	4,999.525	4,989.372	229.799	
14	A14	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.909	27.828	0.065	4,709.555	4,989.372	229.799	
15	A15	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.894	27.828	0.065	4,757.323	4,989.372	229.799	
16	A16	☐		10K	RNAse P	UNKNOWN	FAM-NFQ-...	26.902	26.907	0.08	9,550.178	9,533.951	524.797	
17	A17	☐		10K	RNAse P	UNKNOWN	FAM-NFQ-...	26.907	26.907	0.08	9,523.245	9,533.951	524.797	
18	A18	☐		10K	RNAse P	UNKNOWN	FAM-NFQ-...	26.955	26.907	0.08	9,206.665	9,533.951	524.797	
19	A19	☐		10K	RNAse P	UNKNOWN	FAM-NFQ-...	26.849	26.907	0.08	9,912.618	9,533.951	524.797	
20	A20	☐		10K	RNAse P	UNKNOWN	FAM-NFQ-...	26.982	26.907	0.08	9,028.357	9,533.951	524.797	
21	A21	☐		10K	RNAse P	UNKNOWN	FAM-NFQ-...	26.991	26.907	0.08	8,977.218	9,533.951	524.797	
22	A22	☐		10K	RNAse P	UNKNOWN	FAM-NFQ-...	26.974	26.907	0.08	9,080.47	9,533.951	524.797	
23	A23	☐		10K	RNAse P	UNKNOWN	FAM-NFQ-...	26.975	26.907	0.08	9,074.082	9,533.951	524.797	
24	A24	☐		10K	RNAse P	UNKNOWN	FAM-NFQ-...	26.915	26.907	0.08	9,464.632	9,533.951	524.797	
25	B1	☐			RNAse P	NTC	FAM-NFQ-...	Undetermi...						
26	B2	☐			RNAse P	STANDARD	FAM-NFQ-...	29.654	29.801	0.084	1,250			
27	B3	☐			RNAse P	STANDARD	FAM-NFQ-...	28.696	28.762	0.071	2,500			
28	B4	☐			RNAse P	STANDARD	FAM-NFQ-...	27.829	27.858	0.055	5,000			
29	B5	☐			RNAse P	STANDARD	FAM-NFQ-...	26.85	26.891	0.053	10,000			
30	B6	☐			RNAse P	STANDARD	FAM-NFQ-...	25.731	25.804	0.047	20,000			
31	B7	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.774	27.828	0.065	5,175.613	4,989.372	229.799	
32	B8	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.796	27.828	0.065	5,097.28	4,989.372	229.799	
33	B9	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.871	27.828	0.065	4,834.493	4,989.372	229.799	
34	B10	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.94	27.828	0.065	4,606.265	4,989.372	229.799	
35	B11	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.935	27.828	0.065	4,623.702	4,989.372	229.799	
36	B12	☐		5K	RNAse P	UNKNOWN	FAM-NFQ-...	27.859	27.828	0.065	4,876.215	4,989.372	229.799	

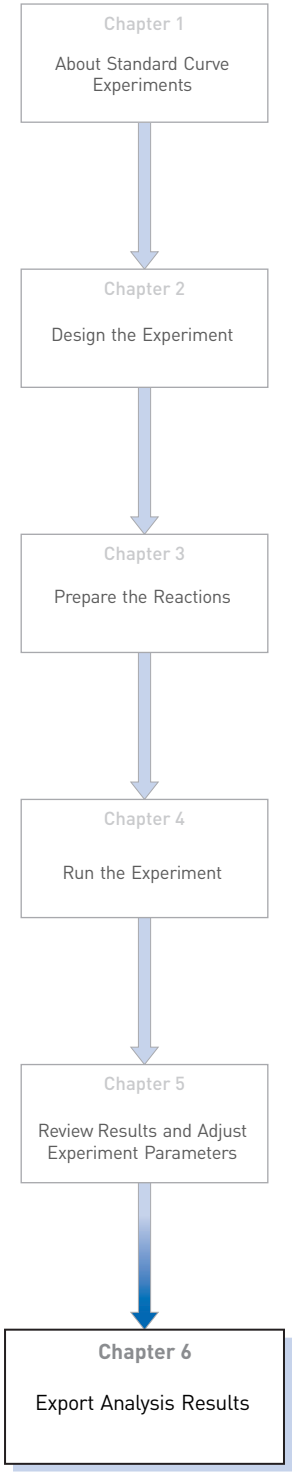
5. Click **Analyze** to reanalyze the experiment data with the outlying well(s) removed from the analysis.

   Note: You can also omit undesirable wells in an experiment from the Plate Layout screen. To omit a well from the Plate Layout screen, right-click the well and select **Omit**.

For more information

For more information on	Refer to	Part number
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*.	127AP05-03

6 Export Analysis Results

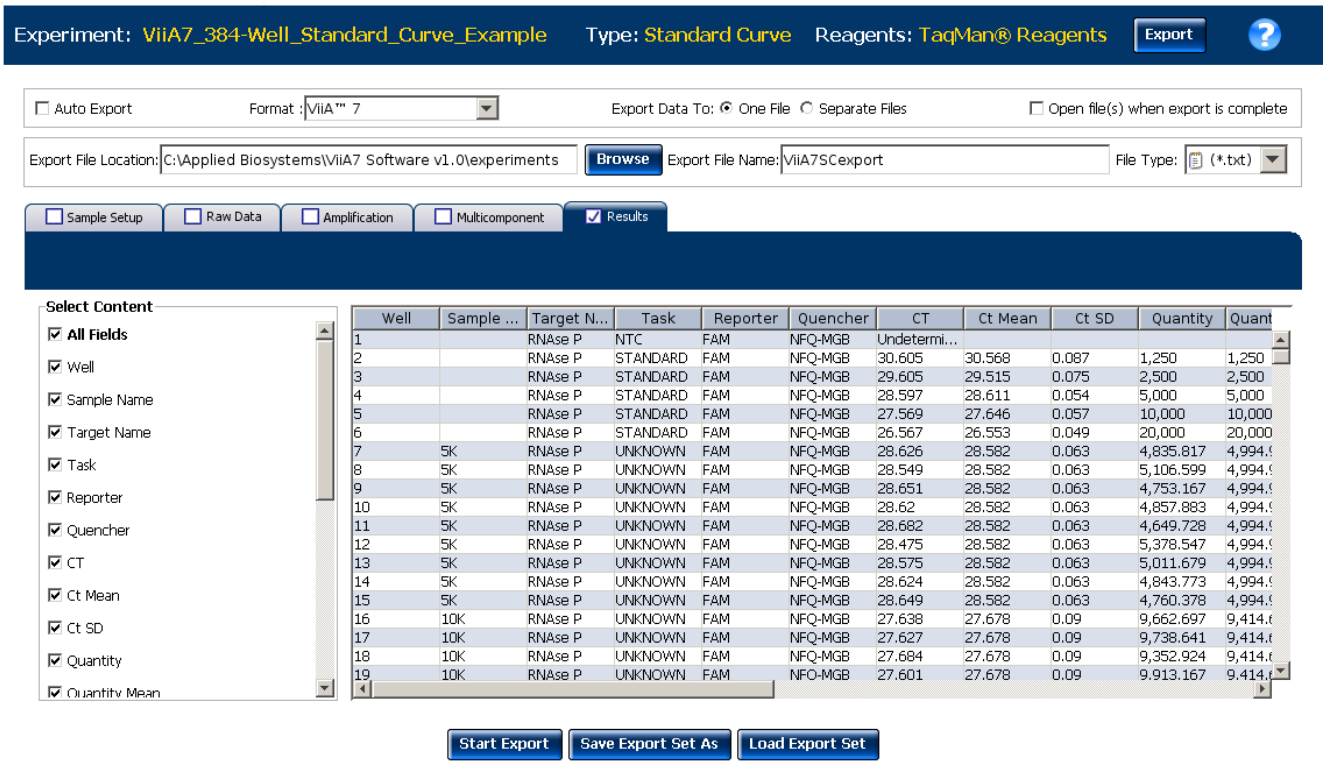


1. Open the Standard Curve example experiment file that you analyzed in Chapter 5.
2. In the Experiment Menu, click **Export.**

   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.
3. Select **ViiA™7 Format**.
4. Complete the Export dialog box as shown below:

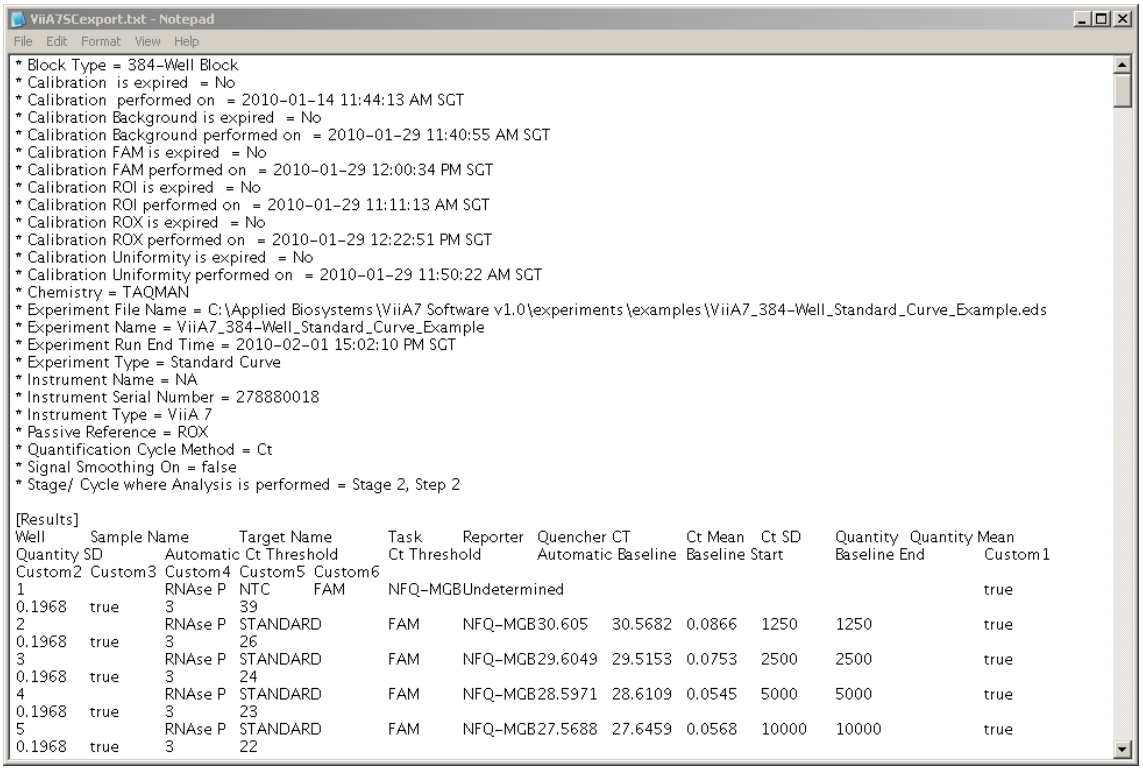
Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	ViiA7SCexport
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\ViiA7 Software v1.1\experiments

Your Export screen should look like this:



Well	Sample ...	Target N...	Task	Reporter	Quencher	CT	Ct Mean	Ct SD	Quantity	Quant
1		RNAse P	NTC	FAM	NFQ-MGB	Undetermi...				
2		RNAse P	STANDARD	FAM	NFQ-MGB	30.605	30.568	0.087	1,250	1,250
3		RNAse P	STANDARD	FAM	NFQ-MGB	29.605	29.515	0.075	2,500	2,500
4		RNAse P	STANDARD	FAM	NFQ-MGB	28.597	28.611	0.054	5,000	5,000
5		RNAse P	STANDARD	FAM	NFQ-MGB	27.569	27.646	0.057	10,000	10,000
6		RNAse P	STANDARD	FAM	NFQ-MGB	26.567	26.553	0.049	20,000	20,000
7	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	28.626	28.582	0.063	4,835.817	4,994.9
8	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	28.549	28.582	0.063	5,106.599	4,994.9
9	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	28.651	28.582	0.063	4,753.167	4,994.9
10	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	28.62	28.582	0.063	4,857.883	4,994.9
11	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	28.682	28.582	0.063	4,649.728	4,994.9
12	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	28.475	28.582	0.063	5,378.547	4,994.9
13	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	28.575	28.582	0.063	5,011.679	4,994.9
14	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	28.624	28.582	0.063	4,843.773	4,994.9
15	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	28.649	28.582	0.063	4,760.378	4,994.9
16	10K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27.638	27.678	0.09	9,662.697	9,414.6
17	10K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27.627	27.678	0.09	9,738.641	9,414.6
18	10K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27.684	27.678	0.09	9,352.924	9,414.6
19	10K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27.601	27.678	0.09	9,913.167	9,414.6

Your exported file when opened in Notepad should look like this:



ViiA7SCexport.txt - Notepad

File Edit Format View Help

```
* Block Type = 384-Well Block
* Calibration  is expired  = No
* Calibration  performed on  = 2010-01-14 11:44:13 AM SGT
* Calibration Background is expired  = No
* Calibration Background performed on  = 2010-01-29 11:40:55 AM SGT
* Calibration FAM is expired  = No
* Calibration FAM performed on  = 2010-01-29 12:00:34 PM SGT
* Calibration ROI is expired  = No
* Calibration ROI performed on  = 2010-01-29 11:11:13 AM SGT
* Calibration ROX is expired  = No
* Calibration ROX performed on  = 2010-01-29 12:22:51 PM SGT
* Calibration Uniformity is expired  = No
* Calibration Uniformity performed on  = 2010-01-29 11:50:22 AM SGT
* Chemistry = TAQMAN
* Experiment File Name = C:\Applied Biosystems\ViiA7 Software v1.0\experiments\examples\ViiA7_384-Well_Standard_Curve_Example.eds
* Experiment Name = ViiA7_384-Well_Standard_Curve_Example
* Experiment Run End Time = 2010-02-01 15:02:10 PM SGT
* Experiment Type = Standard Curve
* Instrument Name = NA
* Instrument Serial Number = 278880018
* Instrument Type = ViiA 7
* Passive Reference = ROX
* Quantification Cycle Method = Ct
* Signal Smoothing On = false
* Stage/ Cycle where Analysis is performed = Stage 2, Step 2

[Results]
Well  Sample Name  Target Name  Task  Reporter  Quencher  CT  Ct Mean  Ct SD  Quantity  Quantity Mean
Quantity SD  Automatic Ct Threshold  Ct Threshold  Automatic Baseline  Baseline Start  Baseline End  Custom1
Custom2  Custom3  Custom4  Custom5  Custom6
1  RNAse P  NTC  FAM  NFQ-MGB  Undetermined  true
0.1968  true  3  39
2  RNAse P  STANDARD  FAM  NFQ-MGB  30.605  30.5682  0.0866  1250  1250  true
0.1968  true  3  26
3  RNAse P  STANDARD  FAM  NFQ-MGB  29.6049  29.5153  0.0753  2500  2500  true
0.1968  true  3  24
4  RNAse P  STANDARD  FAM  NFQ-MGB  28.5971  28.6109  0.0545  5000  5000  true
0.1968  true  3  23
5  RNAse P  STANDARD  FAM  NFQ-MGB  27.5688  27.6459  0.0568  10000  10000  true
0.1968  true  3  22
```

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BOOKLET 3

Running Relative Standard Curve and Comparative C_T Experiments

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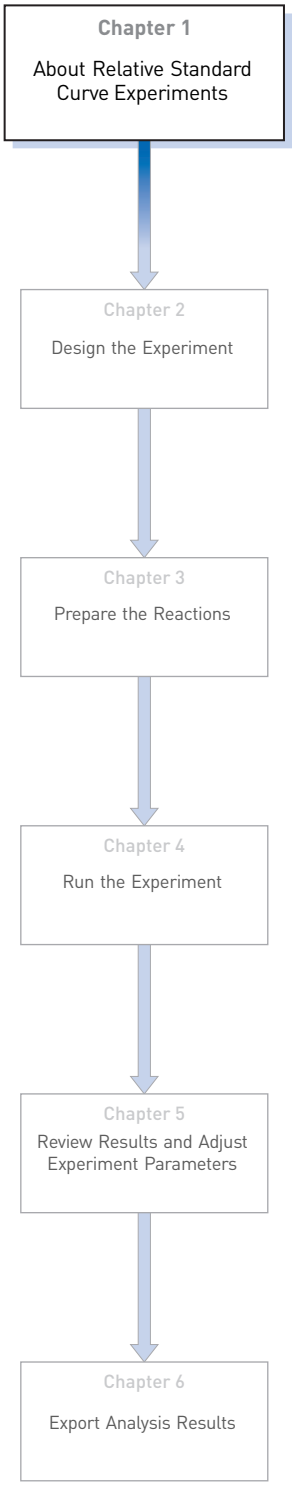
PART I

Running Relative Standard Curve Experiments

1 About Relative Standard Curve Experiments



This chapter covers:

- About Relative Standard Curve experiments. 12
- About the example experiment 13

IMPORTANT! First-time users of the ViiA™ 7 System, please read Booklet 1, *Getting Started with ViiA™ 7 System Experiments* and Booklet 7, *ViiA™ 7 System Experiments - Appendixes* of this binder thoroughly. The booklets provide information and general instructions that are applicable to all the experiments described in this binder.

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems ViiA™ 7 Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ ViiA™ 7 Software Help**.

About Relative Standard Curve experiments

The Relative Standard Curve method is used to determine relative target quantity in samples. The ViiA™ 7 Software measures amplification of the target and of the endogenous control in samples, in a reference sample, and in a standard dilution series. Measurements are normalized using the endogenous control. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates target quantity and endogenous control quantity in the samples and the reference sample. For each sample and reference sample, the target quantity is normalized by endogenous control quantity (quantity of target/ quantity of endogenous control). The normalized quotient from samples is divided by the quotient from the reference sample to get relative quantification (fold change). The software determines the relative quantity of target in each sample by comparing target quantity in each sample to target quantity in the reference sample.

Relative Standard Curve experiments are commonly used to:

- Compare expression levels of a gene in different tissues.
- Compare expression levels of a gene in a treated sample and an untreated sample.
- Compare expression levels of wild-type alleles and mutated alleles.
- Analyze the gene expression changes over time under specific treatment conditions.

Assemble required components

- **Sample** – The tissue group that you are testing for a target gene.
- **Reference sample (also called a calibrator)** – The sample used as the basis for relative quantification results. For example, in a study of drug effects on gene expression, an untreated control is an appropriate reference sample.
- **Standard** – A sample that contains known quantities of the target; used in quantification experiments to generate standard curves.
- **Standard dilution series** – A set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards.
- **Endogenous control** – A gene that is used to normalize template input differences, and sample-to-sample or run-to-run variation.
- **Replicates** – The total number of identical reactions containing identical components and identical volumes.
- **Negative controls** – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

PCR options

When performing real-time PCR, choose between:

- Singleplex and multiplex PCR (below)

  and

- 1-step and 2-step RT-PCR (page 13)

Singleplex and Multiplex PCR

You can perform a PCR reaction using either:

- **Singleplex PCR** – In singleplex PCR a single primer and probe set is present in the reaction tube or well. Only one target or endogenous control can be amplified per reaction.

  Or

- **Multiplex PCR** – In multiplex PCR, two or more primer and probe sets are present in the reaction tube or well. Each set amplifies a specific target or endogenous control. Typically, a probe labeled with FAM™ dye detects the target and a probe labeled with VIC® dye detects the endogenous control.

IMPORTANT! SYBR® Green reagents cannot be used for multiplex PCR.

1- and 2-Step RT-PCR

You can perform reverse transcription (RT) and PCR in a single reaction (1-step) or in separate reactions (2-step). The reagent configuration you use depends on whether you are performing 1- or 2-step RT-PCR:

- **1-step PCR**– In 1-step RT-PCR, RT and PCR take place in one buffer system. Using one buffer system provides the convenience of a single-tube preparation for RT and PCR amplification. However, you cannot use Fast PCR Master Mix or the carryover prevention enzyme, AmpErase® UNG (uracil-N-glycosylase), to perform 1-step RT-PCR.
- **2-step PCR**– 2-step RT-PCR is performed in two separate reactions: First, total RNA is reverse-transcribed into cDNA, then the cDNA is amplified by PCR. This method is useful for detecting multiple transcripts from a single cDNA template or for storing cDNA aliquots for later use. The AmpErase® UNG enzyme can be used to prevent carryover contamination.

About the example experiment

To illustrate how to perform a Relative Standard Curve, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with the ViiA™ 7 System.

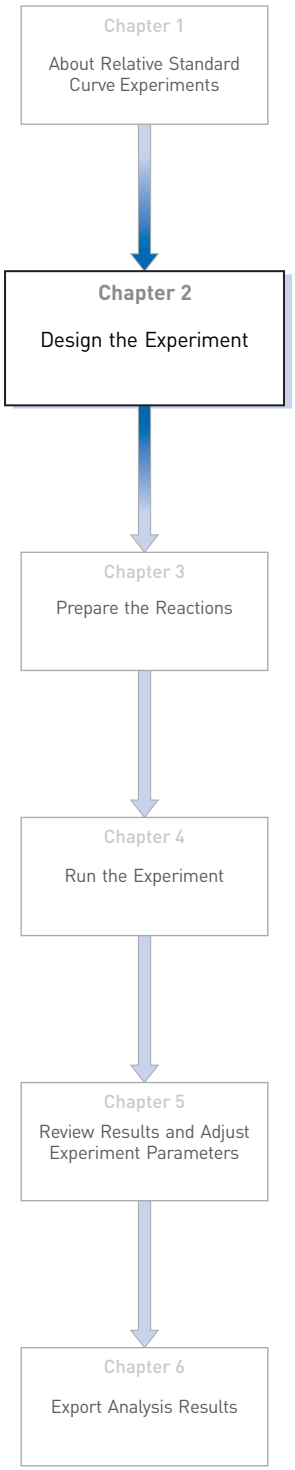
The objective of the Relative Standard Curve example experiment is to compare the expression of the FAS transcriptional factor (an oncoprotein that activates the transcription of growth-associated genes) in Human cDNA tissues.

In the Relative Standard Curve example experiment:

- The samples are kidney, liver, brain, and heart.
- The target is FAS.
- The endogenous control is HPRT.

- The reference sample is brain.
- One standard curve is set up for FAS. The standard used for the standard dilution series is a Human cDNA sample of known total concentration.
- One standard curve is set up for HPRT (endogenous control). The standard used for the standard dilution series is a Human Male Raji cDNA sample of known total concentration.
- Reactions are set up for **2-step RT-PCR**. The Invitrogen VILO Kit is used for reverse transcription; the TaqMan® Gene Expression Master Mix (2×) is used for PCR.
- Select primer and probe sets from the Applied Biosystems TaqMan® Gene Expression Assays product line:
  - For the target assay (FAS), select assay ID Hs00907759_m1.
  - For the endogenous control assay (HPRT), select assay ID Hs99999909_m1.

2 Design the Experiment



This chapter explains how to design the example experiment from the Experiment Setup menu.

This chapter covers:

- Define the experiment properties. 16
- Define targets, samples, and biological replicates 17
- Assign targets, samples, and biological groups 19
- Set up the run method 21
- Order materials for the experiment 22
- Tips for designing your own experiment 22
- For more information. 23

Note: To automatically export the analyzed data to a specified location, select the Auto Export task from the Export screen, before running the experiment. For more information on Auto Export, refer to Chapter 1 in Booklet 1, Getting Started with ViiA™ 7 System Experiments.

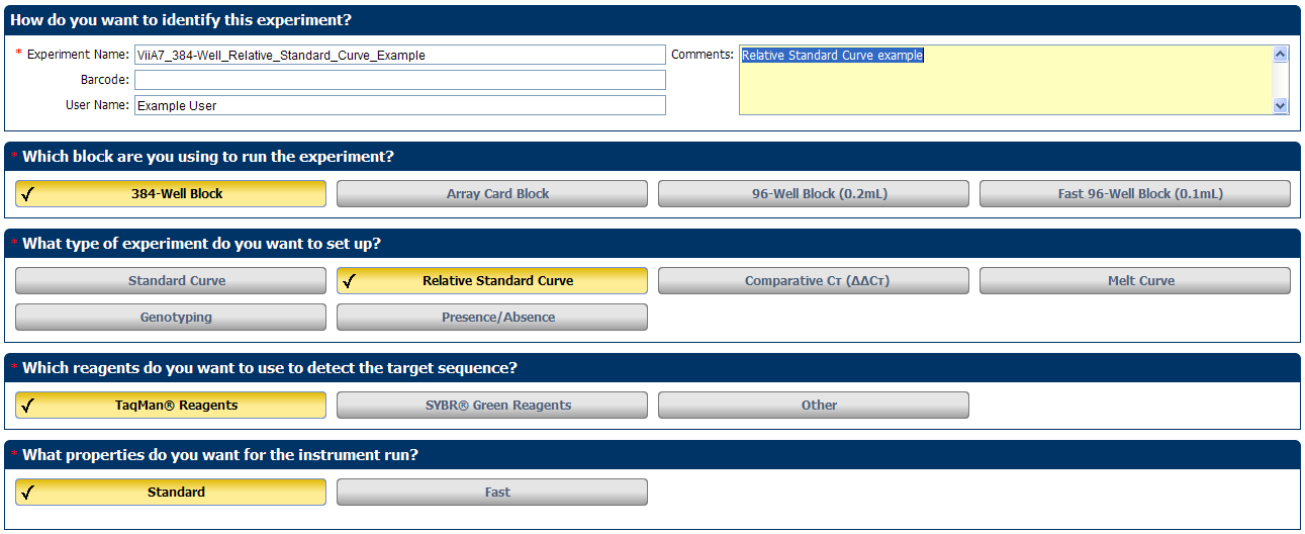
Define the experiment properties

Click **Experiment Setup** ▸ **Experiment Properties** to create a new experiment in the ViiA™ 7 Software. Enter:

Field or Selection	Entry
Experiment Name	ViiA7_384-Well_Relative_Standard_Curve_Example
Barcode	Leave field empty
User Name	Example User
Comments	Relative Standard Curve example
Block	384-Well Block
Experiment Type	Relative Standard Curve
Reagents	TaqMan® Reagents
Ramp speed	Standard

Save the experiment.

Your Experiment Properties screen should look like this:



Define targets, samples, and biological replicates

Click **Define** to access the Define screen. Enter:

1. Targets

Target name	Reporter	Quencher	Color
FAS	FAM	NFQ-MGB	
HPRT	VIC	NFQ-MGB	

2. Samples

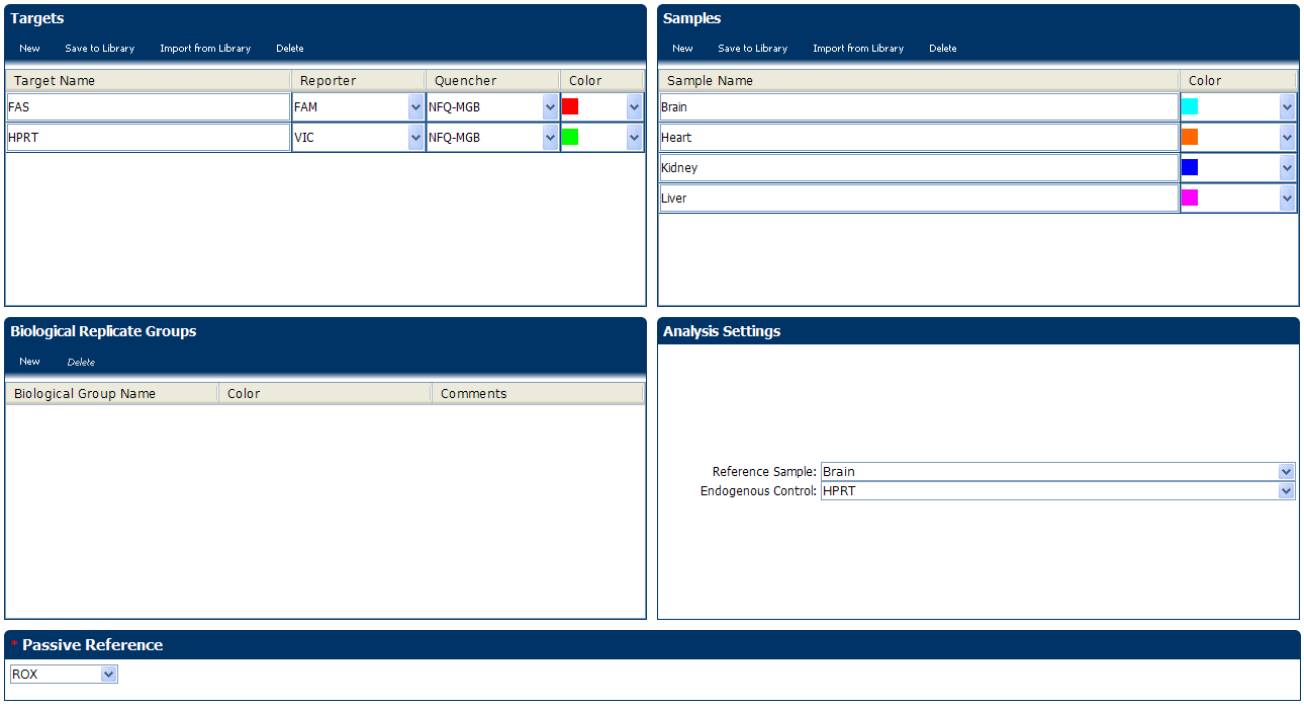
Sample name	Color
Brain	
Heart	
Kidney	
Liver	

3. Dye to be used as a Passive Reference
   ROX

4. Analysis Settings

Field	Select
Reference Sample	Brain
Endogenous Control	HPRT

Your Define screen should look like this:



Note: This example experiment does not define biological replicate groups. Leave Biological Replicate Groups blank.

Assign targets, samples, and biological groups

Click **Assign** to access the Assign screen.

Note: To automatically set up and assign standards, click

to open the Define and Set Up Standards dialog box.

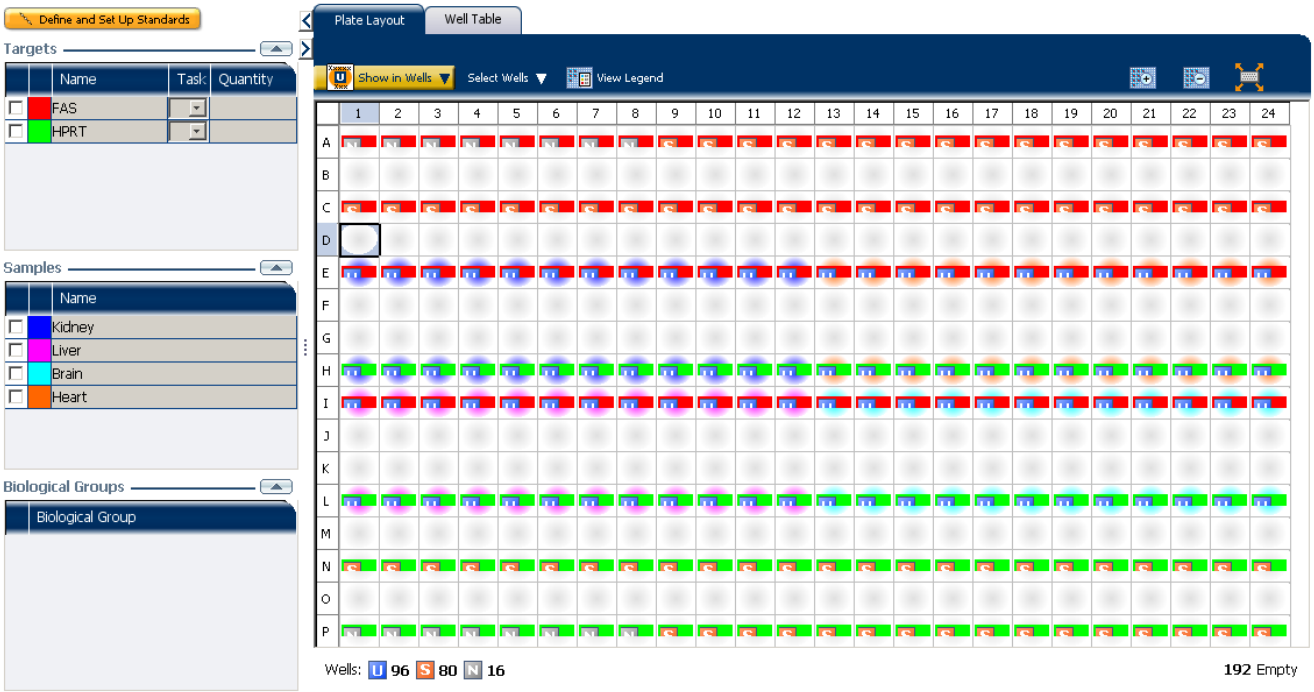
To assign the targets and samples:

1. Set up the standards.
2. For the first standard for the FAS target:
   a. Click-drag to select wells A9-A16.
   b. Check check box next to FAS in the Targets list.
   c. Select S in the Task drop-down menu.
   d. Enter 625 in the Quantity column.
   e. Repeat steps a through c for each of the standards for the FAS target, selecting the wells listed in the table below, and entering the corresponding quantity.
3. Repeat step 2 for each standard for the HPRT target.

Target name	Well number	Task	Quantity	Sample name
FAS	A1 - A8	Negative	None	None
	E1 - E12	Unknown	Determined by run	Kidney
	E13 - E24	Unknown	Determined by run	Heart
	I1 - 112	Unknown	Determined by run	Liver
	I13 - I24	Unknown	Determined by run	Brain
	A9 - A16	Standard	625	None
	A17 - A24	Standard	2,500	None
	C1 - C8	Standard	312.50	None
	C9 - C16	Standard	1,250	None
	C17 - C24	Standard	5,000	None

Target name	Well number	Task	Quantity	Sample name
HPRT	P1 - P8	Negative	None	None
	H1 - H12	Unknown	Determined by run	Kidney
	H13 - H 24	Unknown	Determined by run	Heart
	L1 - L12	Unknown	Determined by run	Liver
	L13 - L24	Unknown	Determined by run	Brain
	N1 - N8	Standard	165.25	None
	N9 - N16	Standard	625	None
	N17 - N24	Standard	2,500	None
	P9 - P16	Standard	312.50	None
	P17 - P24	Standard	1,250	None

Your Assign screen should look like this:



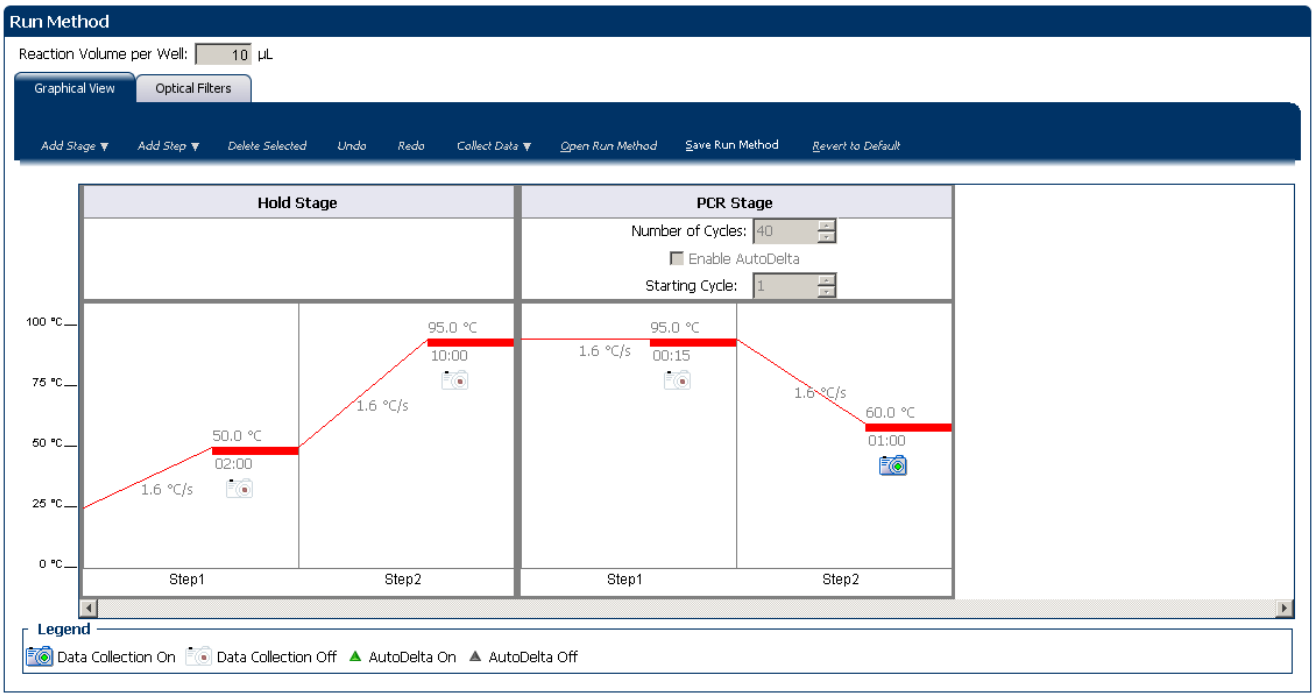
Set up the run method

Click **Run Method** to access the Run Method screen. Set the thermal profile under the Graphical View tab. Enter:

- Reaction Volume Per Well: 10µL
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Hold Stage	Step 1	1.6 °C/s	50 °C	2 minutes
	Step 2	1.6 °C/s	95 °C	10 minutes
PCR Stage	Step 1	1.6 °C/s	95 °C	15 seconds
Number of Cycles: 40 Enable AutoDelta: Unchecked (default) Starting Cycle: Disabled when Enable AutoDelta is unchecked	Step 2	1.6 °C/s	60 °C	1 minute

Your Run Method screen should look like this:

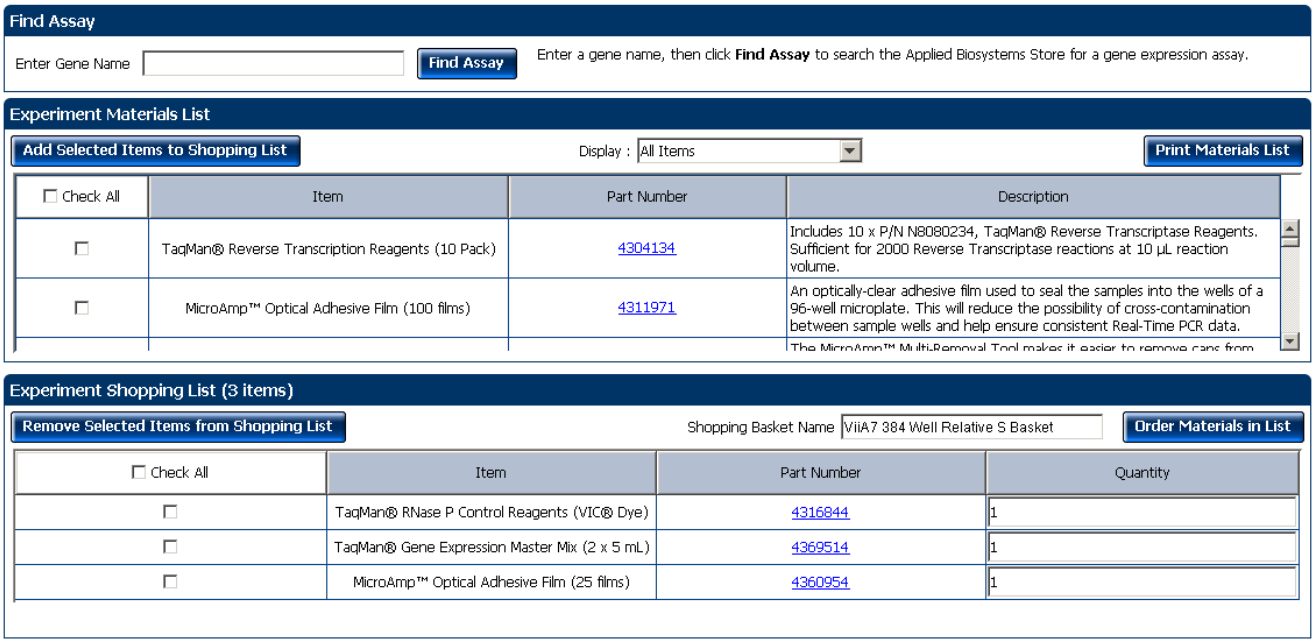


Order materials for the experiment

The recommended materials are:

- MicroAmp® Optical 384-Well Reaction Plate
- MicroAmp® Optical Adhesive Film
- TaqMan® Gene Expression Master Mix (2X)
- FAS target assay
- HPRT target assay

Your Materials List screen should look like this:



Tips for designing your own experiment

Applied Biosystems recommends that you:

- Set up a standard curve for each target assay in the reaction plate.
- Identify each target assay with a unique name and color. You can enter up to 100 characters in the Target Name field.
- Identify each sample using a unique name and color. You can enter up to 100 characters in the Sample Name field.
- Select an endogenous control for each sample. The endogenous control is a target that is present in all samples under investigation. It should be expressed equally in all sample types, regardless of treatment or tissue origin (examples of endogenous controls are β-actin, GAPDH, and 18S ribosomal RNA [18S rRNA]). The endogenous control is used to normalize the PCR results; the endogenous control corrects for variable sample mass, nucleic acid extraction efficiency, reverse transcription efficiency, and pipette calibration errors.

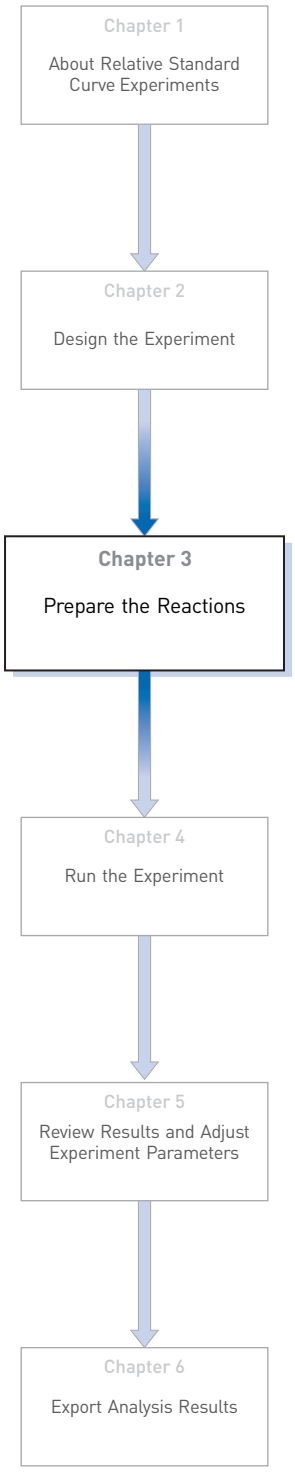
  Note: Each sample type (for example, each tissue in a study comparing multiple tissues) requires an endogenous control.

- Enter at least five dilution points for each standard curve in the reaction plate.
- Enter at least three replicates (identical reactions) for each point in the standard curve and for each sample reaction.
- Enter at least three negative control reactions for each target assay.
- Carefully consider the appropriate range of standard quantities for your assay because the range of standard quantities affects the amplification efficiency calculations:
  - For more accurate measurements of amplification efficiency, use a broad range of standard quantities, spanning between 5 and 6 logs. If you do so, use a PCR product or a highly concentrated template, such as a cDNA clone.
  - If you have a limited amount of cDNA template and/or if the target is a low-copy number transcript, or known to fall within a given range, a narrow range of standard quantities may be necessary.
- Minimally run a five-point curve of 1:10 dilutions to minimize the effects of small pipetting errors.
- Select a reference sample from your previously defined samples. Amplification results from the samples and from the reference sample are compared to determine relative expression.
- Select an endogenous control from your previously defined target assays. Amplification results from the endogenous control are used to normalize the amplification results from the target for differences in the amount of template added to each reaction.

For more information

For more information on...	Refer to...	Part number
Consumables	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments* Appendix A in Booklet 7, *ViiA™ 7 System Experiments - Appendixes*	4441434
Using other quantification methods	Booklet 2, *Running Standard Curve Experiments* and Part 2 of Booklet 3, *Running Relative Standard Curve and Comparative C_T Experiments*.	4441434
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*	127AP05-03
Using Alternative Setup	Chapter 2 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

3 Prepare the Reactions



This chapter explains how to prepare the PCR reactions for the Relative Standard Curve example experiment.

This chapter covers:

- Assemble required materials 26
- Prepare the template 26
- Prepare the sample dilutions 26
- Prepare the standard dilution series for FAS and HPRT assays 27
- Prepare the reaction mix (“cocktail mix”). 28
- Prepare the reaction plate 29
- Tips for preparing reactions for your own experiments. 32
- For more information. 32

Assemble required materials

- Items listed in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*
- Samples - Total RNA isolated from kidney, heart, liver, and brain tissues.
- Example experiment reaction mix components:
  - TaqMan® Gene Expression Master Mix (2×)
  - FAS Assay Mix (20×)
  - HPRT Assay Mix (20×)

Prepare the template

Prepare the template for the PCR reactions (both samples and standards) using one of the Invitrogen VILO kits, SuperScript® VILO™ cDNA Synthesis Kit (PN 4453650).

Example experiment settings

For the Relative Standard Curve example experiment, the template for the PCR reactions is cDNA reverse-transcribed from total RNA samples using the Invitrogen VILO kits.

To prepare the template

Use the Invitrogen VILO kits to reverse-transcribe cDNA from the total RNA samples. Follow the procedures in the *Invitrogen VILO Kits Protocol* (PN 100002284) to:

1. Isolate total RNA from cells using an Ambion® sample preparation kit appropriate to the tissue or cell type.
2. Quantify and perform quality control on the RNA.
3. Convert the RNA to cDNA via reverse transcription.

Prepare the sample dilutions

For the Relative Standard Curve example experiment, no more than 10% of your reaction should consist of the undiluted cDNA.

1. Label a separate microcentrifuge tube for each diluted sample:
   - Kidney
   - Heart
   - Liver
   - Brain

2. Add the required volume of water (diluent) to each empty tube:

Tube	Sample name	Diluent volume (µL)
1	Kidney	76
2	Heart	76
3	Liver	76
4	Brain	76

3. Add the required volume of sample stock (100 ng/µL) to each empty tube:

Tube	Sample name	Diluent volume (µL)
1	Kidney	4
2	Heart	4
3	Liver	4
4	Brain	4

4. Vortex each diluted sample for 3 to 5 seconds, then centrifuge the tubes briefly.

5. Place the diluted samples on ice until you prepare the reaction plate.

Prepare the standard dilution series for FAS and HPRT assays

The same standard materials are used to prepare the exact same dilutions for both the target genes. The prepared standards are then used to generate the two standard curves.

- The stock concentration for cDNA is 100 ng/µL.
- The volumes calculated for both the FAS and HPRT assays are:

Standard name (labeled tube)	Dilution point	Source	Source volume (µL)	Diluent volume (µL)	Total volume (µL)	Standard concentration (ng/µL)
Std. 1	1	Stock	20	20	40	100
Std. 2	2	Dilution 1	20	20	40	50
Std. 3	3	Dilution	20	20	40	25
Std. 4	4	Dilution 3	20	20	40	12.5
Std. 5	5	Dilution 4	20	20	40	6.25

1. Label ten separate microcentrifuge tubes for each diluted standard:
   - FAS (FAS Std. 1 - FAS Std. 5)
   - HPRT (HPRT Std. 1 - HPRT Std. 5)

2. Prepare five standard dilutions each for FAS and HPRT:

   Note: For dilution 1, first vortex the stock for 3 to 5 seconds, then centrifuge both the Std. 1 tubes briefly before pipetting 10 µL stock into each Std. 1 tube.

3. For each subsequent dilution, add source to the standard:
   a. Use a new pipette tip to add 10 µL of source to the FAS and HPRT tubes containing the standard.
   b. Vortex the tubes for 3 to 5 seconds, then centrifuge the tubes briefly.

4. Place the standards on ice until you prepare the reaction plate.

Prepare the reaction mix ("cocktail mix")

1. Label an appropriately sized tube for each reaction mix:
   - FAS Reaction Mix
   - HPRT Reaction Mix

2. For the FAS assay, add the required volumes of each component to the FAS Reaction Mix tube:

Component	Volume (µL) for 1 reaction	Volume (µL) for 96 reactions (plus 10% excess)
TaqMan® Gene Expression Master Mix (2X)	5	530
FAS Assay Mix (20X)	0.5	53
Water	3.5	371
Total Reaction Mix Volume	9	954

3. For the HPRT assay, add the required volumes of each component to the HPRT Reaction Mix tube:

Component	Volume (µL) for 1 reaction	Volume (µL) for 96 reactions (plus 10% excess)
TaqMan® Gene Expression Master Mix (2X.)	5	530
HPRT Assay Mix (20X	0.5	53
Water	3.5	371
Total Reaction Mix Volume	9	954

4. Mix the reaction in each tube by gently pipetting up and down, then cap each tube.

5. Centrifuge the tubes briefly to remove air bubbles.

6. Place the reaction mixes on ice until you prepare the reaction plate.

   Note: Do not add the sample or standard at this time.

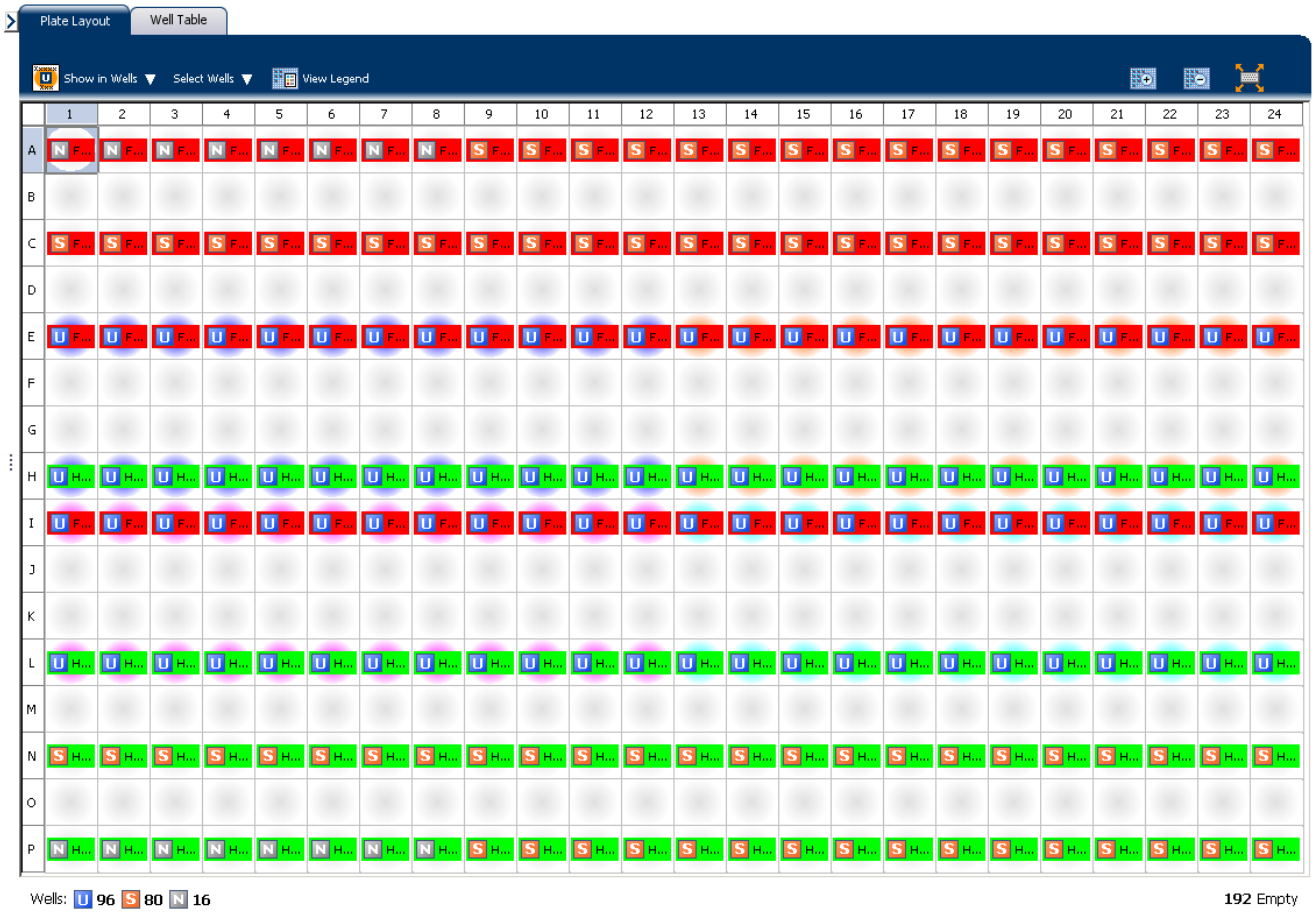
Prepare the reaction plate

Example experiment reaction plate components

The reaction plate for the Relative Standard Curve example experiment contains:

- A MicroAmp® Optical 384-Well Reaction Plate
- Reaction volume: 10µL/well
- 96 Unknown wells U
- 80 Standard wells S
- 16 Negative control wells N
- 192 Empty wells

The plate layout for the example experiment looks like this:



To prepare the reaction plate components

1. For each target, prepare the negative control reactions:
   a. To an appropriately sized tube, add the volumes of reaction mix and water listed below.

Tube	Reaction mix	Reaction mix volume (µL)	Water volume (µL)
1	FAS Reaction Mix	79.2	8.8
2	HPRT Reaction Mix	79.2	8.8

   b. Mix the reaction by gently pipetting up and down, then cap the tube.
   c. Centrifuge the tube briefly to remove air bubbles.
   d. Add 10 µL of the negative control reaction to the appropriate wells in the reaction plate.

2. For each replicate group, prepare the standard reactions:
   a. To appropriately sized tubes, add the volumes of reaction mix and standard listed below.

Tube	Standard rection	Reaction mix	Reaction mix voulme (µL)	Standard	Standard volume (µL)
1	FAS Std. 1	FAS Reaction Mix	79.2	FAS Std. 1	8.8
2	FAS Std. 2	FAS Reaction Mix	79.2	FAS Std. 2	8.8
3	FAS Std. 3	FAS Reaction Mix	79.2	FAS Std. 3	8.8
4	FAS Std. 4	FAS Reaction Mix	79.2	FAS Std. 4	8.8
5	FAS Std. 5	FAS Reaction Mix	79.2	FAS Std. 5	8.8
6	HPRT Std. 1	HPRT Reaction Mix	79.2	HPRT Std. 1	8.8
7	HPRT Std. 2	HPRT Reaction Mix	79.2	HPRT Std. 2	8.8
8	HPRT Std. 3	HPRT Reaction Mix	79.2	HPRT Std. 3	8.8
9	HPRT Std. 4	HPRT Reaction Mix	79.2	HPRT Std. 4	8.8
10	HPRT Std. 5	HPRT Reaction Mix	79.2	HPRT Std. 5	8.8

   b. Mix the reactions by gently pipetting up and down, then cap the tubes.
   c. Centrifuge the tubes briefly to remove air bubbles.

d. Add 10 µL of the standard reaction to the appropriate wells in the reaction plate.

3. For each replicate group, prepare the reactions for the unknowns:

   a. To appropriately sized tubes, add the volumes of reaction mix and sample listed below:

Tube	Unknown reaction	Reaction mix	Reaction mix volume (µL)	Sample	Sample volume (µL)
1	FAS Kidney	FAS Reaction Mix	118.8	Kidney	13.2
2	FAS Heart	FAS Reaction Mix	118.8	Heart	13.2
3	FAS Liver	FAS Reaction Mix	118.8	Liver	13.2
4	FAS Brain	FAS Reaction Mix	118.8	Brain	13.2
5	HPRT Kidney	HPRT Reaction Mix	118.8	Kidney	13.2
6	HPRT Heart	HPRT Reaction Mix	118.8	Heart	13.2
7	HPRT Liver	HPRT Reaction Mix	118.8	Liver	13.2
8	HPRT Brain	HPRT Reaction Mix	118.8	Brain	13.2

   b. Mix the reactions by gently pipetting up and down, then cap the tubes.

   c. Centrifuge the tubes briefly to remove air bubbles.

   d. Add 10 µL of the unknown (sample) reaction to the appropriate wells in the reaction plate.

4. Seal the reaction plate with optical adhesive film.

5. Centrifuge the reaction plate briefly to remove air bubbles.

6. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.

7. Until you are ready to perform the run, place the reaction plate at 4 °C, in the dark.

Tips for preparing reactions for your own experiments

Tips for preparing templates

When you prepare your own Relative Standard Curve experiment, Applied Biosystems recommends the following templates:

- **Complementary DNA (cDNA)** – cDNA reverse-transcribed from total RNA samples.
- **Genomic DNA (gDNA)** – Purified gDNA already extracted from tissue or sample

Tips for preparing sample dilutions

When you prepare your own Relative Standard Curve experiment, for optimal performance of TaqMan® Gene Expression Assays or Custom TaqMan® Gene Expression Assays, use 10 to 100 ng of cDNA template per 10µL reaction.

Tips for preparing the reaction mix

If your experiment includes more than one target assay, prepare the reaction mix for each target assay separately.

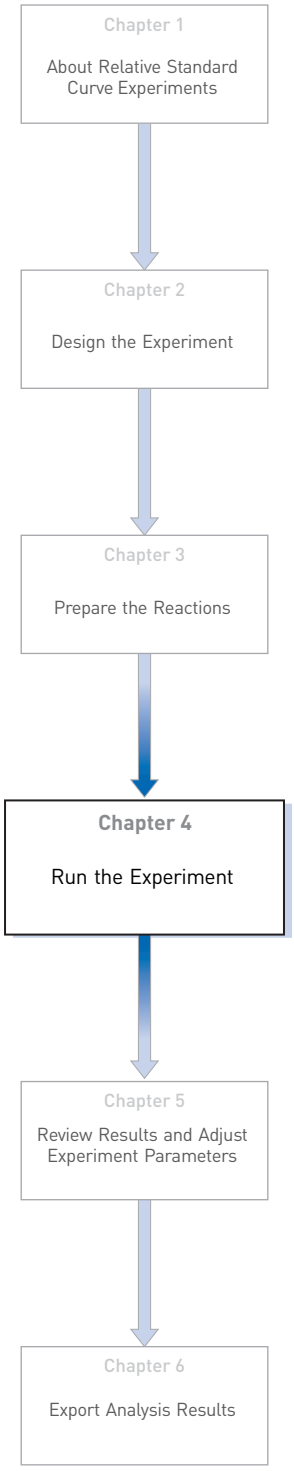
Tips for preparing the reaction plate

When you prepare your own Relative Standard Curve experiment, make sure the arrangement of the PCR reactions matches the plate layout displayed in the ViiA™ 7 Software.

For more information

For more information on...	Refer to...	Part number
Assigning the reaction plate components	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

4 Run the Experiment



This chapter explains how to run the example experiment on the ViiA™ 7 Instrument.

This chapter covers:

- Start the run 34
- Monitor the run 34

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the ViiA™ 7 Instrument is in operation.

Start the run

1. Open the Relative Standard Curve example file that you created using instructions in Chapter 2.
2. Load the reaction plate into the instrument.
3. Start the run.

Monitor the run

Monitor the example experiment run:

- From the ViiA™ 7 Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the ViiA™ 7 Software (to monitor an experiment started from another computer or from the ViiA™ 7 Instrument touchscreen).
- From the ViiA™ 7 Instrument touchscreen.

From the Instrument Console of the ViiA™ 7 Software

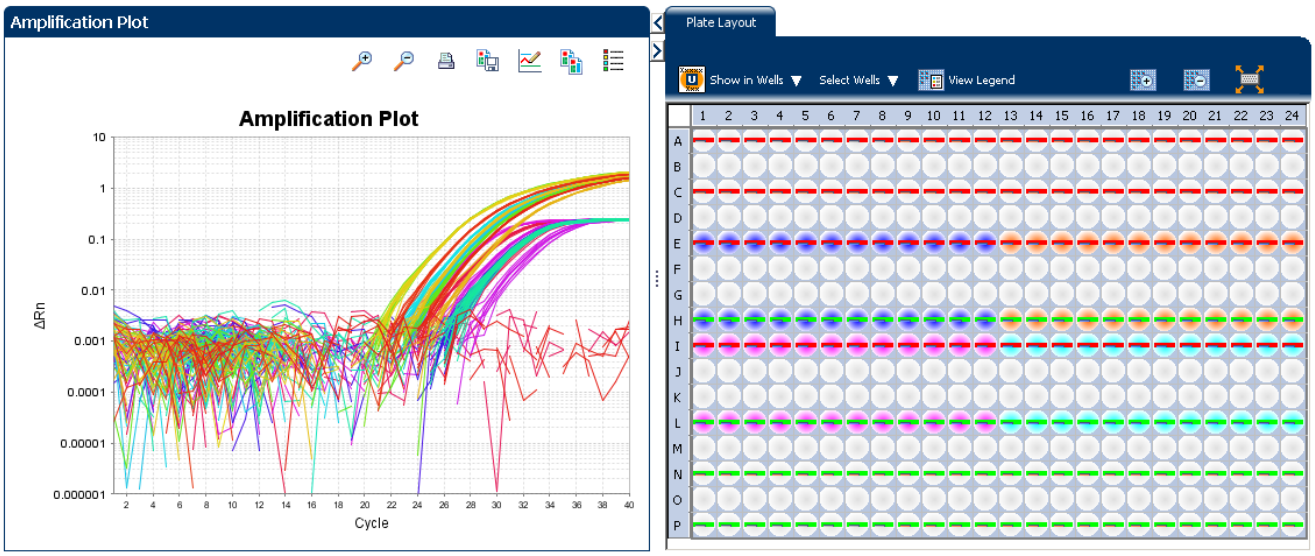
1. In the Instrument Console screen, select the instrument icon.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. In the Manage Instrument screen, click **Monitor Running Experiment** to access the Run screen.

View the Amplification Plot

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the ViiA™ 7 Software for potential problems.

To view data in the Amplification Plot screen, click **Amplification Plot** from the Run Experiment Menu, and select the Plate Layout tab, then select the wells that you want to view.

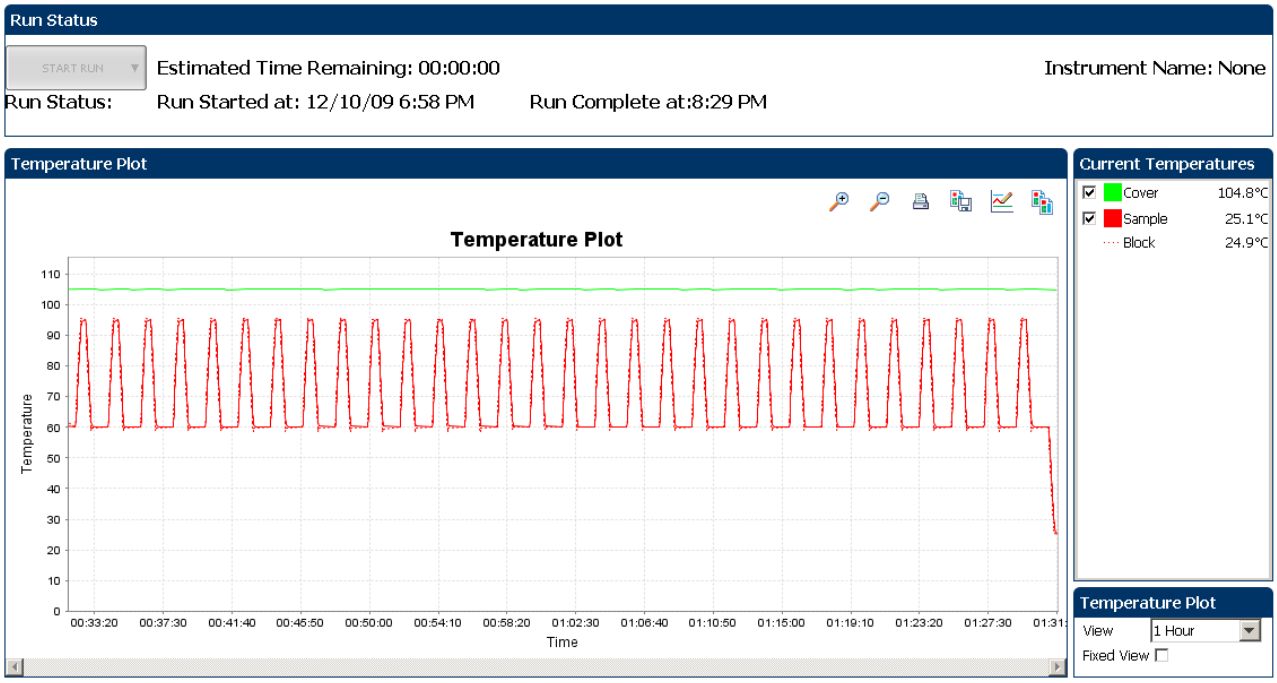
The figure below shows the Amplification Plot screen as it appears at the end of the example experiment.



View the Temperature Plot

To view data in the Temperature Plot screen, click **Temperature Plot** from the Run Experiment Menu.

The figure below shows the Temperature Plot screen as it appears during the example experiment.

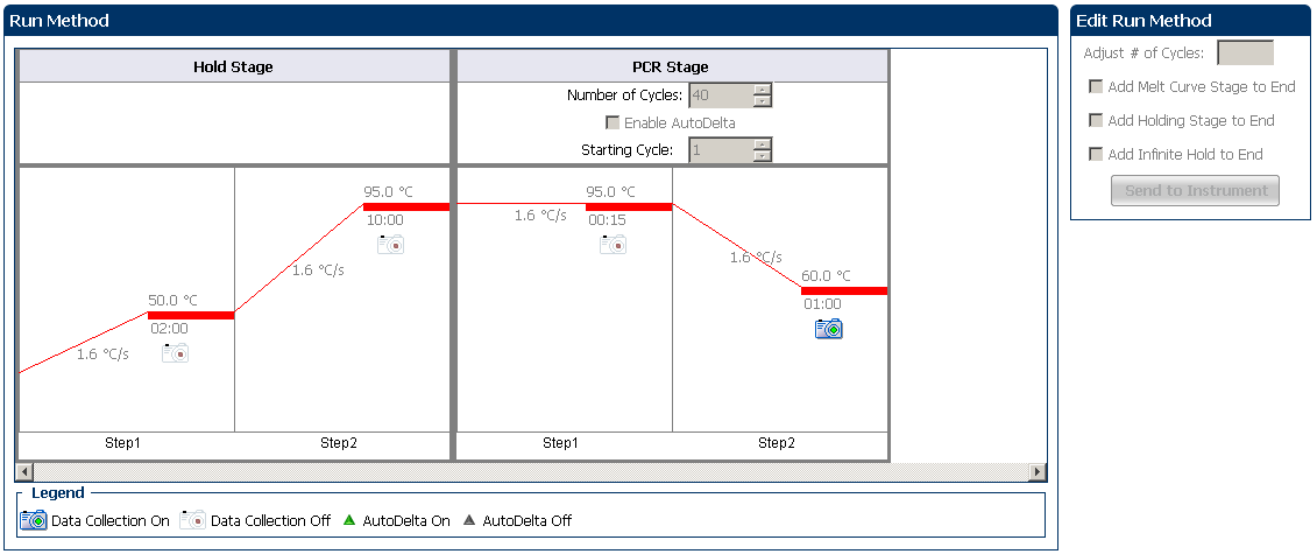


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

View the Run Method

To view data in the Run Method screen, click **Run Method** from the Run Experiment Menu.

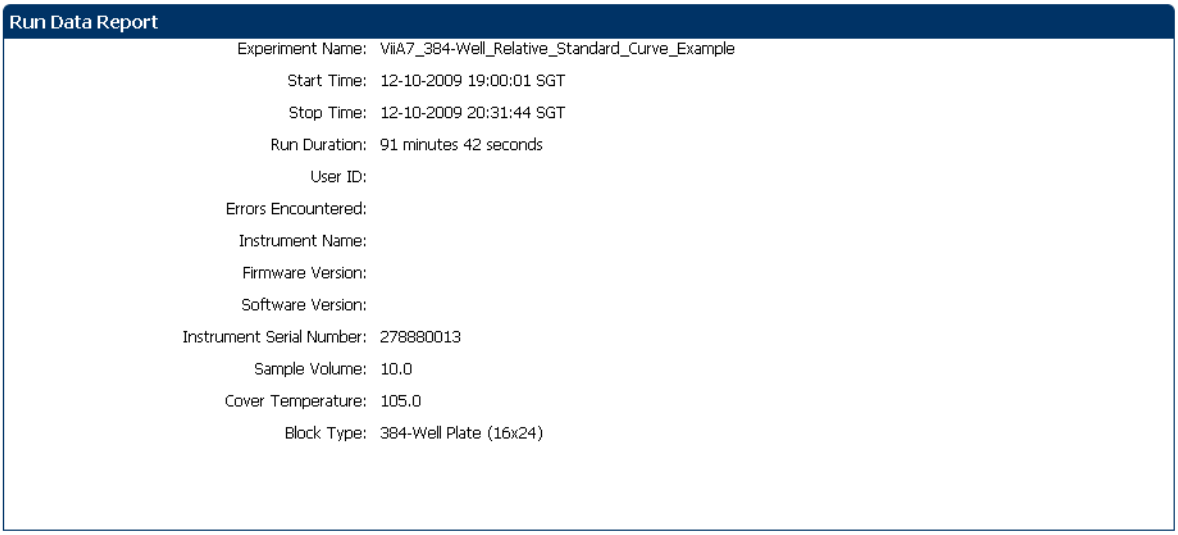
The figure below shows the Run Method screen as it appears in the example experiment.



View run data

To view the run data, click **View Run Data** from the Run Experiment Menu.

Your View Run Data screen should look like this:

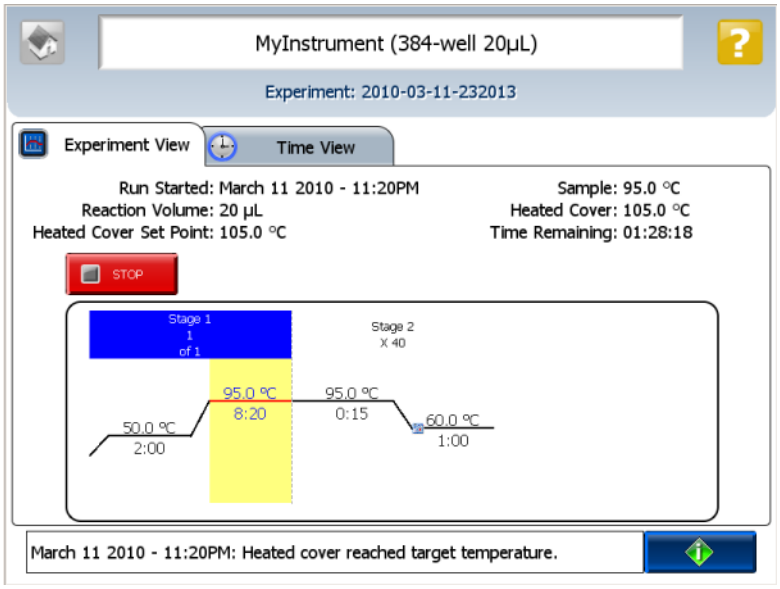


From the ViiA™ 7 Instrument touchscreen

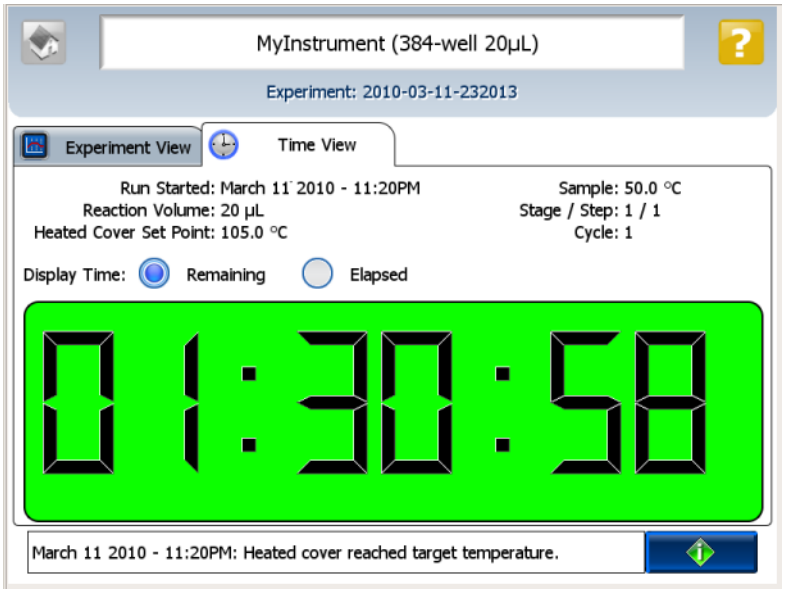
You can also view the progress of the run from the touchscreen of the ViiA™ 7 Instrument.

The Run Method screen on the **ViiA™ 7 Instrument** touchscreen looks like this:

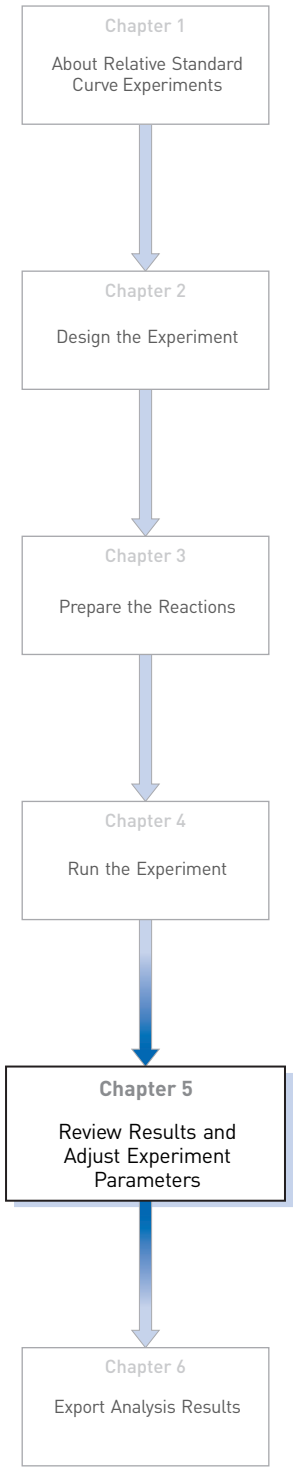
Experiment view



Time view



5 Review Results and Adjust Experiment Parameters



In Section 5.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 5.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

- **Section 5.1 Review Results 41**
- Analyze the example experiment. 41
- View the Standard Curve Plot 41
- Assess amplification results using the Amplification Plot. 44
- Assess the gene expression profile using the Gene Expression Plot. 51
- Identify well problems using the Well Table 53
- Confirm accurate dye signal using the Multicomponent Plot. 55
- Determine signal accuracy using the Raw Data Plot 57
- View the endogenous control profile using the QC Plot 59
- Review the QC flags in the QC Summary 60
- For more information. 62
- **Section 5.2 Adjust parameters for re-analysis of your own experiments 63**
- Adjust analysis settings 63
- Improve C_T precision by omitting wells. 68
- For more information. 69

5.1

Section 5.1 Review Results

Analyze the example experiment

1. Open the example experiment file that you ran in Chapter 4.
2. Click **Analyze**. The software analyzes the data using the default analysis settings. You can also access the experiment to analyze from the Home screen.

View the Standard Curve Plot

The Standard Curve Plot screen displays the standard curve for samples designated as standards. The ViiA™ 7 Software calculates the quantity of an unknown target from the standard curve.

Example experiment standard curve values

In the standard curve example experiment, you review the Standard Curve Plot screen for the following regression coefficient values:

- Slope/amplification efficiency
- R^2 value (correlation coefficient)
- C_T values

To view and assess the Standard Curve plot

1. From the Experiment Menu pane, select **Analysis ▸ Standard Curve**.

   Note: If no data are displayed, click **Analyze**.

2. Display all 384 wells in the Standard Curve Plot screen by clicking the upper left corner of the plate layout in the Plate Layout tab.
3. Enter the Plot Settings:

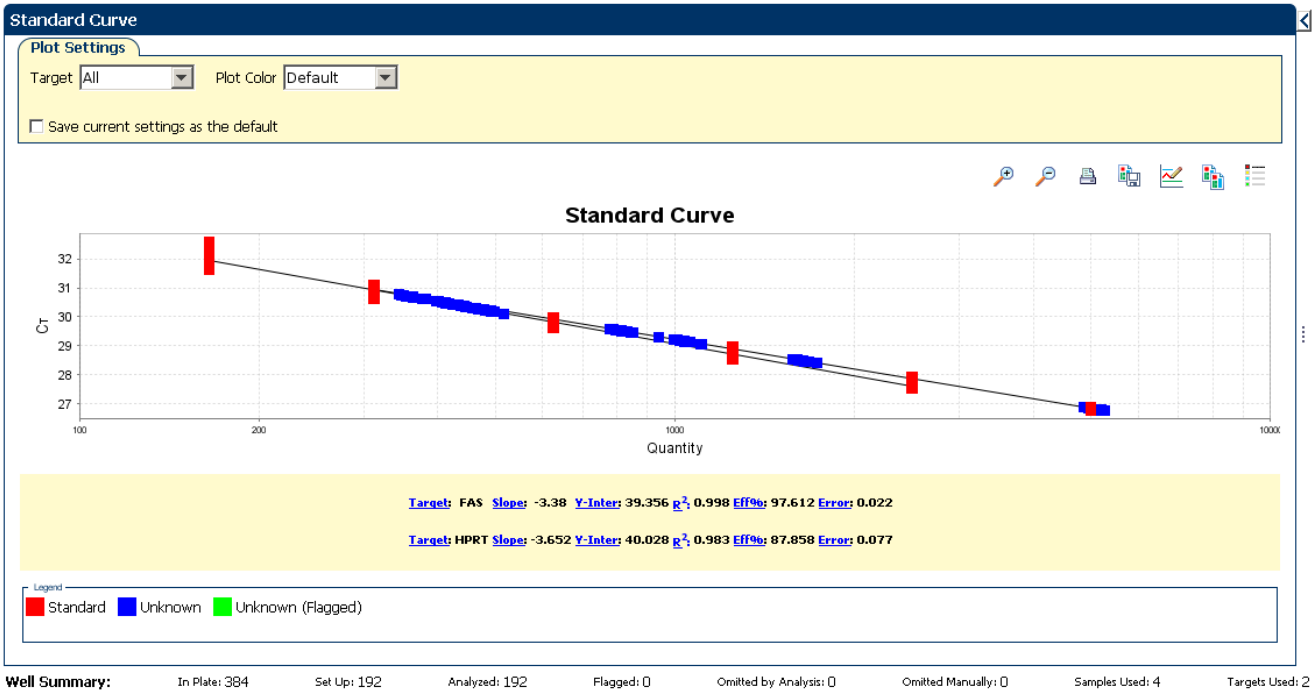
Menu	Selection
Target	All
Plot Color	Default

Menu	Selection
(This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.)	Check (default)

4. View the values displayed below the standard curve. In the example experiment, the values for each target fall within the acceptable ranges:

Target	Slope	R^2 Value	Amplification efficiency (Eff%)
FAS	–3.38	0.998	97.612
HPRT	–3.652	0.983	87.858

5. Check that all samples are within the standard curve. In the example experiment, all samples (blue dots) are within the standard curve (red dots).



6. Check the C_T values:

7. Click the **Well Table** tab.

8. From the Group By drop-down menu, select **Replicate**.

9. Look at the values in the C_T column. In the example experiment, the C_T values fall within the expected range (>8 and <35).

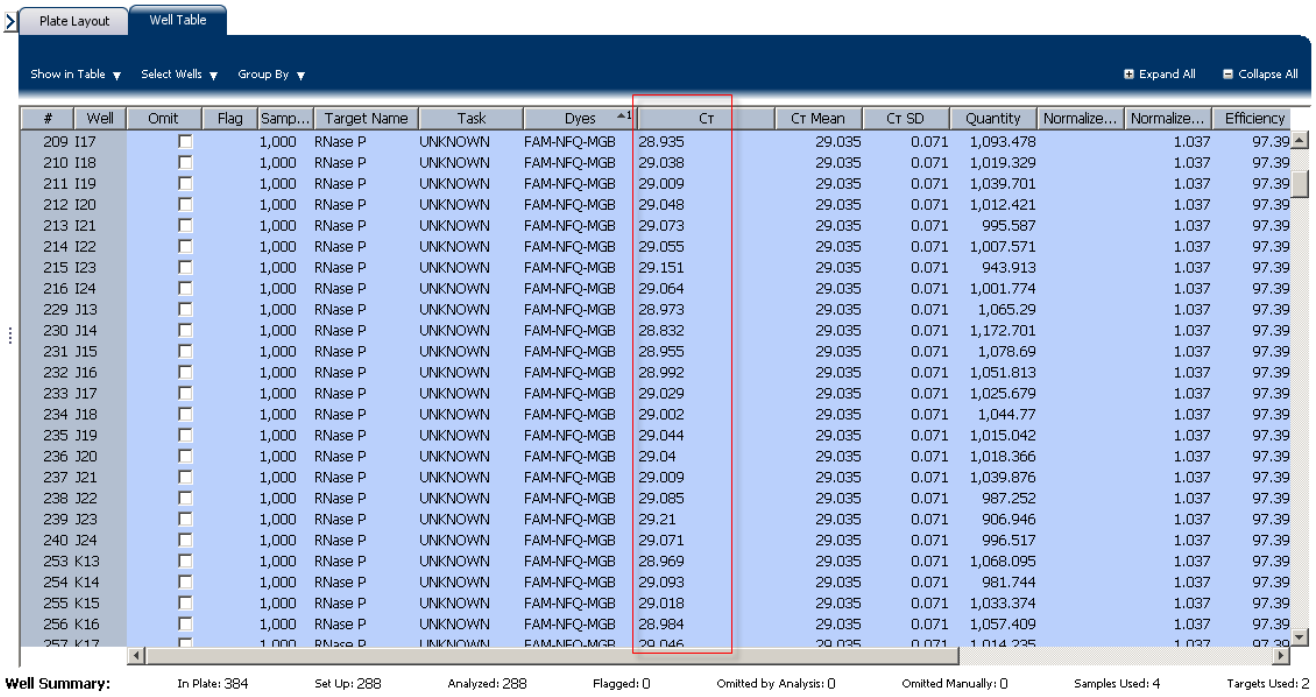


Plate Layout | Well Table

Show in Table | Select Wells | Group By | Expand All | Collapse All

#	Well	Omit	Flag	Samp...	Target Name	Task	Dyes	CT	CT Mean	CT SD	Quantity	Normalize...	Normalize...	Efficiency
209	I17			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.935	29.035	0.071	1,093.478		1.037	97.39
210	I18			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.038	29.035	0.071	1,019.329		1.037	97.39
211	I19			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.009	29.035	0.071	1,039.701		1.037	97.39
212	I20			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.048	29.035	0.071	1,012.421		1.037	97.39
213	I21			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.073	29.035	0.071	995.587		1.037	97.39
214	I22			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.055	29.035	0.071	1,007.571		1.037	97.39
215	I23			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.151	29.035	0.071	943.913		1.037	97.39
216	I24			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.064	29.035	0.071	1,001.774		1.037	97.39
229	J13			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.973	29.035	0.071	1,065.29		1.037	97.39
230	J14			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.832	29.035	0.071	1,172.701		1.037	97.39
231	J15			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.955	29.035	0.071	1,078.69		1.037	97.39
232	J16			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.992	29.035	0.071	1,051.813		1.037	97.39
233	J17			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.029	29.035	0.071	1,025.679		1.037	97.39
234	J18			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.002	29.035	0.071	1,044.77		1.037	97.39
235	J19			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.044	29.035	0.071	1,015.042		1.037	97.39
236	J20			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.04	29.035	0.071	1,018.366		1.037	97.39
237	J21			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.009	29.035	0.071	1,039.876		1.037	97.39
238	J22			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.085	29.035	0.071	987.252		1.037	97.39
239	J23			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.21	29.035	0.071	906.946		1.037	97.39
240	J24			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.071	29.035	0.071	996.517		1.037	97.39
253	K13			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.969	29.035	0.071	1,068.095		1.037	97.39
254	K14			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.093	29.035	0.071	981.744		1.037	97.39
255	K15			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.018	29.035	0.071	1,033.374		1.037	97.39
256	K16			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.984	29.035	0.071	1,057.409		1.037	97.39
257	K17			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.046	29.035	0.071	1,014.235		1.037	97.39

Well Summary: In Plate: 384 Set Up: 288 Analyzed: 288 Flagged: 0 Omitted by Analysis: 0 Omitted Manually: 0 Samples Used: 4 Targets Used: 2

Tips for analyzing your own experiments

When you analyze your own Relative Standard Curve experiment, look for:

- **Slope/amplification efficiency values** – The amplification efficiency is calculated using the slope of the regression line in the standard curve. A slope close to –3.3 indicates optimal, 100% PCR amplification efficiency. Factors that affect amplification efficiency:
  - Range of standard quantities – For more accurate and precise efficiency measurements, use a broad range of standard quantities, 5 to 6 logs (10^5 to 10^6 fold).
  - Number of standard replicates – For more accurate efficiency measurements, include replicates to decrease the effects of pipetting inaccuracies.
  - PCR inhibitors – PCR inhibitors in the reaction can alter amplification efficiency.
- **R^2 values (correlation coefficient)** – The R^2 value is a measure of the closeness of fit between the regression line and the individual C_T data points of the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points. An R^2 value >0.99 is desirable.
- **C_T values** – The threshold cycle (C_T) is the PCR cycle number at which the fluorescence level meets the threshold.
  - A C_T value >8 and <35 is desirable.
  - A C_T value <8 indicates that there is too much template in the reaction.
  - A C_T value >35 indicates a low amount of target in the reaction; for C_T values >35, expect a higher standard deviation.

If your experiment does not meet the guidelines above, troubleshoot as follows:

- Omit wells (see "Improve C_T precision by omitting wells" on page 68).
  Or
- Rerun the experiment.

Assess amplification results using the Amplification Plot

Amplification plots available for viewing

The Amplification Plot screen displays amplification of all samples in the selected wells. There are three plots available:

- **ΔRn vs Cycle** – ΔRn is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. This plot displays ΔRn as a function of cycle number. Use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle** – Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays Rn as a function of cycle number. Use this plot to identify and examine irregular amplification.
- **C_T vs Well** – C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. Use this plot to locate outlying amplification (outliers).

Each plot can be viewed as a linear or log10 graph type.

Purpose

The purpose of viewing the amplification plot for the example experiment is to identify:

- Correct baseline and threshold values
- Outliers

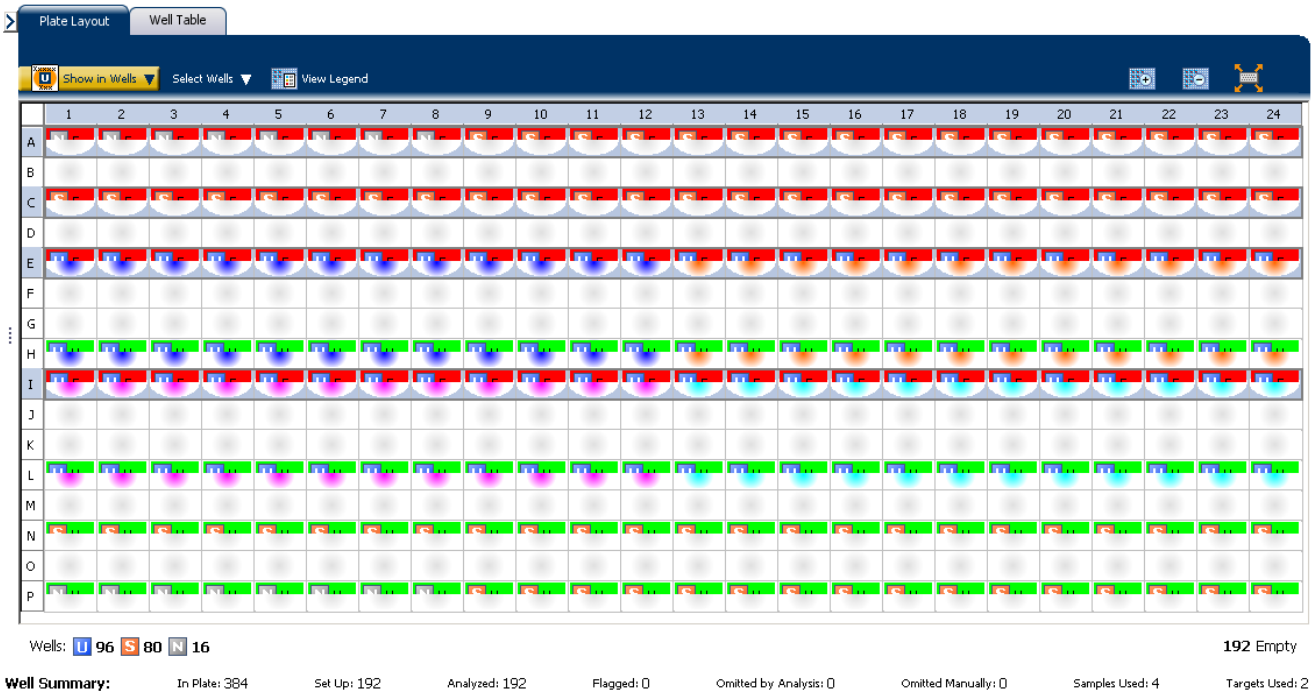
View the Amplification Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the FAS wells in the Amplification Plot screen:
   a. Click the **Plate Layout** tab.
   b. From the Select Wells drop-down menu, select **Target**, then **FAS**.

The Plate Layout screen should look like this:

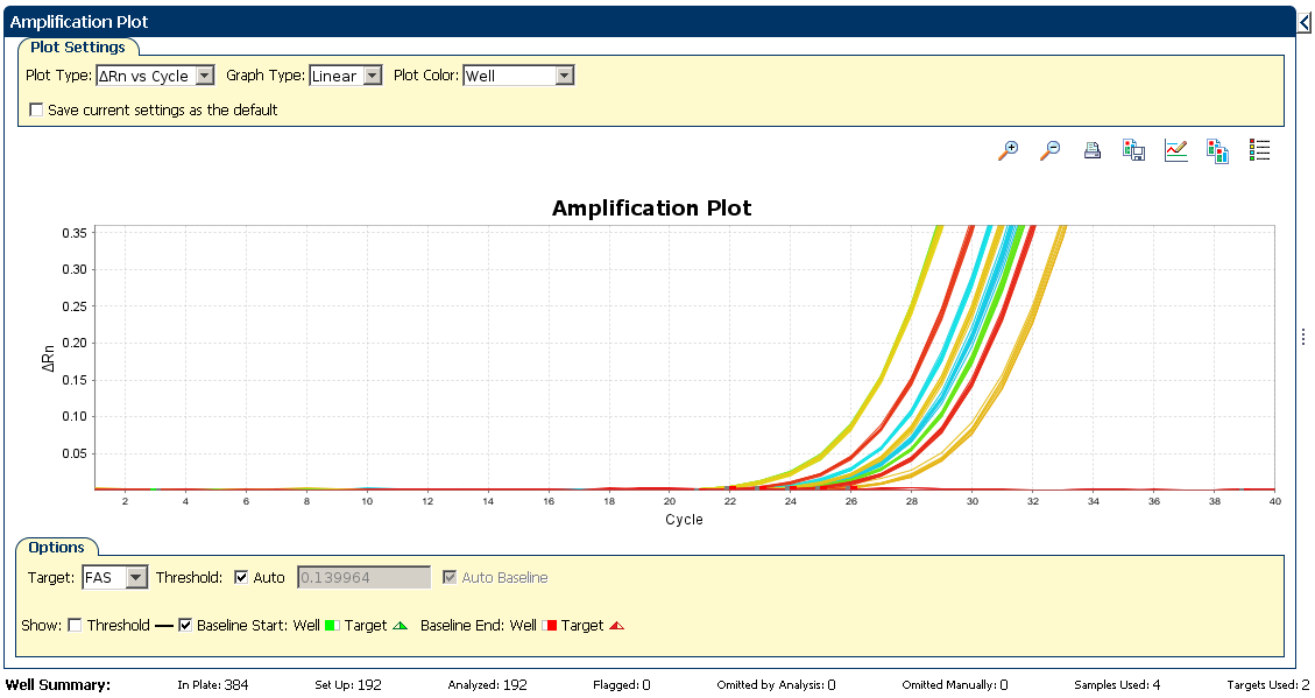


3. In the Amplification Plot screen, enter:

Menu	Selection
Plot Type	ΔRn vs Cycle (default)
Plot Color	Well (default)
(This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.)	Check (default)

4. View the baseline values:
   a. From the Graph Type drop-down menu, select **Linear**.
   b. Select the **Baseline** check box to show the start cycle and end cycle.

c. Verify that the baseline is set correctly: The end cycle should be set a few cycles before the cycle number where significant fluorescent signal is detected. In the example experiment, the baseline is set correctly.

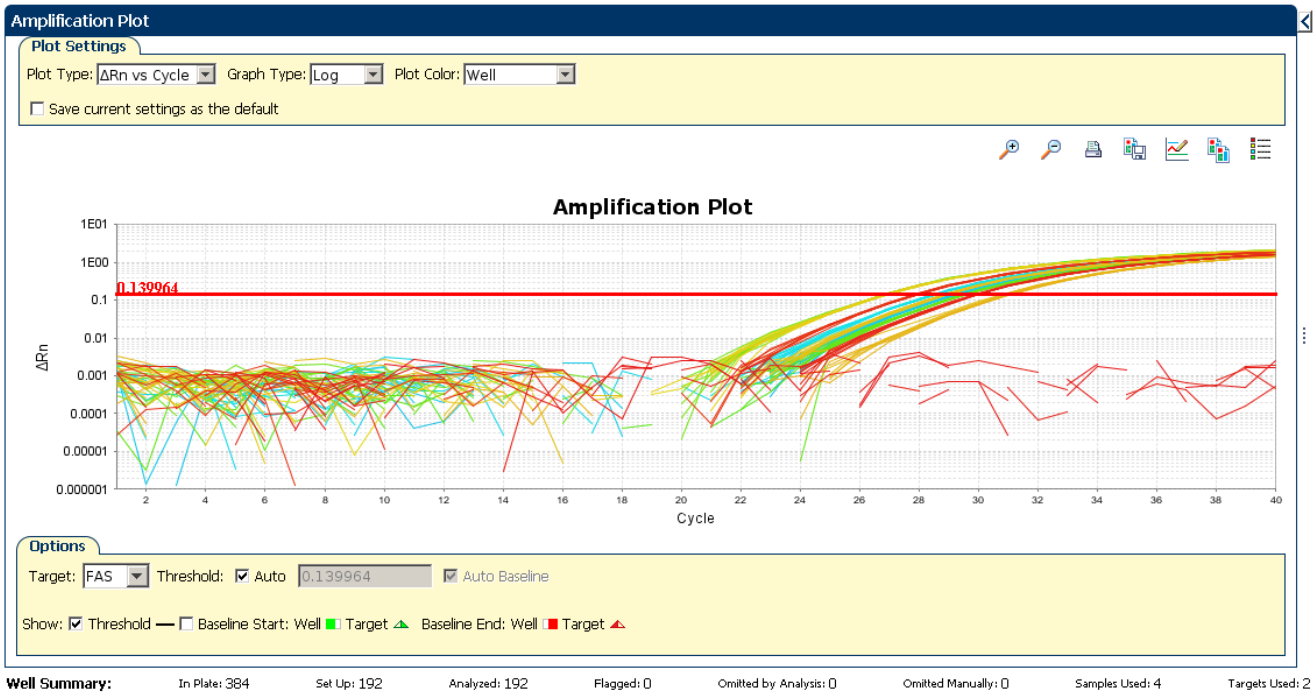


5. View the threshold values:

Menu	Select
Graph Type	Log
Target	FAS

a. Select the **Threshold** check box to show the threshold.

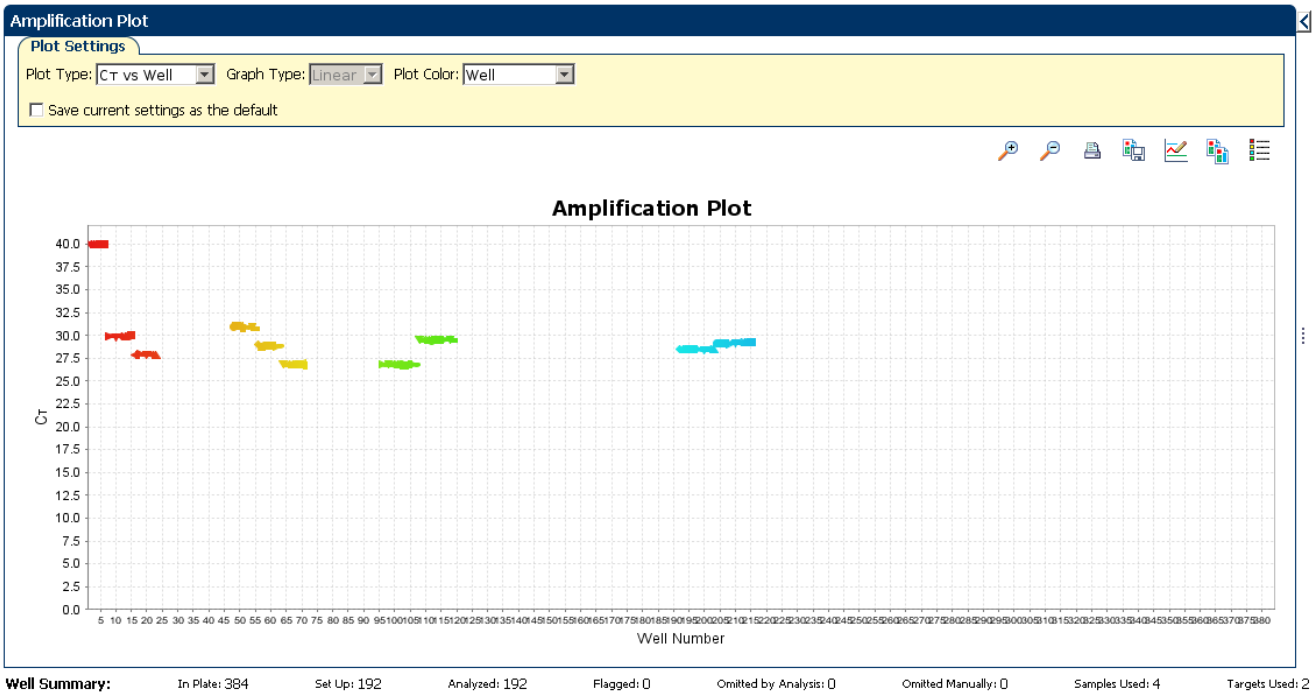
b. Verify that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.



6. Locate outliers:

a. From the Plot Type drop-down menu, select **C_T vs Well**.

b. Look for outliers from the amplification plot. In the example experiment, there are no outliers for FAS.



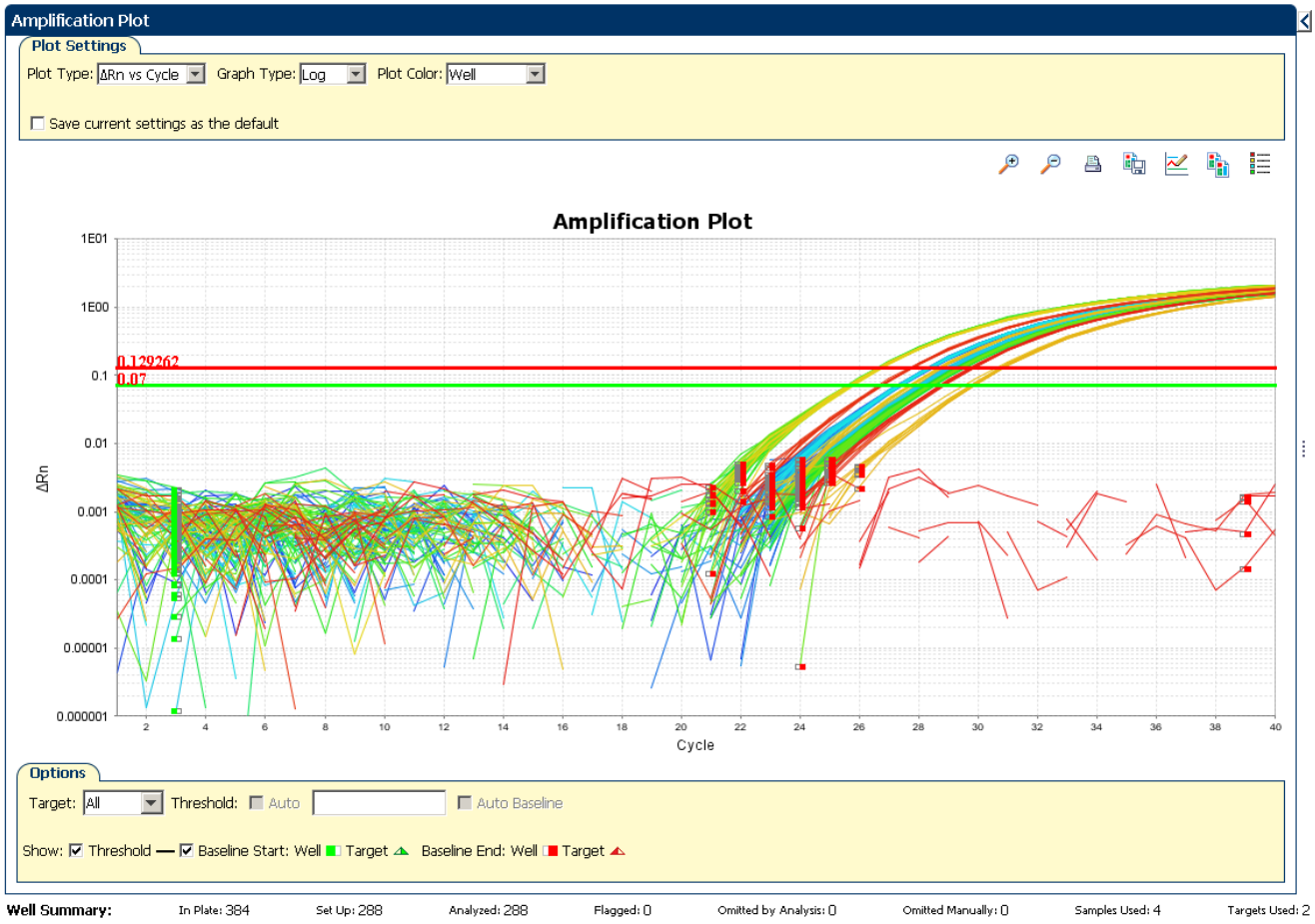
7. Repeat steps 2 through 6 for the HPRT wells.

Tips for analyzing your own experiments

When you analyze your own Relative Standard Curve experiment, look for:

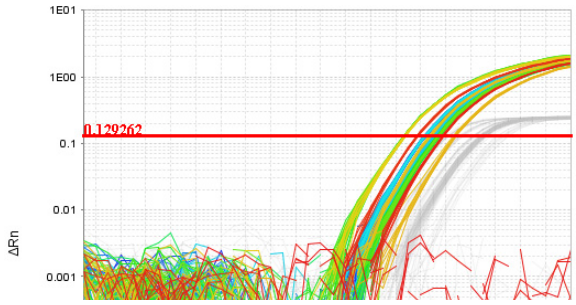
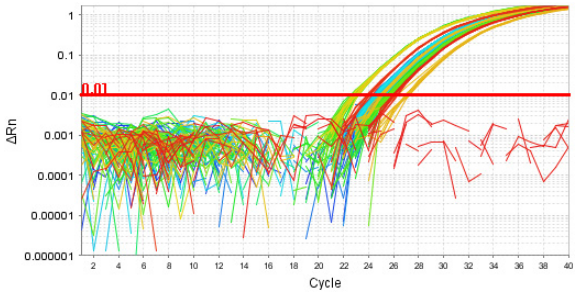
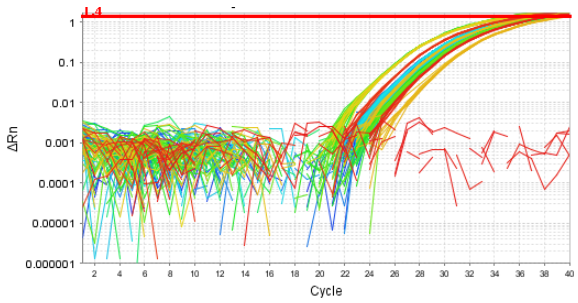
- **Outliers**
- **A typical amplification plot** – The ViiA™ 7 Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear phase
  - Exponential (geometric phase)
  - Baseline

  A typical amplification plot should look like this:

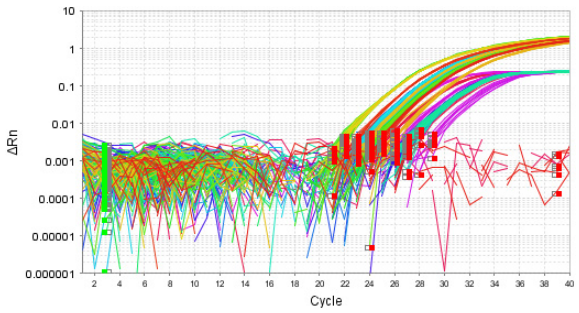
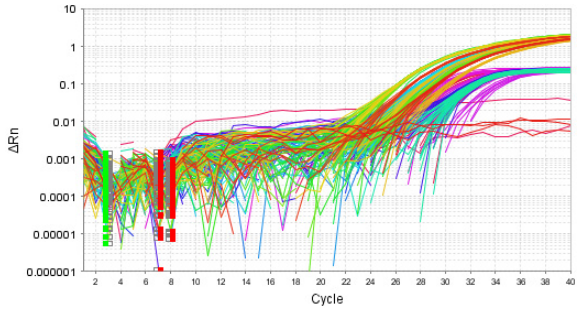
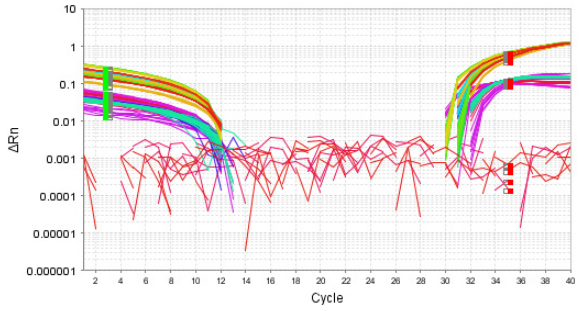


IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the ViiA™ 7 Software. Therefore, Applied Biosystems recommends that you examine the Amplification Plot screen and review the assigned baseline and threshold values for each well after analysis completes.

- **Correct threshold values.**

Threshold Set Correctly The threshold is set in the exponential phase of the amplification curve. Threshold settings above or below the optimum increase the standard deviation of the replicate groups.	
Threshold Set Too Low The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the exponential phase of the curve.	
Threshold Set Too High The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar down into the exponential phase of the curve.	

- **Correct baseline values**

Baseline Set Correctly The amplification curve begins after the maximum baseline.	
Baseline Set Too Low The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.	
Baseline Set Too High The amplification curve begins before the maximum baseline. Decrease the End Cycle value.	

If your experiment does not meet the guidelines above, troubleshoot as follows:

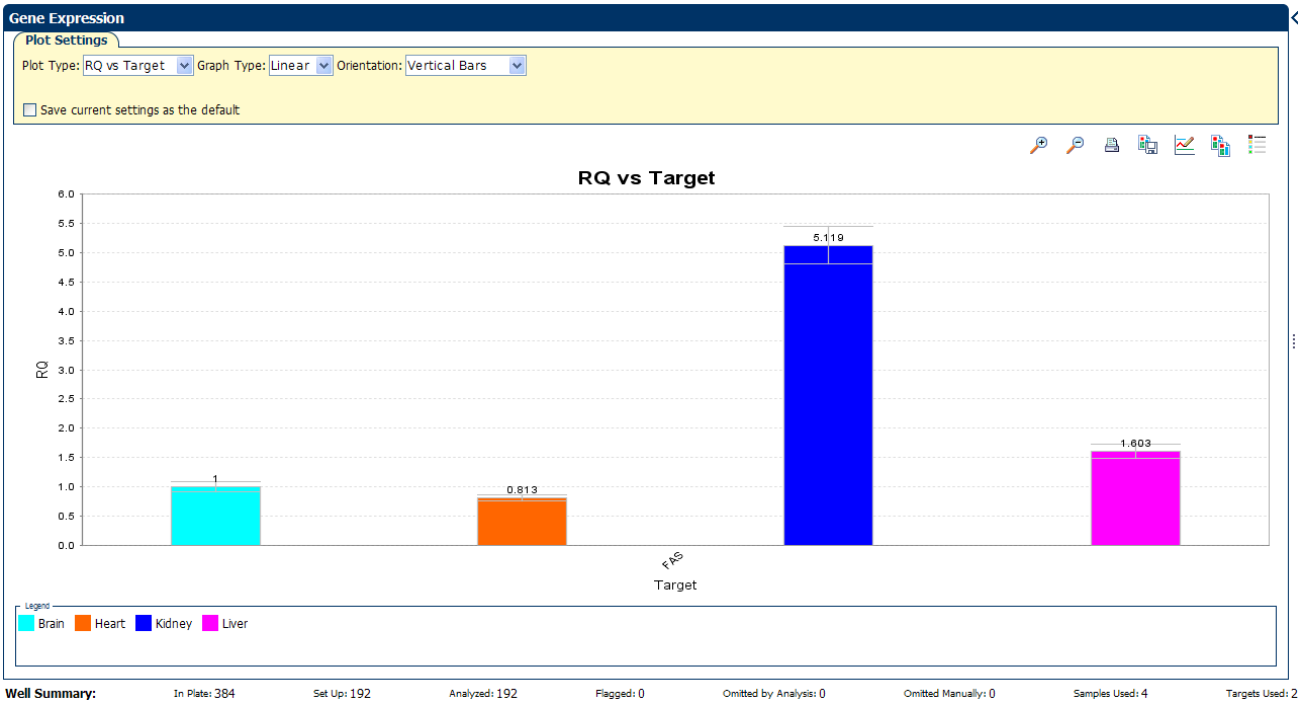
- Omit wells (see "Improve C_T precision by omitting wells" on page 68).

  Or
- Manually adjust the baseline and/or threshold (see "Adjust analysis settings" on page 63).

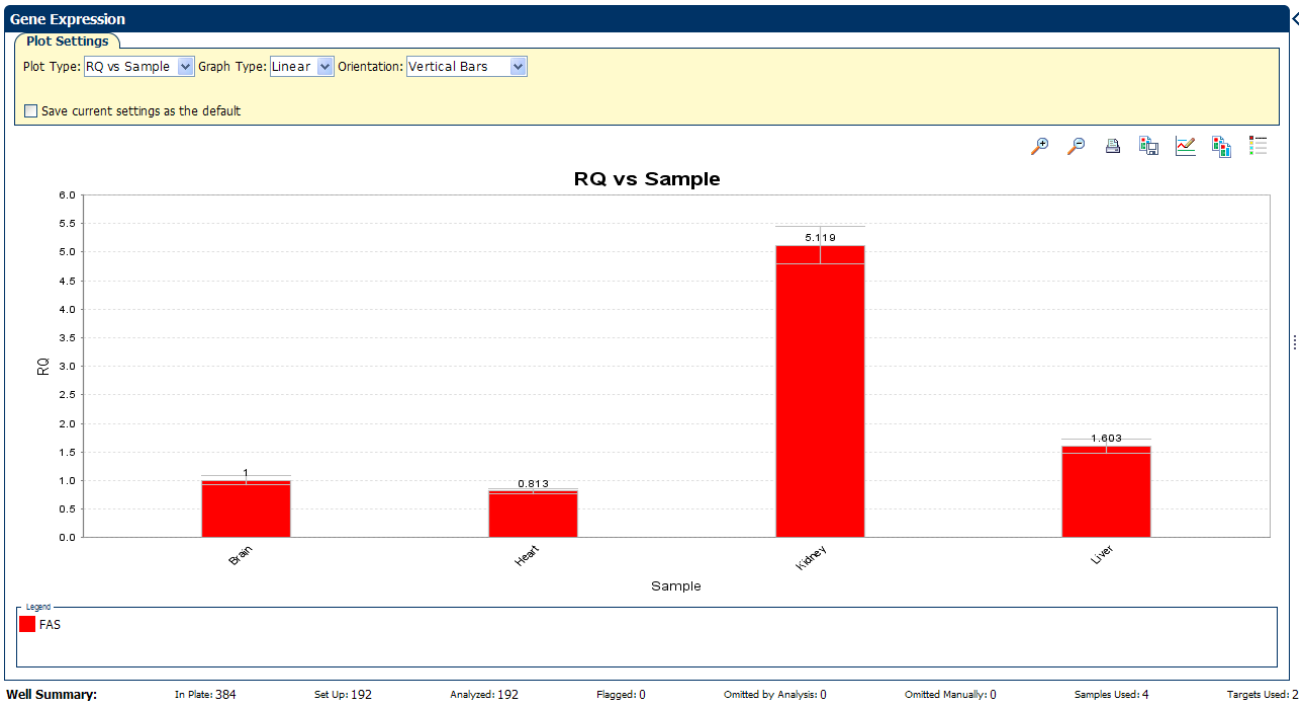
Assess the gene expression profile using the Gene Expression Plot

The Gene Expression Plot screen displays the results of relative quantification calculations in the gene expression profile. There are two plots available:

- **RQ vs Target** – Groups the relative quantification (RQ) values by target. Each sample is plotted for each target. You can view the plot as the linear, log10, Ln, and log2 graph types. The Gene Expression plot when viewed as a linear graph type looks like this:



- **RQ vs Sample** – Groups the relative quantification (RQ) values by sample. Each target is plotted for each sample. You can view the plot as the linear, log10, Ln, and log2 graph types. The Gene Expression plot when viewed as a linear graph type looks like this:



Example experiment values

Review each target in the Gene Expression Plot screen for the expression level (or fold change) of the target sample relative to the reference sample.

View the Gene Expression Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Gene Expression**.

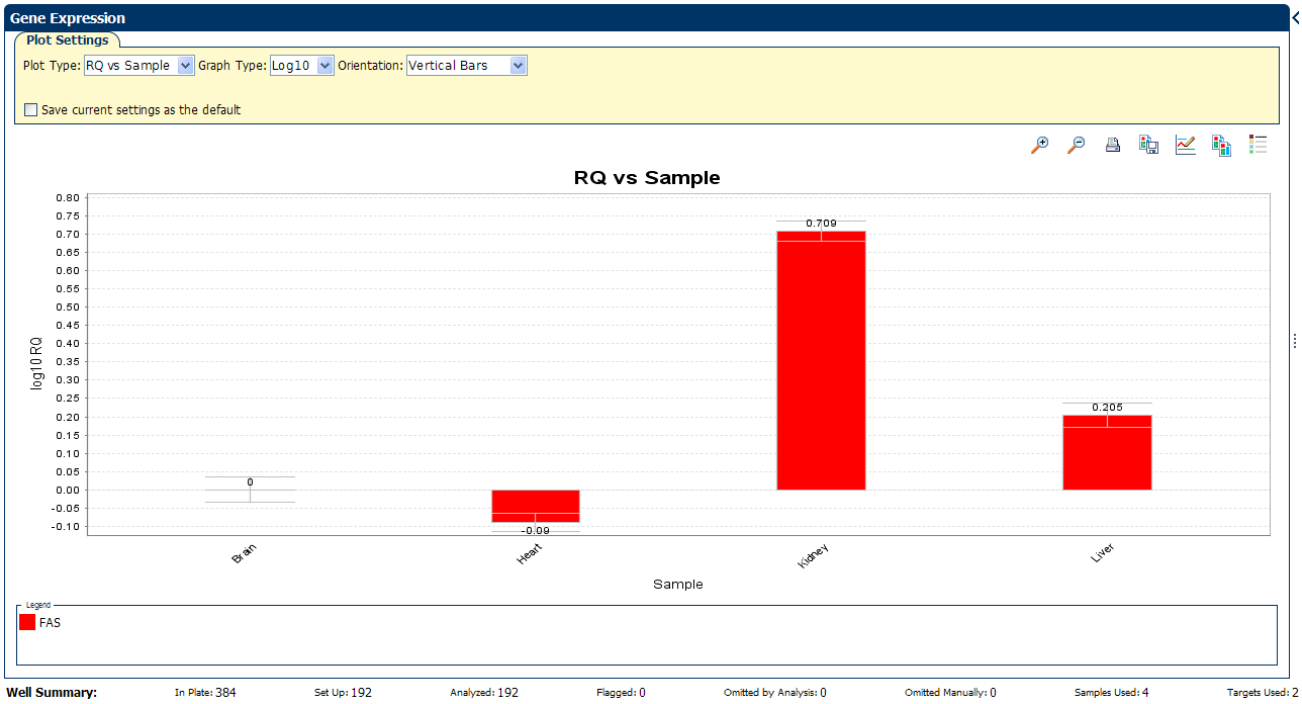
   Note: If no data are displayed, click **Analyze**.

2. In the Gene Expression Plot screen:
   a. From the Plot Type drop-down menu, select **RQ vs Sample**.
   b. From the Graph Type drop-down menu, select **Log10**.
   c. From the Orientation drop-down menu, select **Vertical Bars**.

3. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

In the example experiment, the expression level of FAS in heart, kidney, and liver is displayed relative to its expression level in the reference sample (brain). Since the reference sample is compared to itself, the relative expression level is 1. When the result is displayed in the Log10 graph type, the expression level of the reference sample appears as 0 in the graph (log10 of 1 = 0).



Assessing the gene expression plot in your own experiments

When you analyze your own Relative Standard Curve experiment, look for differences in gene expression (as a fold change) relative to the reference sample.

Identify well problems using the Well Table

The Well Table displays data for each well in the reaction plate, including:

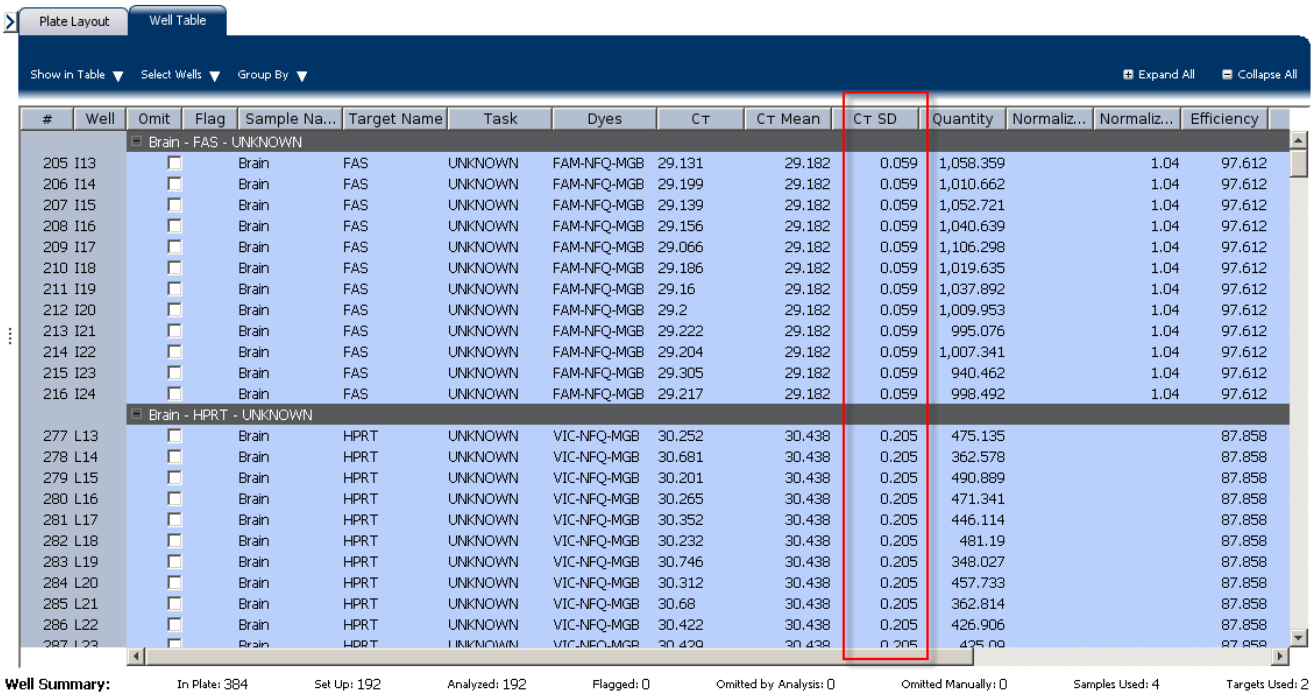
- The sample name, target name, task, and dyes
- The calculated threshold cycle (C_T), normalized fluorescence (Rn), and quantity values
- Flags

Example experiment values and flags

Review the Well Table to evaluate the C_T precision of the replicate groups.

View the well table

1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**, then click the **Well Table** tab.
2. From the Group By drop-down menu, select **Replicate**.
3. Look at the C_T SD column to evaluate the C_T precision of the replicate groups. In the example experiment, the C_T SD have the expected value of < 0.5.



#	Well	Omit	Flag	Sample Na...	Target Name	Task	Dyes	CT	CT Mean	CT SD	Quantity	Normaliz...	Normaliz...	Efficiency
		Brain - FAS - UNKNOWN												
205	I13			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.131	29.182	0.059	1,058.359		1.04	97.612
206	I14			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.199	29.182	0.059	1,010.662		1.04	97.612
207	I15			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.139	29.182	0.059	1,052.721		1.04	97.612
208	I16			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.156	29.182	0.059	1,040.639		1.04	97.612
209	I17			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.066	29.182	0.059	1,106.298		1.04	97.612
210	I18			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.186	29.182	0.059	1,019.635		1.04	97.612
211	I19			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.16	29.182	0.059	1,037.892		1.04	97.612
212	I20			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.2	29.182	0.059	1,009.953		1.04	97.612
213	I21			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.222	29.182	0.059	995.076		1.04	97.612
214	I22			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.204	29.182	0.059	1,007.341		1.04	97.612
215	I23			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.305	29.182	0.059	940.462		1.04	97.612
216	I24			Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.217	29.182	0.059	998.492		1.04	97.612
		Brain - HPRT - UNKNOWN												
277	L13			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.252	30.438	0.205	475.135			87.858
278	L14			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.681	30.438	0.205	362.578			87.858
279	L15			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.201	30.438	0.205	490.889			87.858
280	L16			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.265	30.438	0.205	471.341			87.858
281	L17			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.352	30.438	0.205	446.114			87.858
282	L18			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.232	30.438	0.205	481.19			87.858
283	L19			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.746	30.438	0.205	348.027			87.858
284	L20			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.312	30.438	0.205	457.733			87.858
285	L21			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.68	30.438	0.205	362.814			87.858
286	L22			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.422	30.438	0.205	426.906			87.858

Well Summary: In Plate: 384 Set Up: 192 Analyzed: 192 Flagged: 0 Omitted by Analysis: 0 Omitted Manually: 0 Samples Used: 4 Targets Used: 2

Note: To show or hide columns in the Well Table, select or deselect respectively the column name from the Show in Table drop-down menu.

Assessing the well table in your own experiments

When you analyze your own Relative Standard Curve experiment, look for standard deviation in the replicate groups (C_T SD values). If needed, omit outliers ("Improve C_T precision by omitting wells" on page 68).

Confirm accurate dye signal using the Multicomponent Plot

The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the Relative Standard Curve example experiment, you review the Multicomponent Plot screen for:

- ROX™ dye (passive reference)
- FAM™ dye (reporter for RNase P)
- VIC® dye (reporter for IPC)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

View the Multicomponent Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Multicomponent Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the unknown and standard wells one at a time in the Multicomponent Plot screen:

   a. Click the **Plate Layout** tab.

   b. Select one well in the plate layout; the well is shown in the Multicomponent Plot screen.

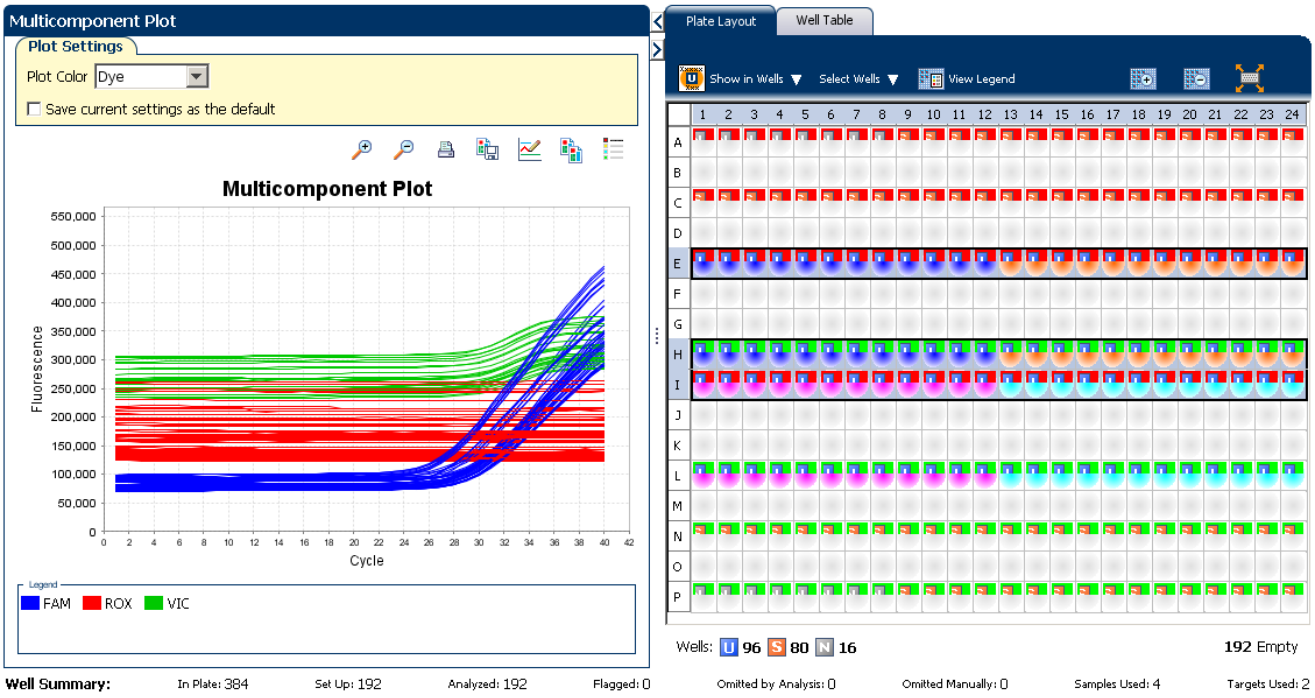
      Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

3. From the Plot Color drop-down menu, select **Dye**.

4. Click **Show a legend for the plot** (default).

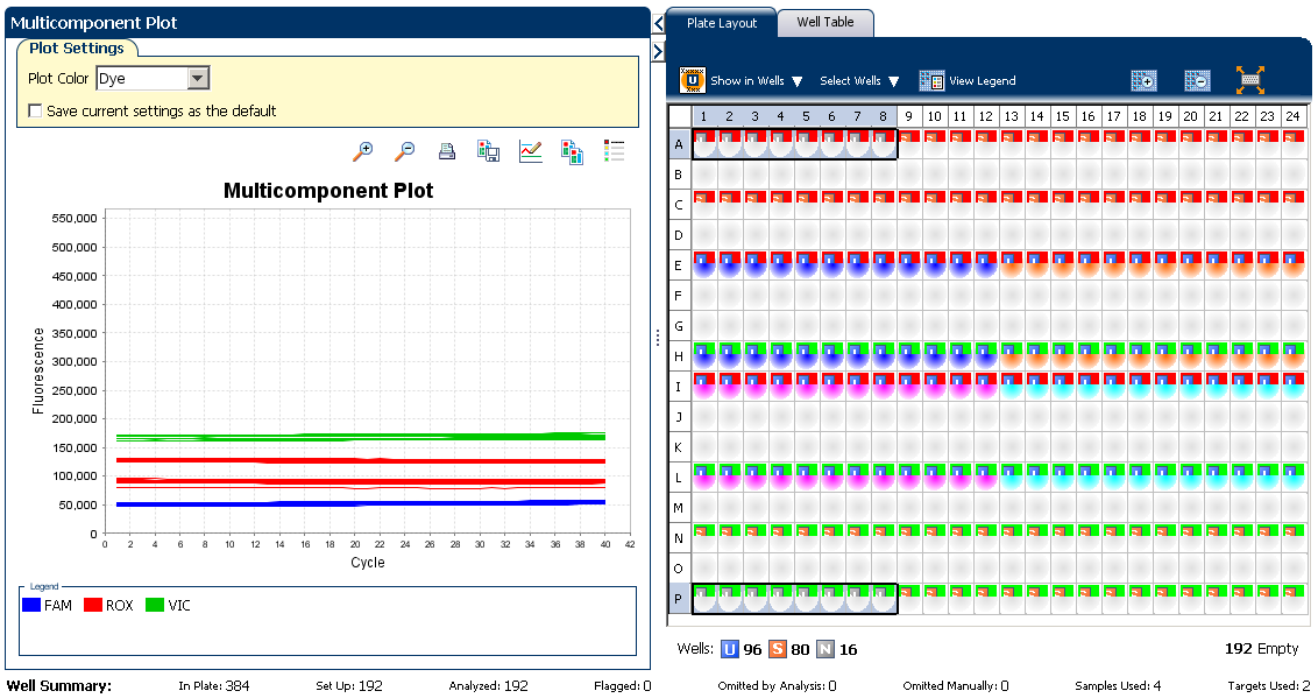
   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

5. Check the FAM and VIC dye signals. In the example experiment, the FAM and VIC dyes signal increase throughout the PCR process, indicating normal amplification.



6. Check the ROX dye signal. In the example experiment, the ROX dye signal remains constant throughout the PCR process indicating typical data.

7. Select the negative control wells one at time and check for amplification. In the example experiment, there is no amplification in any of the negative control wells.



Tips for confirming dye accuracy in your own experiment

When you analyze your own Relative Standard Curve experiment, look for:

- **Passive reference** – The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye** – The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- **Irregularities in the signal** – There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative control wells** – There should not be any amplification in the negative control wells.

Determine signal accuracy using the Raw Data Plot

The Raw Data Plot screen displays the raw fluorescence signal (not normalized) for each optical filter for the selected wells during each cycle of the real-time PCR.

About the example experiment

In the Relative Standard Curve example experiment, you review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the Raw Data Plot

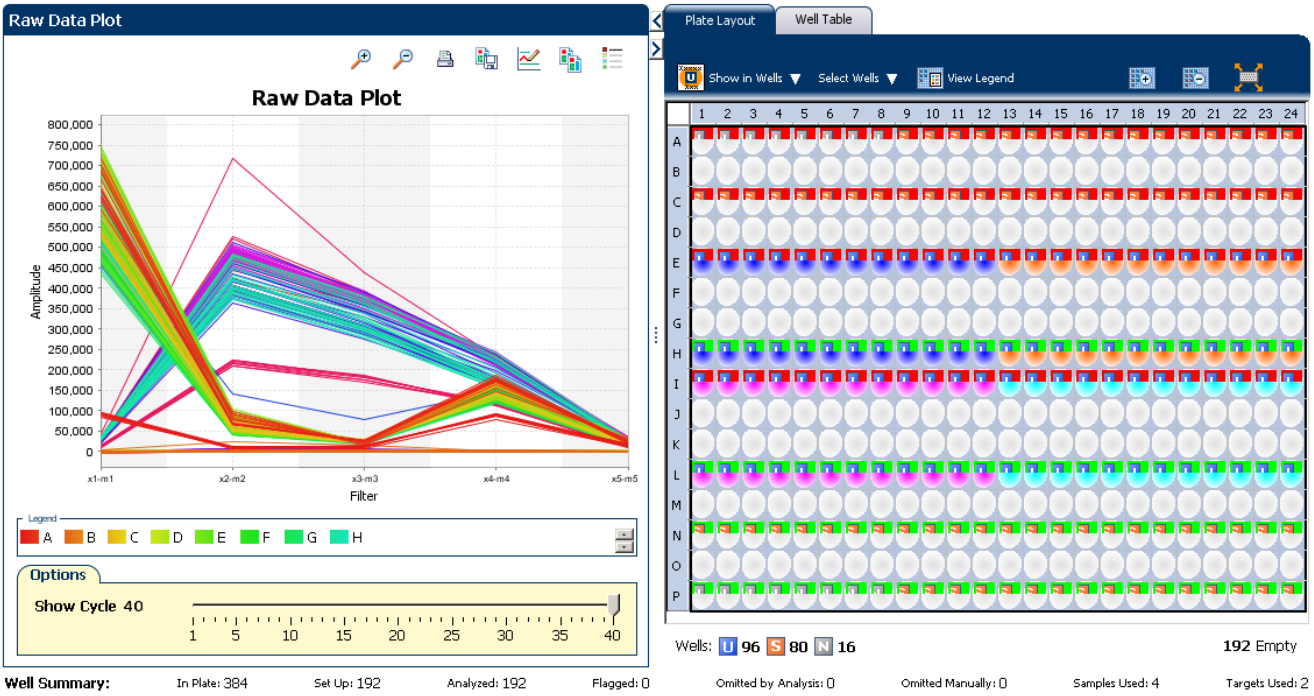
1. From the Experiment Menu pane, select **Analysis** ▸ **Raw Data Plot**.

   Note: If no data are displayed, click **Analyze**.

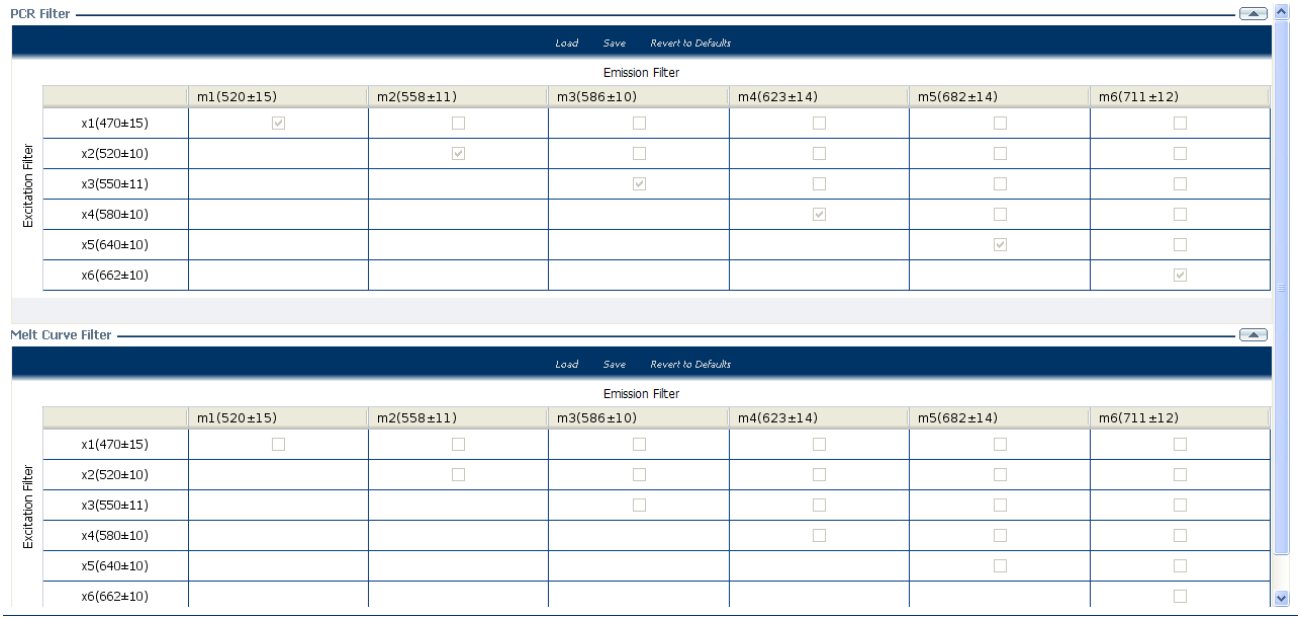
2. Display all 384 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the Plate Layout tab.

3. Click **Show a legend for the plot** (default). The legend displays the color code for each row of the reaction plate (see the legend in the Raw Data Plot shown below).

4. Click and drag the Show Cycle pointer from cycle 1 to cycle 40. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.



The filters used for the example experiment are:



Tips for determining signal accuracy in your own experiment

When you analyze your own Relative Standard Curve experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

View the endogenous control profile using the QC Plot

In the Relative Standard Curve experiment, the QC Plot screen displays the Endogenous Control Profile plot for all the targets in the experiment. The QC Plot serves as a tool to help users choose the best endogenous control for that experiment. The endogenous control profile plot is a visual display of the C_T level of the endogenous control across the sample. You can view up to four endogenous controls at a time. The sample is plotted on the X-axis, and the C_T is plotted on the Y-axis. The expression is viewed as a color and shape combination in the plot. Endogenous controls are also known as reference genes.

To view the QC Plot:

1. From the Experiment Menu pane, select **Analysis ▸ QC Plot**.

   Note: If no data are displayed, click **Analyze**.

2. In the QC Plot screen, click **Target Table** to select a target to profile:
   a. In the Candidate Control column, select the check box of the target of the endogenous control profile to plot. In the example experiment, the endogenous control is HPRT.
   b. Select a color from the Color drop-down menu.
   c. Select a shape from the Shape drop-down menu.

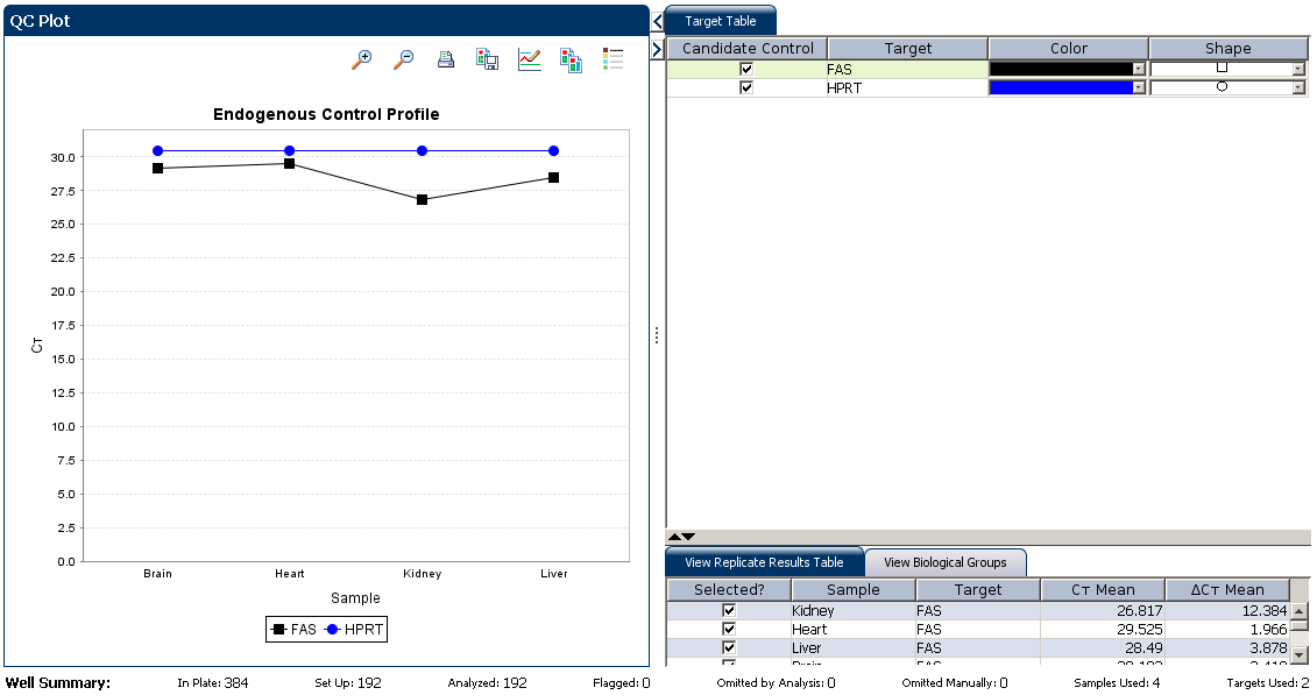
3. Click the **View Replicate Results Table**.

4. Select the check box of the samples you want to plot.

5. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

The QC Plot in the Relative Standard Curve example experiment looks like this. Note that the endogenous control, HPRT is expressed at the same level in all the four samples:



This example experiment does not define Biological Groups.

Review the QC flags in the QC Summary

The QC Summary screen displays a list of the ViiA™ 7 Software flags, including the flag frequency and location for the open experiment.

View the QC Summary

1. From the Experiment Menu pane, select **Analysis ▸ QC Summary**.

   Note: If no data are displayed, click **Analyze**.

2. Review the Flags Summary.

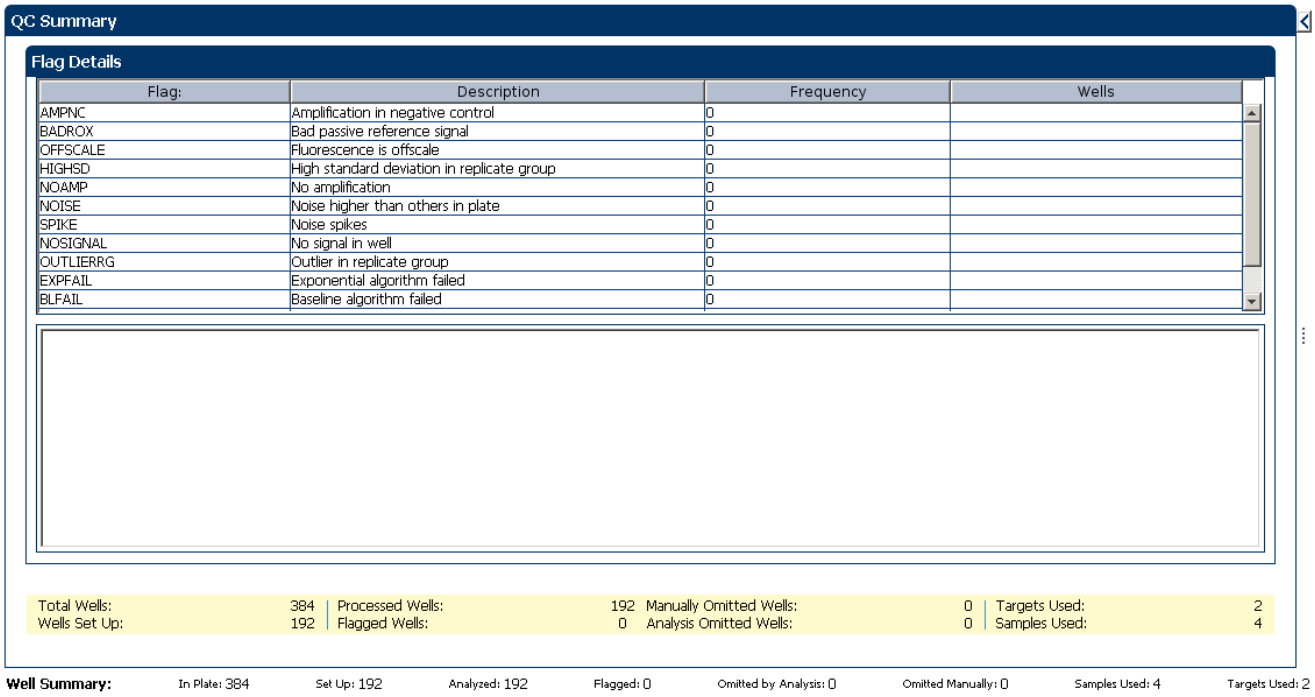
   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment. If the frequency is > 0, the flag appears somewhere in the experiment; the well position is listed in the Wells column.

   In the example experiment, there are no flagged wells.

3. In the Flag Details table, click each flag with a frequency > 0 to display detailed information about the flag. In the example experiment, the Frequency column displays 0 for all flags.

4. *(Optional)* For those flags with frequency > 0, click the troubleshooting link to view information on correcting the flag.

The QC Summary screen for the example experiment looks like this:



Possible flags

The flags listed below may be triggered by the experiment data.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
Secondary analysis flags	
OUTLIERRG	Outlier in replicate group

Flag	Description
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the Frequency column for each flag).

For more information

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with ViiA™7 System Experiments*.	4441434

5.2 Section 5.2 Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

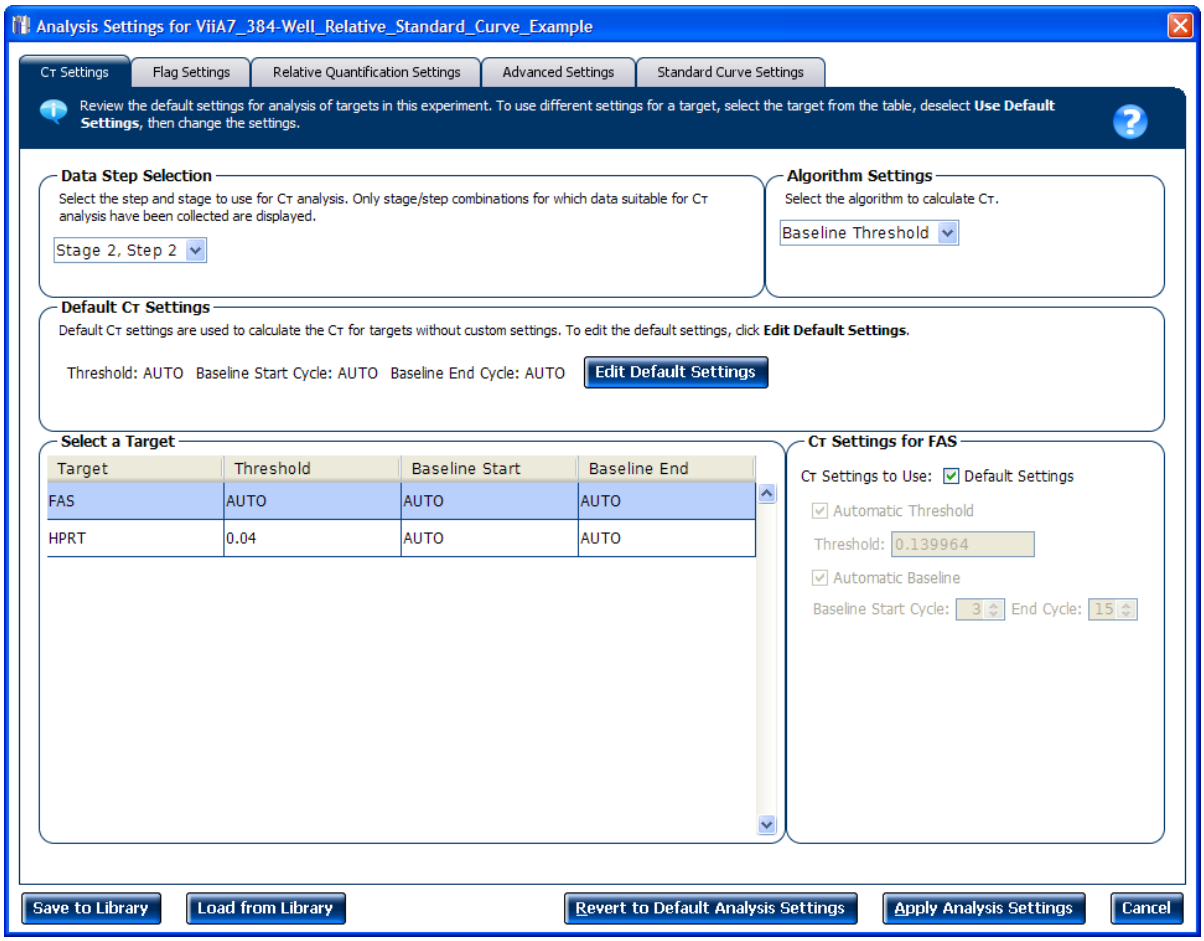
The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, and advanced options.

If the default analysis settings in the ViiA™ 7 Software are not suitable for your own experiment, you can change the settings in the Analysis Settings dialog box, then reanalyze your experiment.

View the analysis settings

1. From the Experiment Menu pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the Analysis Settings dialog box.
   In the example experiment, the default analysis settings are used for each tab:
   - C_T Settings
   - Flag Settings
   - Relative Quantification Settings
   - Advanced Settings
   - Standard Curve Settings

The Analysis Settings dialog box for a Relative Standard Curve experiment looks like this:



3. View and, if necessary, change the analysis settings (see "Adjust analysis settings" below).

   Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see About the Analysis Settings Library in Booklet 1, *Getting Started with ViiA™ 7* System Experiments.

4. Click **Apply Analysis Settings** to apply the current analysis settings.

   Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

C_T Settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method.

- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for gene quantification.

The Relative Threshold algorithm lets you compare the data on a per-well or per-target basis. This setting is ideal for analyzing a single gene across samples or a single sample across genes with no dependence on targets, thereby reducing variability. Any settings for baseline or threshold do not affect the analysis when you use the Relative Threshold algorithm.

- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the targets that do not have custom settings. To edit the default settings, click **Edit Default Settings**.

- **C_T Settings for Target**

  When you manually set the threshold and baseline, Applied Biosystems recommends:

Setting	Recommendation
Threshold	Enter a value for the threshold so that the threshold is: • Above the background. • Below the plateau and linear regions of the amplification curve. • Within the exponential phase of the amplification curve.
Baseline	Select the Start Cycle and End Cycle values so that the baseline ends before significant fluorescent signal is detected.

Note: This setting is applicable only to the Baseline Threshold algorithm.

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag Settings

Use the Flag Settings tab to:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the ViiA™ 7 Software.

To adjust the flag settings

1. In the Use column, select the check boxes for flags to apply during analysis.
2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

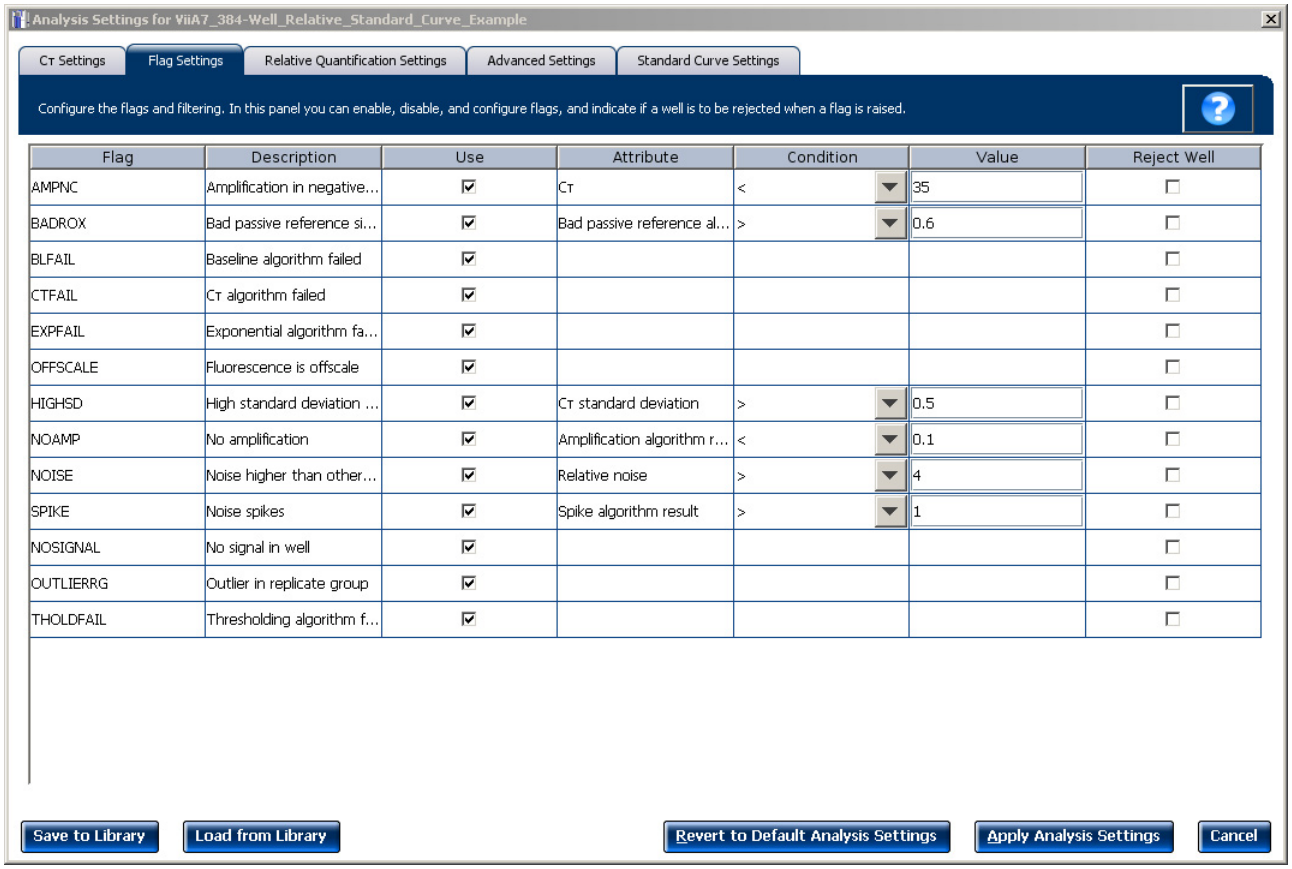
   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations may change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.

4. Click **Apply Analysis Settings** in the Analysis Settings dialog box. If the run status is complete, the data are reanalyzed.

The Flag Settings tab looks like this:



Relative Quantification Settings

Use the Relative Quantification Settings tab to:

- Change the type of analysis, singleplex or multiplex.
- Change the reference sample and/or endogenous control.
- Reject Outliers with ΔC_T values less than or equal to the entered value.

  Note: The Outlier Rejection settings apply only to multiplex reactions.

- Select the algorithm to use to determine the relative quantification minimum and maximum values (error bars):
  - **Confidence Level** - Select to calculate the RQ minimum and maximum values based on the selected confidence level. Select the confidence level to use.
  - **Standard Deviations** - Select to calculate the RQ minimum and maximum values based on the selected number of standard deviations. Select the number of standard deviations to use.

Advanced Settings

Use the Advanced Settings tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold setting.

To use custom baseline settings for a well-target combination:

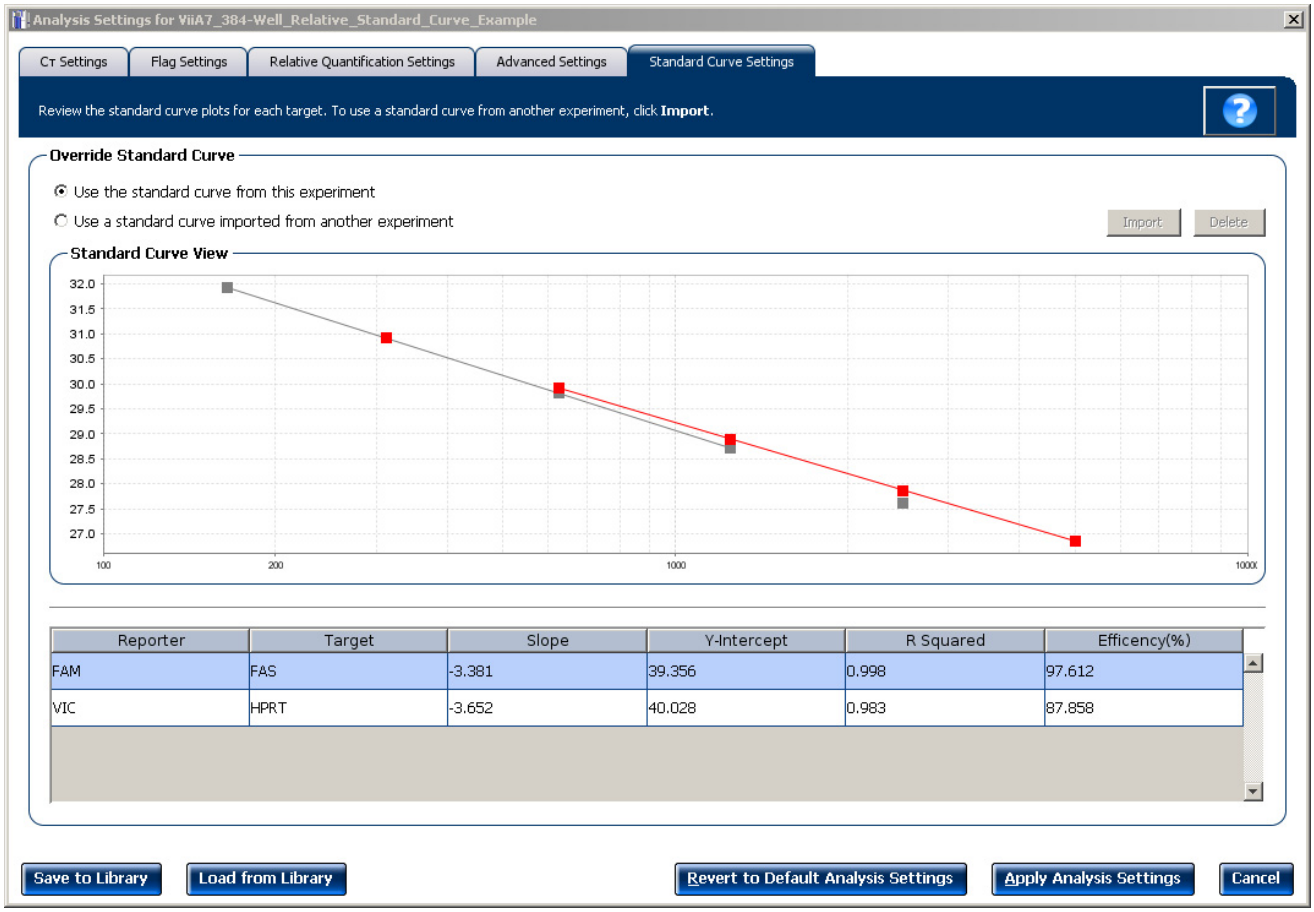
1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** check box.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, then enter the baseline start cycle and baseline end cycle.

Standard Curve Settings

Use this tab to review the settings of the current standard curve experiment or to import the standard curve from an external experiment (with the same samples and targets) and apply it to this current experiment.

Note: The run method must be the same. Applied Biosystems recommends using the standard curve from the current experiment.

For the example experiment, the settings from the current experiment have been used.



Improve C_T precision by omitting wells

Experimental error may cause some wells to be amplified insufficiently or not at all. These wells typically produce C_T values that differ significantly from the average for the associated replicate wells. If included in the calculations, these outliers can result in erroneous measurements; to ensure C_T precision, omit the outliers from the analysis.

Note: In the Relative Standard Curve example experiment, there are no outliers. No wells need to be removed from analysis.

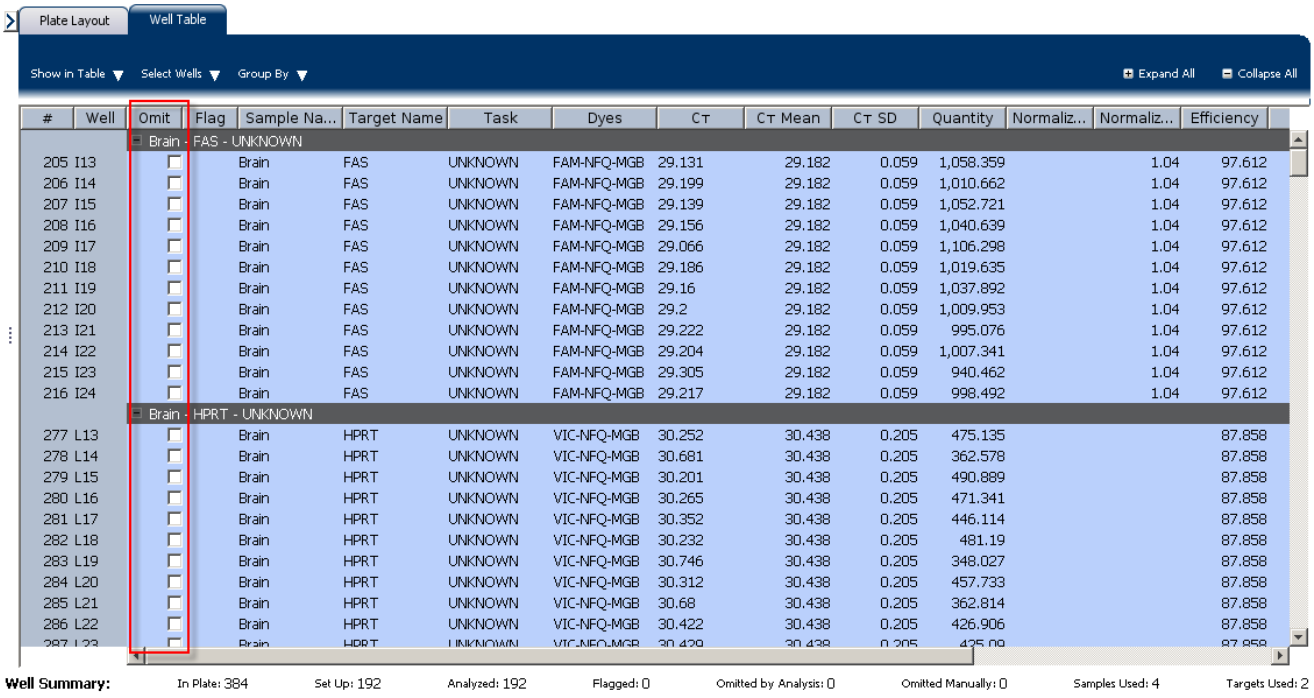
1. From the Experiment Menu pane, select **Analysis ▸ Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.

2. In the Amplification Plot screen, select **C_T vs. Well** from the Plot Type drop-down menu.

3. Select the **Well Table** tab, select replicates to omit:

4. In the Well Table:
   a. From the Group By drop-down menu, select **Replicate**.
   b. Look for outliers in the replicate group (make sure they are flagged).
   c. Select the **Omit** check box next to outlying well(s), as shown below.



#	Well	Omit	Flag	Sample Na...	Target Name	Task	Dyes	CT	CT Mean	CT SD	Quantity	Normaliz...	Normaliz...	Efficiency
			Brain - FAS - UNKNOWN											
205	I13	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.131	29.182	0.059	1,058.359		1.04	97.612
206	I14	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.199	29.182	0.059	1,010.662		1.04	97.612
207	I15	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.139	29.182	0.059	1,052.721		1.04	97.612
208	I16	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.156	29.182	0.059	1,040.639		1.04	97.612
209	I17	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.066	29.182	0.059	1,106.298		1.04	97.612
210	I18	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.186	29.182	0.059	1,019.635		1.04	97.612
211	I19	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.16	29.182	0.059	1,037.892		1.04	97.612
212	I20	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.2	29.182	0.059	1,009.953		1.04	97.612
213	I21	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.222	29.182	0.059	995.076		1.04	97.612
214	I22	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.204	29.182	0.059	1,007.341		1.04	97.612
215	I23	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.305	29.182	0.059	940.462		1.04	97.612
216	I24	☐		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.217	29.182	0.059	998.492		1.04	97.612
			Brain - HPRT - UNKNOWN											
277	L13	☐		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.252	30.438	0.205	475.135			87.858
278	L14	☐		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.681	30.438	0.205	362.578			87.858
279	L15	☐		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.201	30.438	0.205	490.889			87.858
280	L16	☐		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.265	30.438	0.205	471.341			87.858
281	L17	☐		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.352	30.438	0.205	446.114			87.858
282	L18	☐		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.232	30.438	0.205	481.19			87.858
283	L19	☐		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.746	30.438	0.205	348.027			87.858
284	L20	☐		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.312	30.438	0.205	457.733			87.858
285	L21	☐		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.68	30.438	0.205	362.814			87.858
286	L22	☐		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.422	30.438	0.205	426.906			87.858

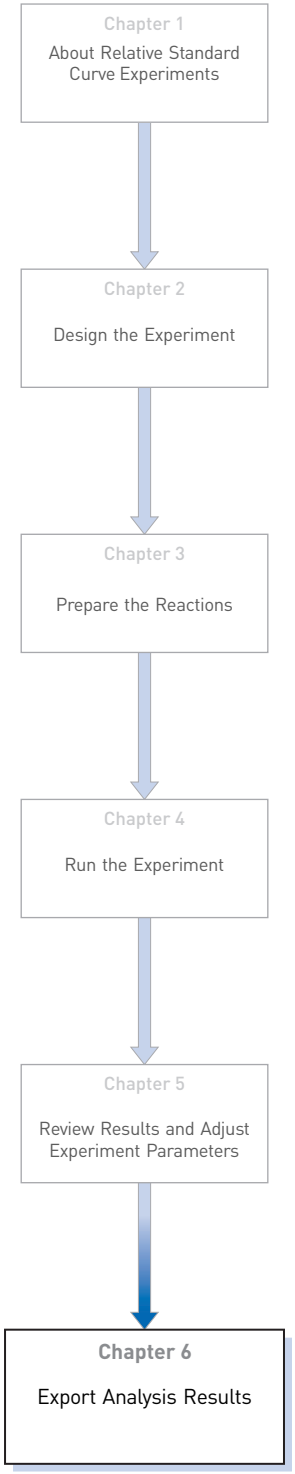
5. Click **Analyze** to reanalyze the experiment data with the outlying well(s) removed from the analysis.

   Note: You can also omit undesirable wells in an experiment from the Plate Layout screen. To omit a well from the Plate Layout screen, right-click the well and select **Omit**.

For more information

For more information on	Refer to	Part number
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*	127AP05-03

6 Export Analysis Results



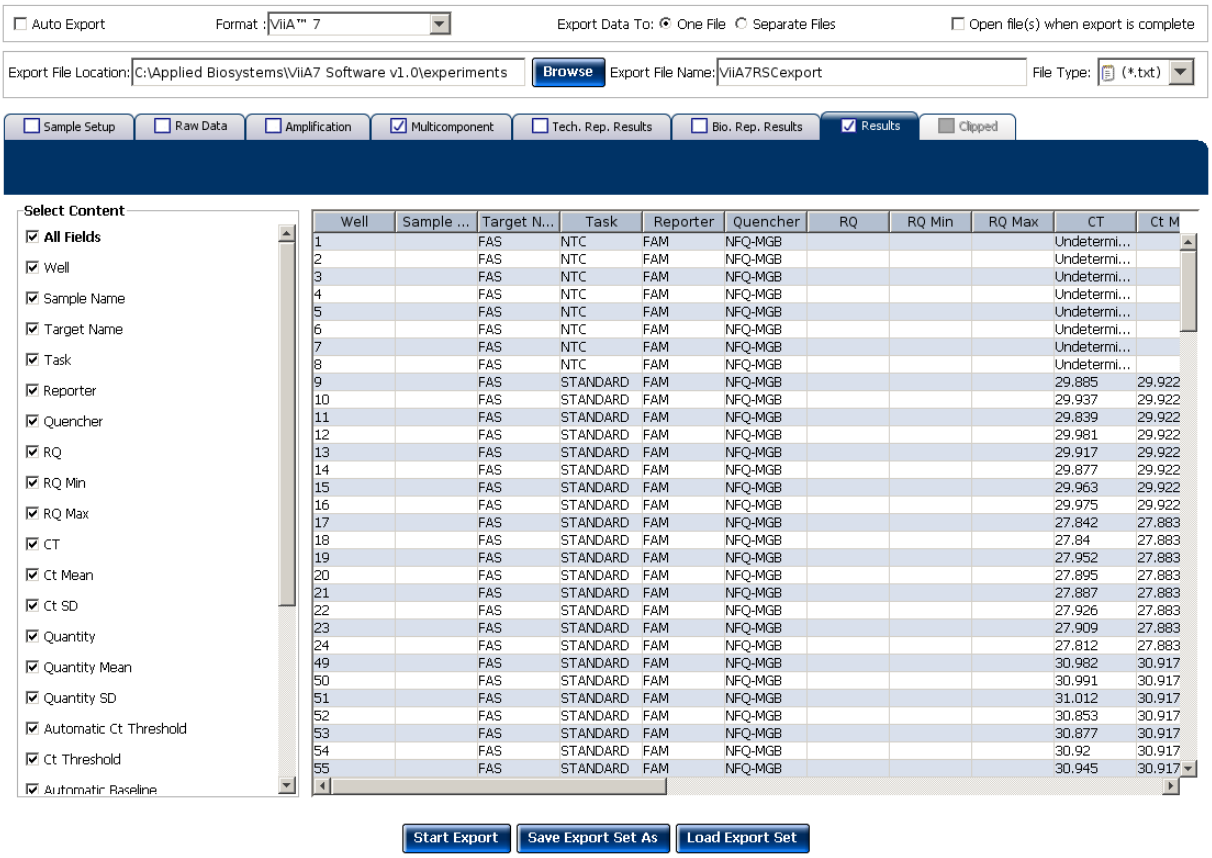
1. Open the Relative Standard Curve example experiment file that you analyzed in Chapter 5.
2. In the Experiment Menu, click **Export.**

   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.
3. Select **ViiA™7 Format**.

   Note: Select **7900** Format if you want to export the Clipped Data.
4. Complete the Export dialog box as shown below:

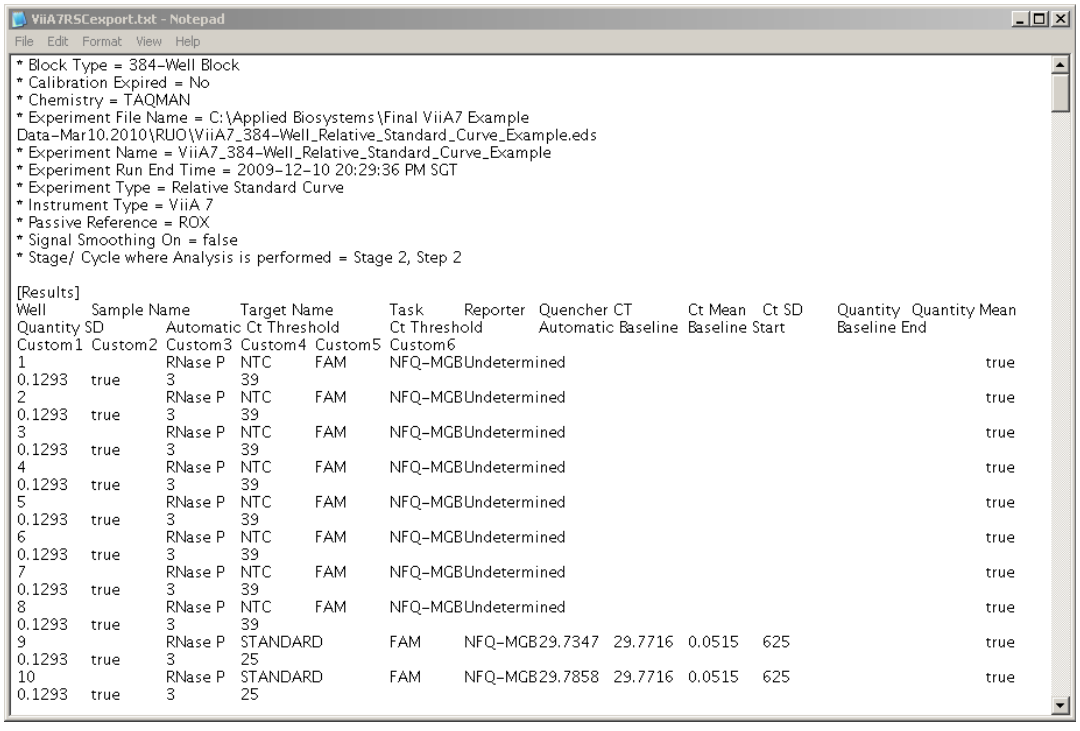
Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	ViiA7RSCexport
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\ViiA7 Software v1.1\experiments

Your Export screen should look like this:



Well	Sample ...	Target N...	Task	Reporter	Quencher	RQ	RQ Min	RQ Max	CT	Ct M
1		FAS	NTC	FAM	NFQ-MGB				Undetermi...	
2		FAS	NTC	FAM	NFQ-MGB				Undetermi...	
3		FAS	NTC	FAM	NFQ-MGB				Undetermi...	
4		FAS	NTC	FAM	NFQ-MGB				Undetermi...	
5		FAS	NTC	FAM	NFQ-MGB				Undetermi...	
6		FAS	NTC	FAM	NFQ-MGB				Undetermi...	
7		FAS	NTC	FAM	NFQ-MGB				Undetermi...	
8		FAS	NTC	FAM	NFQ-MGB				Undetermi...	
9		FAS	STANDARD	FAM	NFQ-MGB				29.885	29.922
10		FAS	STANDARD	FAM	NFQ-MGB				29.937	29.922
11		FAS	STANDARD	FAM	NFQ-MGB				29.839	29.922
12		FAS	STANDARD	FAM	NFQ-MGB				29.981	29.922
13		FAS	STANDARD	FAM	NFQ-MGB				29.917	29.922
14		FAS	STANDARD	FAM	NFQ-MGB				29.877	29.922
15		FAS	STANDARD	FAM	NFQ-MGB				29.963	29.922
16		FAS	STANDARD	FAM	NFQ-MGB				29.975	29.922
17		FAS	STANDARD	FAM	NFQ-MGB				27.842	27.883
18		FAS	STANDARD	FAM	NFQ-MGB				27.84	27.883
19		FAS	STANDARD	FAM	NFQ-MGB				27.952	27.883
20		FAS	STANDARD	FAM	NFQ-MGB				27.895	27.883
21		FAS	STANDARD	FAM	NFQ-MGB				27.887	27.883
22		FAS	STANDARD	FAM	NFQ-MGB				27.926	27.883
23		FAS	STANDARD	FAM	NFQ-MGB				27.909	27.883
24		FAS	STANDARD	FAM	NFQ-MGB				27.812	27.883
49		FAS	STANDARD	FAM	NFQ-MGB				30.982	30.917
50		FAS	STANDARD	FAM	NFQ-MGB				30.991	30.917
51		FAS	STANDARD	FAM	NFQ-MGB				31.012	30.917
52		FAS	STANDARD	FAM	NFQ-MGB				30.853	30.917
53		FAS	STANDARD	FAM	NFQ-MGB				30.877	30.917
54		FAS	STANDARD	FAM	NFQ-MGB				30.92	30.917
55		FAS	STANDARD	FAM	NFQ-MGB				30.945	30.917

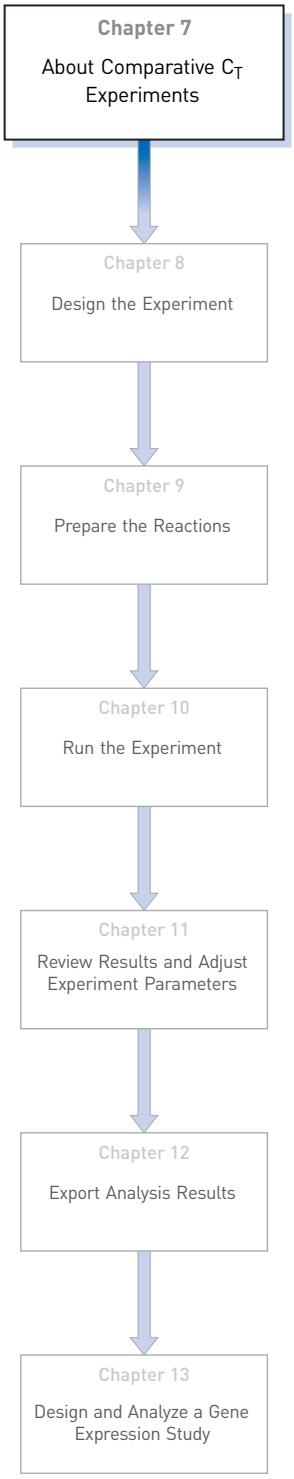
Your exported file when opened in Notepad should look like this:



PART II

Running Comparative C_T Experiments

7 About Comparative C_T Experiments



This chapter covers:

- About Comparative C_T experiments 76
- About the example experiment 77

IMPORTANT! First-time users of the ViiA™ 7 System, please read Booklet 1, *Getting Started with ViiA™ 7 System Experiments* and Booklet 7, *ViiA™ 7 System Experiments - Appendixes* of this binder thoroughly. The booklets provide information and general instructions that are applicable to all the experiments described in this binder.

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems ViiA™ 7 Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ ViiA™ 7 Software Help**.

About Comparative C_T experiments

The Comparative CT ($\Delta\Delta C_T$) method is used to determine the relative target quantity in samples. With the comparative C_T method, the ViiA™ 7 Software measures amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the endogenous control. The software determines the relative quantity of target in each sample by comparing normalized C_T (ΔC_T) in each sample to normalized C_T (ΔC_T) in the reference sample.

Comparative C_T experiments are commonly used to:

- Compare expression levels of a gene in different tissues.
- Compare expression levels of a gene in a treated sample and an untreated sample.
- Compare expression levels of wild-type alleles and mutated alleles.
- Analyze the gene expression changes over time under specific treatment conditions.

Assemble required components

- **Sample** – The tissue group that you are testing for a target gene.
- **Reference sample (also called a calibrator)**– The sample used as the basis for relative quantification results. For example, in a study of drug effects on gene expression, an untreated control is an appropriate reference sample.
- **Endogenous control** – A gene that is used to normalize template input differences, and sample-to-sample or run-to-run variation.
- **Replicates** – The total number of identical reactions containing identical components and identical volumes.
- **Negative controls** – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

PCR options

When performing real-time PCR, choose between:

- Singleplex and multiplex PCR (below)

  and

- 1-step and 2-step RT-PCR (page 77)

Singleplex and Multiplex PCR

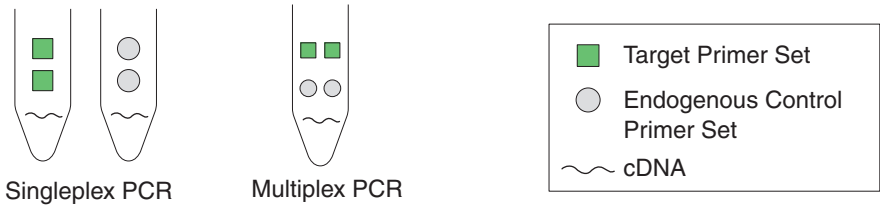
You can perform a PCR reaction using either:

- **Singleplex PCR** – In singleplex PCR a single primer and probe set is present in the reaction tube or well. Only one target or endogenous control can be amplified per reaction.

  Or

- **Multiplex PCR** – In multiplex PCR, two or more primer and probe sets are present in the reaction tube or well. Each set amplifies a specific target or endogenous control. Typically, a probe labeled with FAM™ dye detects the target and a probe labeled with VIC® dye detects the endogenous control.

IMPORTANT! SYBR® Green reagents cannot be used for multiplex PCR.



1- and 2-Step RT-PCR

You can perform reverse transcription (RT) and PCR in a single reaction (1-step) or in separate reactions (2-step). The reagent configuration you use depends on whether you are performing 1- or 2-step RT-PCR:

- **1-step RT-PCR**– In 1-step RT-PCR, RT and PCR take place in one buffer system. Using one buffer system provides the convenience of a single-tube preparation for RT and PCR amplification. However, you cannot use Fast PCR Master Mix or the carryover prevention enzyme, AmpErase® UNG (uracil-N-glycosylase), to perform 1-step RT-PCR.
- **2-step RT-PCR**– 2-step RT-PCR is performed in two separate reactions: First, total RNA is reverse-transcribed into cDNA, then the cDNA is amplified by PCR. This method is useful for detecting multiple transcripts from a single cDNA template or for storing cDNA aliquots for later use. The AmpErase® UNG enzyme can be used to prevent carryover contamination.

About the example experiment

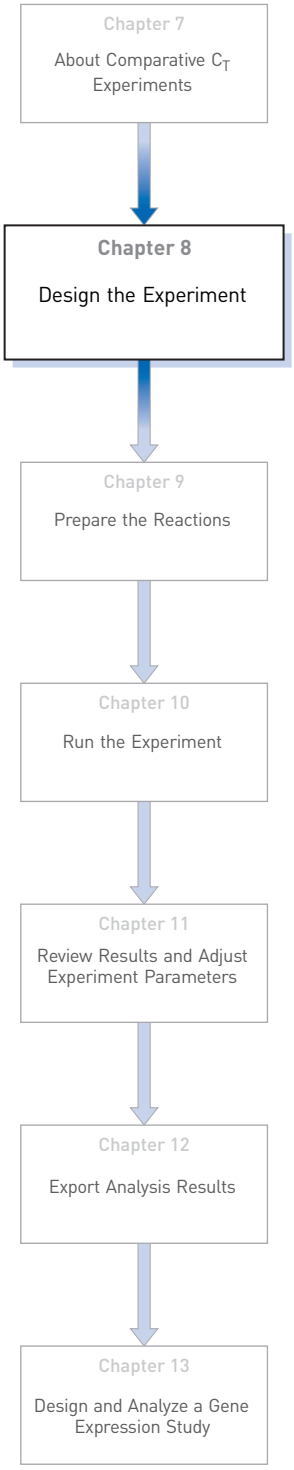
To illustrate how to perform comparative C_T experiment, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with the ViiA™ 7 System.

The objective of the comparative C_T example experiment is to compare the expression of GH1, LPIN1, LIPC, GAPDH, and ACTB in liver, heart, brain, and lung tissues.

- The samples are liver, heart, lung, and brain tissues.
- The targets are GH1, LPIN1, LIPC, GAPDH, and ACTB.
- The reference sample is brain.
- The endogenous control is ACTB.
- The experiment is designed for singleplex PCR, where the targets and endogenous control assays are performed in separate wells.

- Reactions are set up for 2-step RT-PCR. The Invitrogen VILO Kit is used for reverse transcription; the TaqMan® Fast Universal PCR Master Mix is used for PCR.
- Primer and probe sets are selected from the Applied Biosystems TaqMan® Gene Expression Assays product line:
  - GH1 Assay Mix: Hs00236859_m1
  - LPIN1 Assay Mix: Hs00299515_m1
  - LIPC Assay Mix: Hs00165106_m1
  - GAPDH Assay Mix: Hs99999905_m1
  - ACTB Assay Mix: Hs99999903_m1

8 Design the Experiment



This chapter explains how to design the example experiment from the Experiment Setup menu.

This chapter covers:

- Define the experiment properties. 80
- Define targets, samples and biological replicates 81
- Assign targets, samples and biological groups 83
- Set up the run method 84
- Order materials for the experiment 85
- Tips for designing your own experiment 86
- For more information. 87

Note: To automatically export the analyzed data to a specified location, select the Auto Export task from the Export screen, before running the experiment. For more information on Auto Export, refer to Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*.

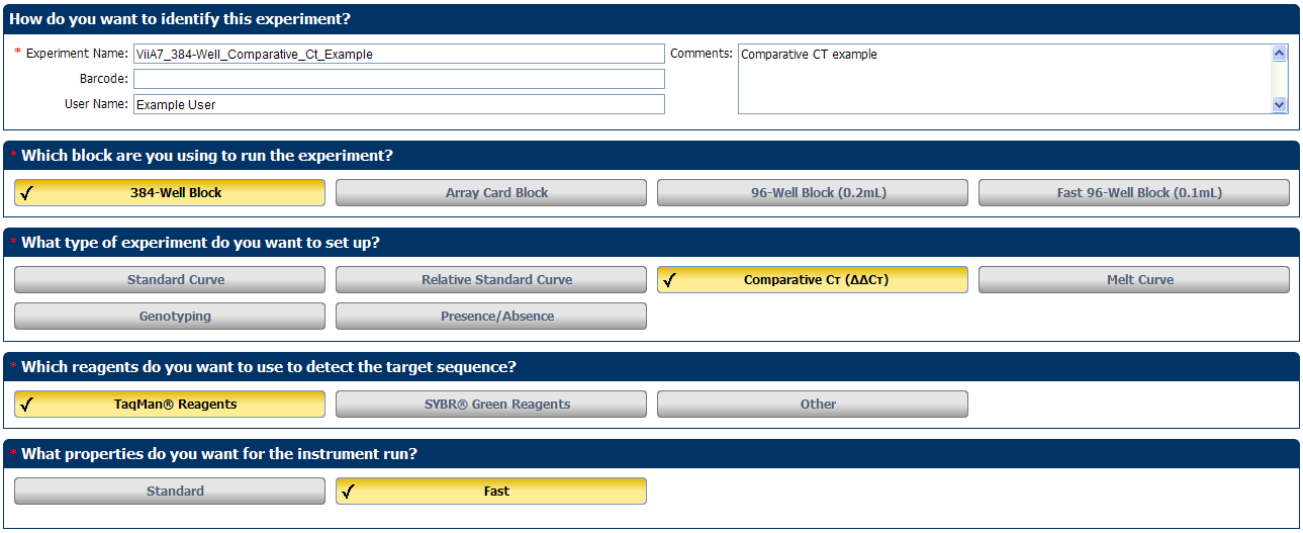
Define the experiment properties

Click **Experiment Setup** ▸ **Experiment Properties** to create a new experiment in the ViiA™ 7 Software. Enter:

Field or Selection	Entry
Experiment Name	ViiA7_384-Well_Comparative_Ct_Example
Barcode	Leave field empty
User Name	Example User
Comments	Comparative C_T example
Block	384-Well Block
Experiment Type	Comparative C_T ($\Delta\Delta C_T$)
Reagents	TaqMan® Reagents
Ramp speed	Fast

Save the experiment.

Your Experiment Properties screen should look like this:



Define targets, samples and biological replicates

Click **Define** to access the Define screen. Enter:

1. Targets

Target name	Reporter	Quencher	Color
ACTB	FAM	NFQ-MGB	
GAPDH	FAM	NFQ-MGB	
GH1	FAM	NFQ-MGB	
LPIN1	FAM	NFQ-MGB	
LIPC	FAM	NFQ-MGB	

2. Samples

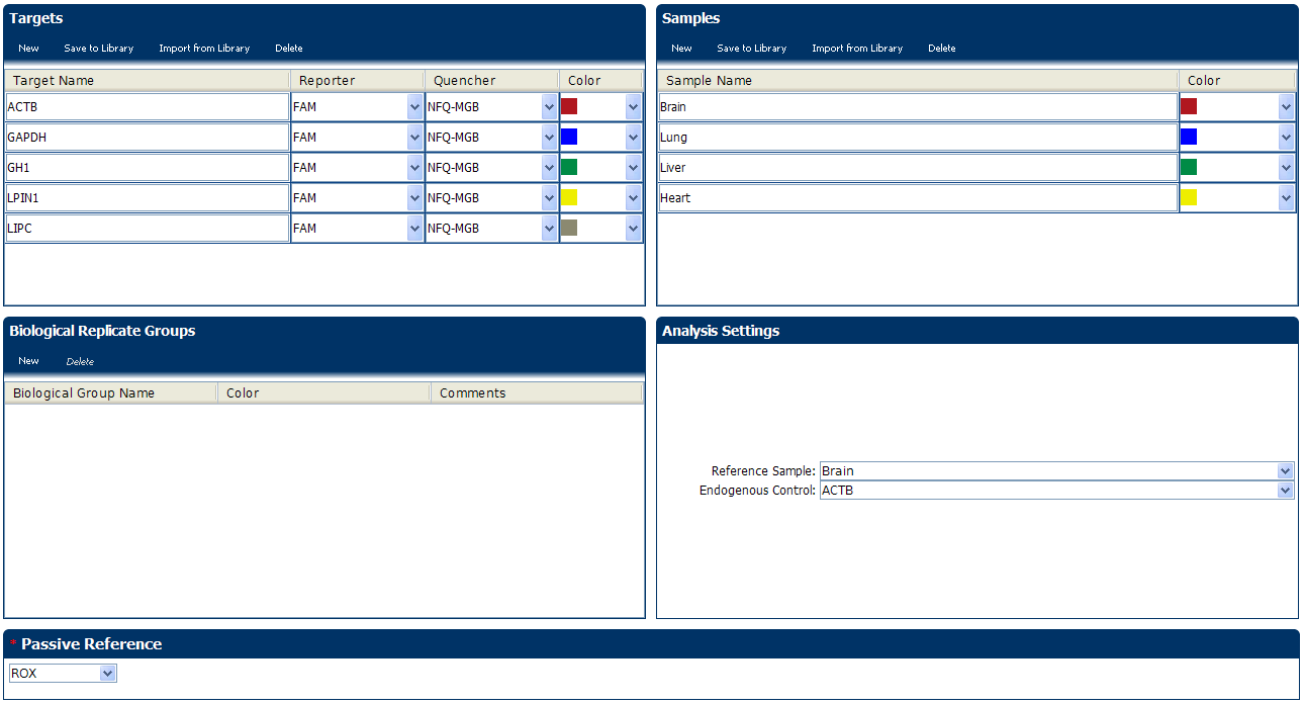
Sample Name	Color
Brain	
Lung	
Liver	
Heart	

3. Dye to be used as a Passive Reference
ROX

4. Analysis Settings

Field	Select
Reference Sample	Brain
Endogenous Control	ACTB

Your Define screen should look like this:



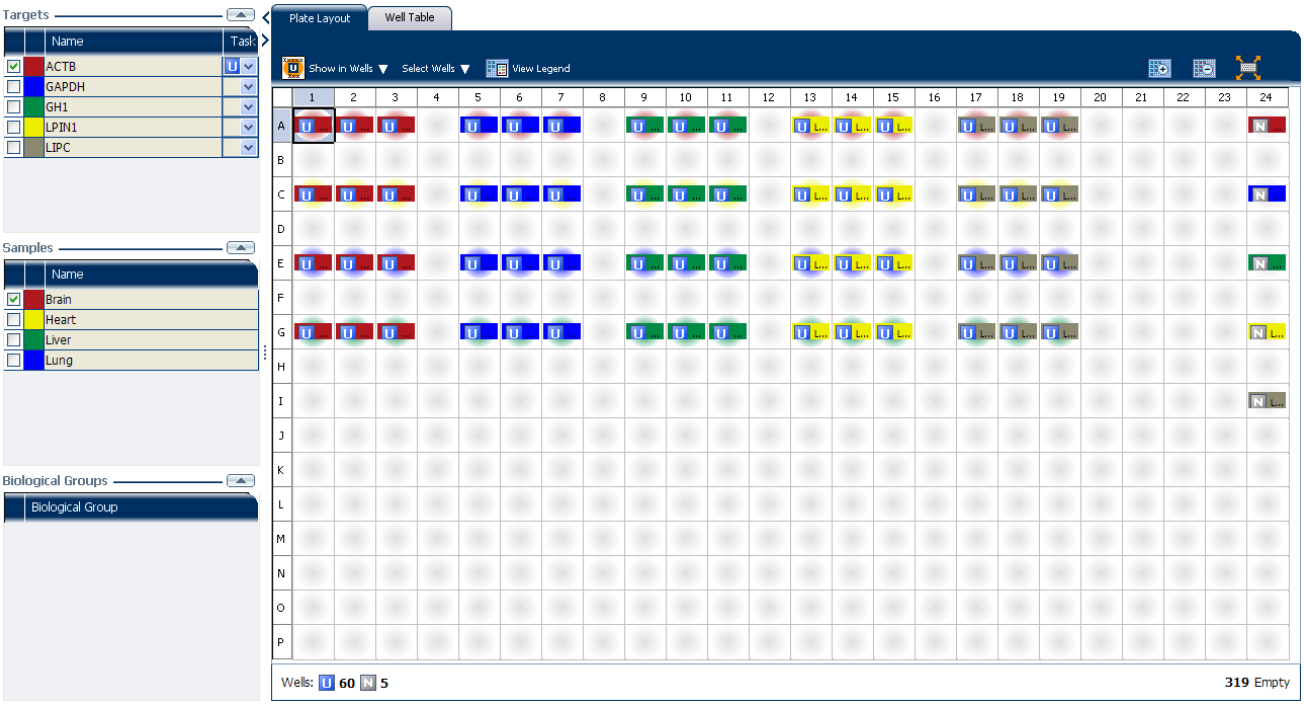
Note: This example experiment does not define biological replicate groups. Leave Biological Replicate Groups blank.

Assign targets, samples and biological groups

Click **Assign** to access the Assign screen. Enter the targets and samples:

Target name	Well number	Task	Sample name
ACTB	A1, A2, A3	Unknown	Brain
	C1, C2, C3	Unknown	Heart
	E1, E2, E3	Unknown	Lung
	G1, G2, G3	Unknown	Liver
	A24	Negative	None
GAPDH	A5, A6, A7	Unknown	Brain
	C5, C6, C7	Unknown	Heart
	E5, E6, E7	Unknown	Lung
	G5, G6, G7	Unknown	Liver
	C24	Negative	None
GH1	A9, A10, A11	Unknown	Brain
	C9, C10, C11	Unknown	Heart
	E9, E10, E11	Unknown	Lung
	G9, G10, G11	Unknown	Liver
	E24	Negative	None
LPIN1	A13, A14, A15	Unknown	Brain
	C13, C14, C15	Unknown	Heart
	E13, E14, E15	Unknown	Lung
	G13, G14, G15	Unknown	Liver
	G24	Negative	None
LIPC	A17, A18, A19	Unknown	Brain
	C17, C18, C19	Unknown	Heart
	E17, E18, E19	Unknown	Lung
	G17, G18, G19	Unknown	Liver
	I24 (Row I, Column 24)	Negative	None

Your Assign screen should look like this:



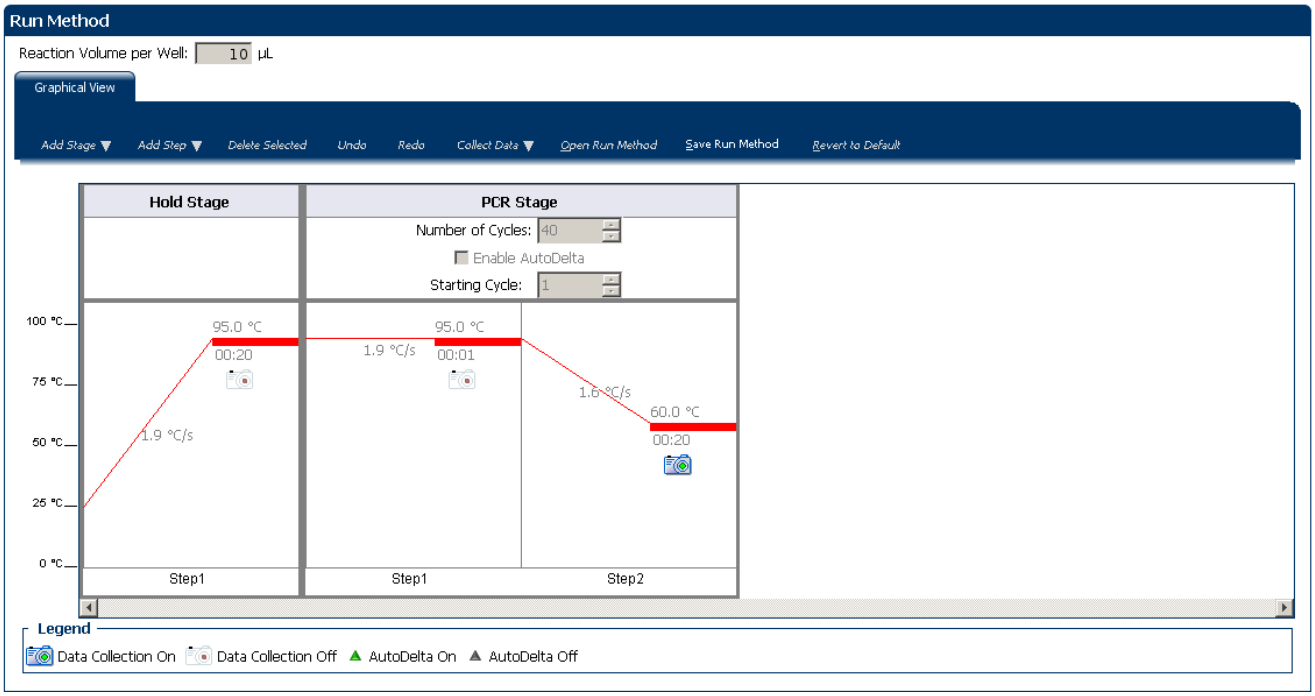
Set up the run method

Click **Run Method** to access the Run Method screen. Set the thermal profile under the Graphical View tab. Enter:

- Reaction Volume Per Well: 10µl
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Hold Stage	Step 1	1.9 °C/s	95 °C	20 seconds
PCR Stage	Step 1	1.9 °C/s	95 °C	1 second
Number of Cycles: 40 Enable AutoDelta: Unchecked (default) Starting Cycle: Disabled when Enable AutoDelta is unchecked	Step 2	1.6 °C/s	60 °C	20 seconds

Your Run Method screen should look like this:

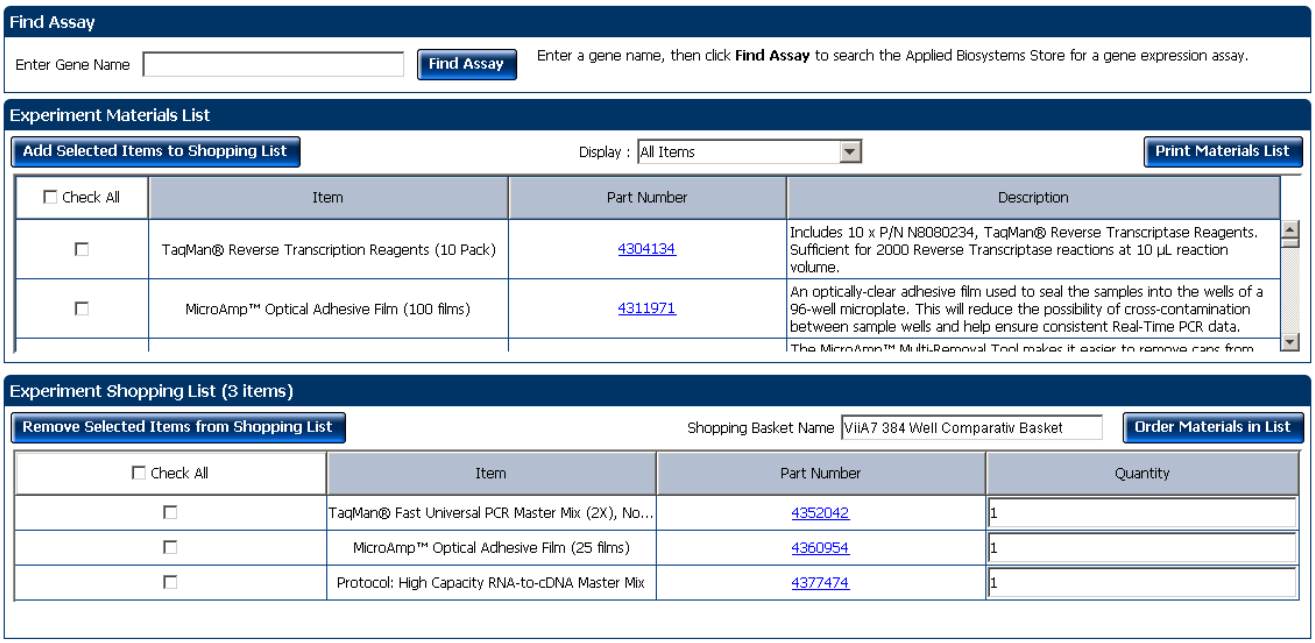


Order materials for the experiment

The recommended materials are:

- MicroAmp® Optical 384-Well Reaction Plate
- MicroAmp® Optical Adhesive Film
- TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG
- GH1 Assay Mix: Hs00236859_m1
- LPIN1 Assay Mix: Hs00299515_m1
- LIPC Assay Mix: Hs00165106_m1
- GAPDH Assay Mix: Hs99999905_m1
- ACTB Assay Mix: Hs99999903_m1

Your Materials List screen should look like this:



Tips for designing your own experiment

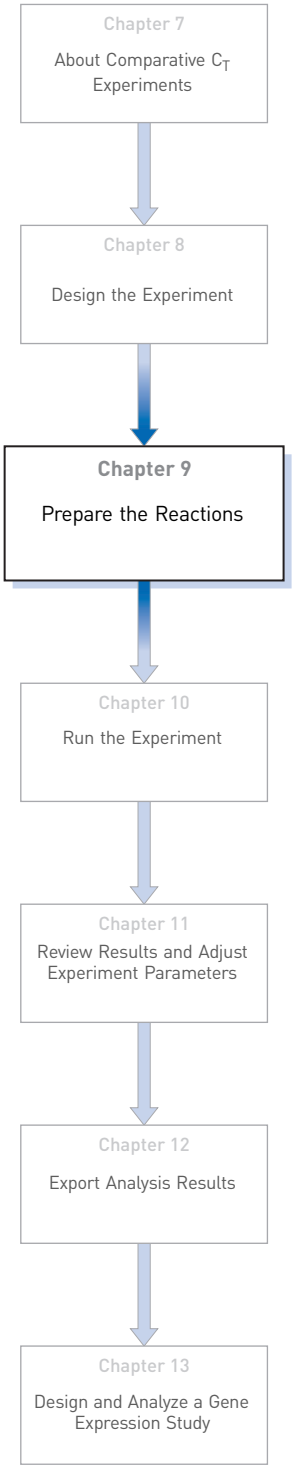
Applied Biosystems recommends that you:

- Identify each target assay with a unique name and color. You can enter up to 100 characters in the Target Name field.
- Identify each sample using a unique name and color. You can enter up to 100 characters in the Sample Name field.
- Select an endogenous control for each sample. The endogenous control is a target that is present in all samples under investigation. It should be expressed equally in all sample types, regardless of treatment or tissue origin (examples of endogenous controls are β-actin, GAPDH, and 18S ribosomal RNA [18S rRNA]). The endogenous control is used to normalize the PCR results; the endogenous control corrects for variable sample mass, nucleic acid extraction efficiency, reverse transcription efficiency, and pipette calibration errors. Note that:
  - Each sample type (for example, each tissue in a study comparing multiple tissues) requires an endogenous control.
  - If samples are spread across multiple plates, each plate must have an endogenous control. Additionally, every plate must include an endogenous control for every sample type on the plate.
- Select an endogenous control from your previously defined target assays. Amplification results from the endogenous control are used to normalize the amplification results from the target for differences in the amount of template added to each reaction.
- Select a reference sample from your previously defined samples. Amplification results from the samples and from the reference sample are compared to determine relative expression.

For more information

For more information on	Refer to	Part number
Consumables	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments* Appendix A in Booklet 7, *ViiA™ 7 System Experiments - Appendixes*	4441434
Using the Standard Curve quantification methods	Booklet 2, *Running Standard Curve Experiments*.	4441434
Using the relative standard curve quantification method	Part 1 of this booklet	4441434
Selecting an endogenous control	Application Note *Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies*	127AP05-03
Reference samples (also known as calibrators) and endogenous controls	*User Bulletin #2: Relative quantification of Gene Expression*	4303859
Using alternative setup	Chapter 2 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

9 Prepare the Reactions



This chapter explains how to prepare the PCR reactions for the Comparative C_T ($\Delta\Delta C_T$) example experiment.

This chapter covers:

- Assemble required materials 90
- Prepare the template 90
- Prepare the sample dilutions 90
- Prepare the reaction mix (“cocktail mix”). 91
- Prepare the reaction plate 92
- Tips for preparing reactions for your own experiments. 93
- For more information. 93

Assemble required materials

- Items listed in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*
- Samples - Total RNA isolated from liver, heart, brain, and lung tissues.
- Example experiment reaction mix components:
  - TaqMan® Fast Universal PCR Master Mix (2×.)
  - ACTB Assay Mix (20×)
  - GAPDH Assay Mix (20×)
  - GH1 Assay Mix (20×)
  - LIPN1 Assay Mix (20×)
  - LIPC Assay Mix (20×)

Prepare the template

Prepare the template for the PCR reactions using the High-Capacity cDNA Reverse Transcription Kit or one of the Invitrogen VILO kits to carry out the reverse transcription.

Example experiment settings

For the Comparative C_T example experiment, the template for the PCR reactions is cDNA reverse-transcribed from total RNA samples using one of the Invitrogen VILO kits, SuperScript® VILO™ cDNA Synthesis Kit (PN 4453650).

Prepare the template

Use the Invitrogen VILO kits to reverse-transcribe cDNA from the total RNA samples. Follow the procedures in the *Invitrogen VILO Kits Protocol* (PN 100002284) to:

1. Prepare the RT master mix.
2. Prepare the cDNA reactions.
3. Perform reverse transcription on a thermal cycler.

Prepare the sample dilutions

For the Comparative C_T example experiment, no more than 10% of your reaction should consist of the undiluted RT product.

1. Label a separate microcentrifuge tube for each diluted sample:
   - Liver
   - Heart
   - Brain
   - Lung

2. Add the required volume of water (diluent) to each empty tube:

Tube	Sample name	Diluent volume (µL)
1	Liver	19
2	Heart	19
3	Brain	19
4	Lung	19

3. Add the required volume of cDNA sample stock (100 ng/µL) to each empty tube:

Tube	Sample name	Volume (µL)
1	Liver	1.0
2	Heart	1.0
3	Brain	1.0
4	Lung	1.0

4. Vortex each diluted sample for 3 to 5 seconds, then centrifuge the tubes briefly.

5. Place the diluted samples on ice until you prepare the reaction plate.

Prepare the reaction mix ("cocktail mix")

1. Label an appropriately sized tube for each reaction mix:
   - ACTB Reaction Mix
   - GAPDH Reaction Mix
   - GH1 Reaction Mix
   - LPIN1 Reaction Mix
   - LIPC Reaction Mix

2. For the ACTB assay, add the required volumes of each component to the ACTB Reaction Mix tube:

Component	Volume (µL) for 1 reaction	Volume (µL) for 13 reactions (plus 10% excess)
TaqMan® Fast Universal PCR Master Mix (2X)	5.0	75.0
ACTB Assay Mix (20X)	0.5	7.5
Water	3.5	52.5
Total Reaction Mix Volume	9.0	135.0

3. Mix the reaction mix in each tube by gently pipetting up and down, then cap each tube.

4. Centrifuge the tubes briefly to remove air bubbles.

5. Place the reaction mixes on ice until you prepare the reaction plate.

6. Repeat steps 2 through 5 for the GAPDH, GH1, LPIN1, and LIPC assays.

   Note: Do not add the sample at this time.

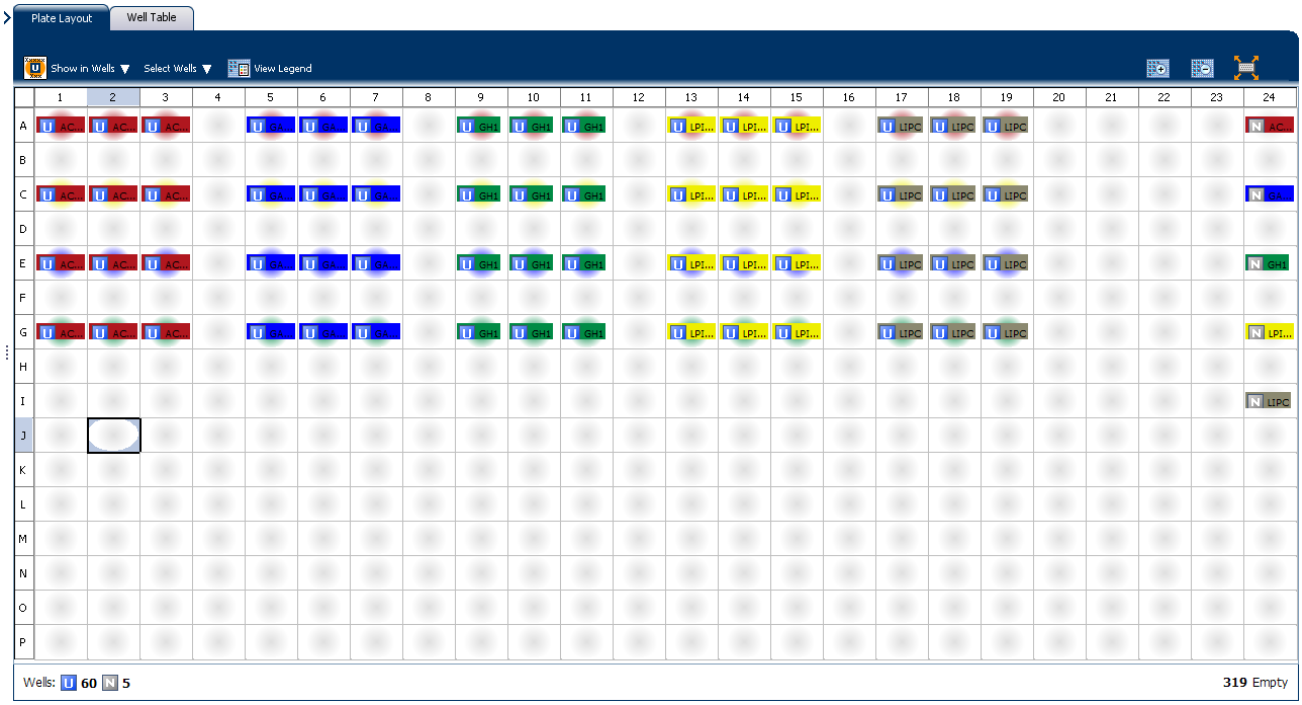
Prepare the reaction plate

Example experiment reaction plate components

The reaction plate for the Comparative C_T example experiment contains:

- A MicroAmp® Optical 384-Well Reaction Plate
- Reaction volume: 10µL/well
- The reaction plate contains:
  - 60 Unknown wells U
  - 5 Negative control wells N
  - 319 Empty wells

The plate layout for the example experiment looks like this:



To prepare the reaction plate components

1. Add 1µL of each cDNA to the appropriate wells.

2. Pipette 1µL of sterile water into the NTC wells.

3. Add 8µL of the appropriate assay-specific cocktail to the wells.

4. Seal the reaction plate with optical adhesive film.
5. Centrifuge the reaction plate briefly to remove air bubbles.
6. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.
7. Until you are ready to perform the run, place the reaction plate at 4 °C, in the dark.

Tips for preparing reactions for your own experiments

Tips for preparing templates

When you prepare your own Comparative C_T experiment, Applied Biosystems recommends the following templates:

- **Complementary DNA (cDNA)** – cDNA reverse-transcribed from total RNA samples.
- **Genomic DNA (gDNA)** – Purified gDNA already extracted from tissue or sample.

Tips for preparing the reaction mix

If your experiment includes more than one target assay, prepare the reaction mix for each target assay separately.

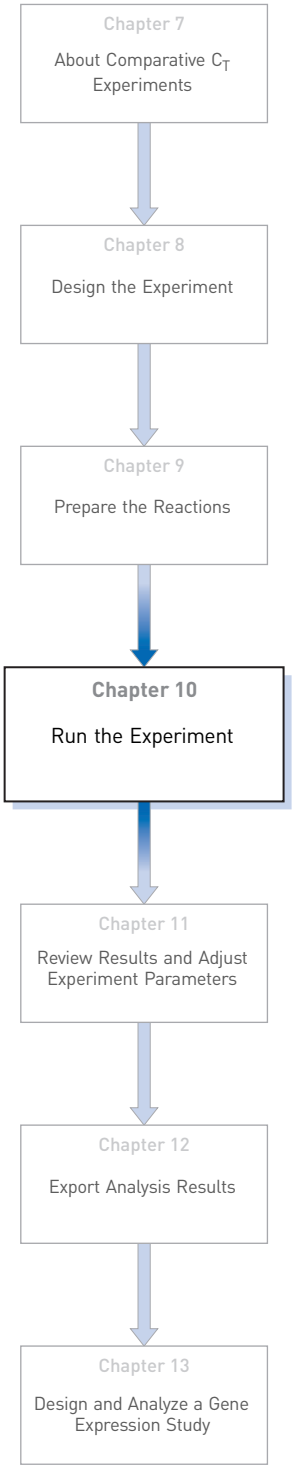
Tips for preparing the reaction plate

When you prepare your own Comparative C_T experiment, make sure the arrangement of the PCR reactions matches the plate layout displayed in the ViiA™ 7 Software.

For more information

For more information on...	Refer to...	Part number
Assigning the reaction plate components	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

10 Run the Experiment



This chapter explains how to run the example experiment on the ViiA™ 7 Instrument.

This chapter covers:

- Start the run 96
- Monitor the run 96

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the ViiA™ 7 Instrument is in operation.

Start the run

1. Open the Comparative C_T example file that you created using instructions in Chapter 8.
2. Load the reaction plate into the instrument.
3. Start the run.

Monitor the run

Monitor the example experiment run:

- From the ViiA™ 7 Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the ViiA™ 7 Software (to monitor an experiment started from another computer or from the ViiA™ 7 Instrument touchscreen).
- From the ViiA™ 7 Instrument touchscreen.

From the Instrument Console of the ViiA™ 7 Software

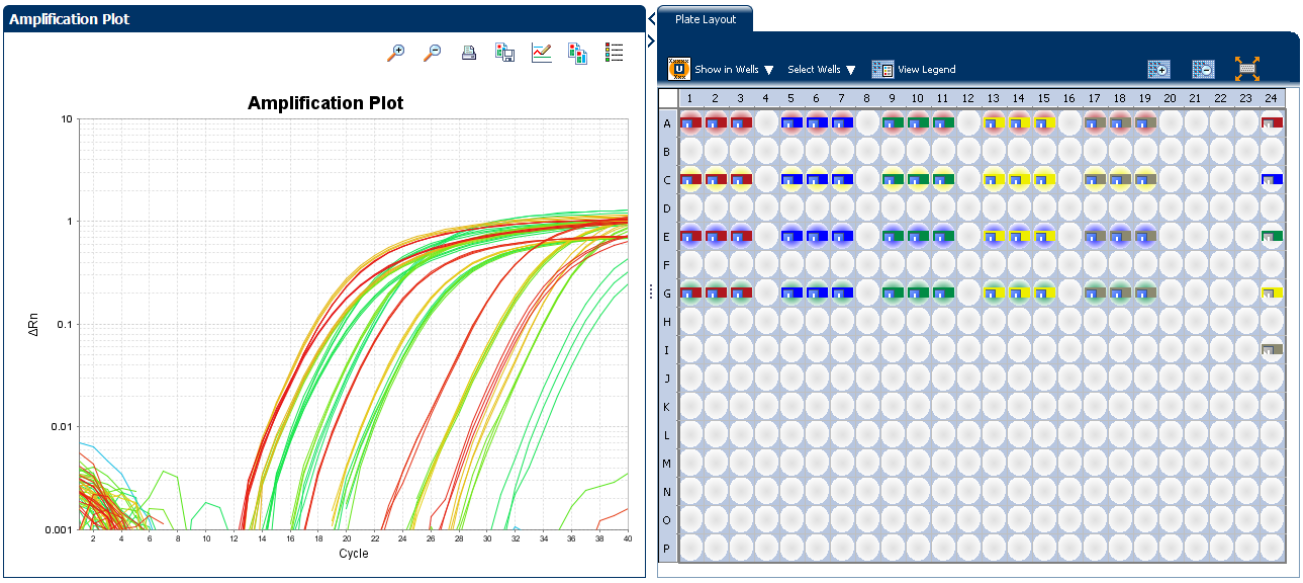
1. In the Instrument Console screen, select the instrument icon.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. In the Manage Instrument screen, click **Monitor Running Experiment** to access the Run screen.

View the Amplification Plot

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the ViiA™ 7 Software for potential problems.

Click **Amplification Plot** from the Run Experiment Menu, select the Plate Layout tab, then select the wells t to view.

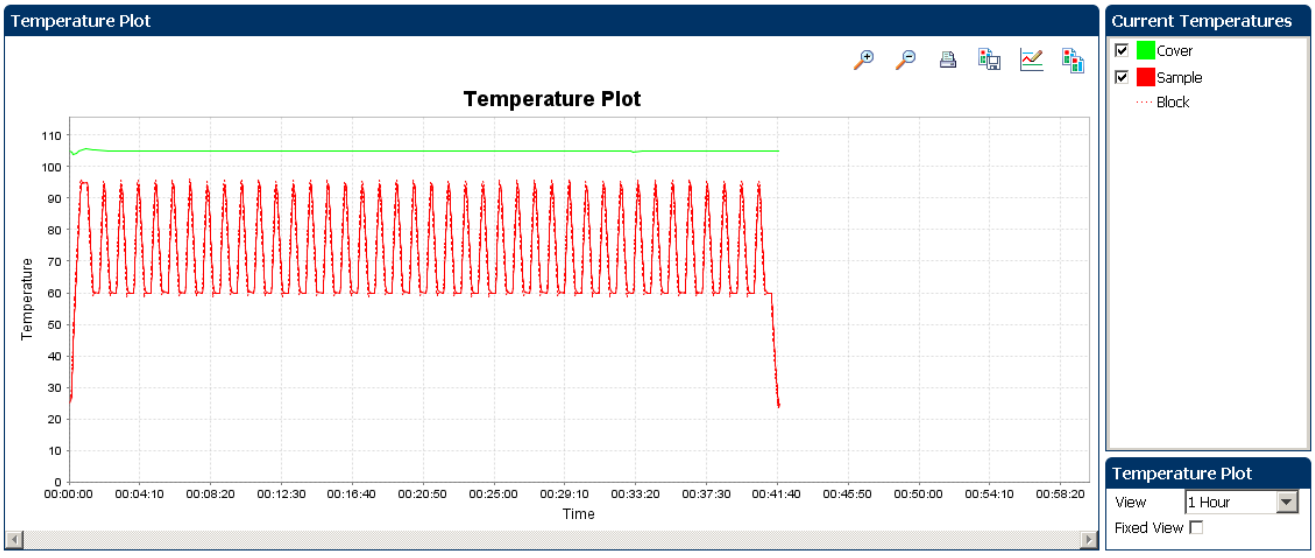
The figure below shows the Amplification Plot screen as it appears at the end of the example experiment.



View the Temperature Plot

Click **Temperature Plot** from the Run Experiment Menu.

The figure below shows the Temperature Plot screen as it appears during the example experiment.

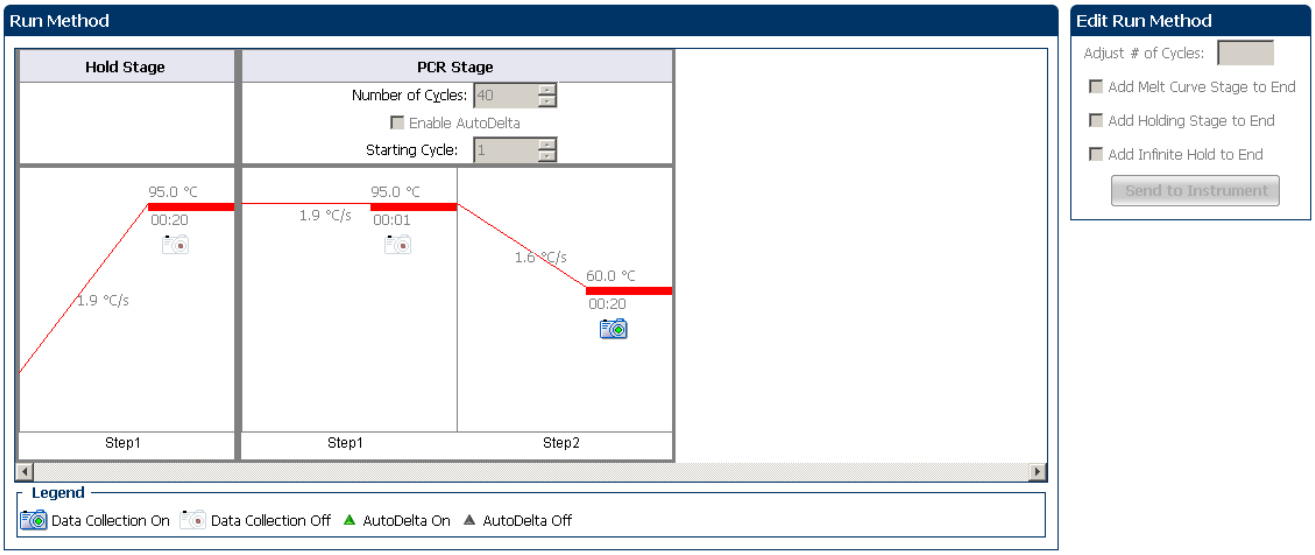


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

View the Run Method

Click **Run Method** from the Run Experiment Menu.

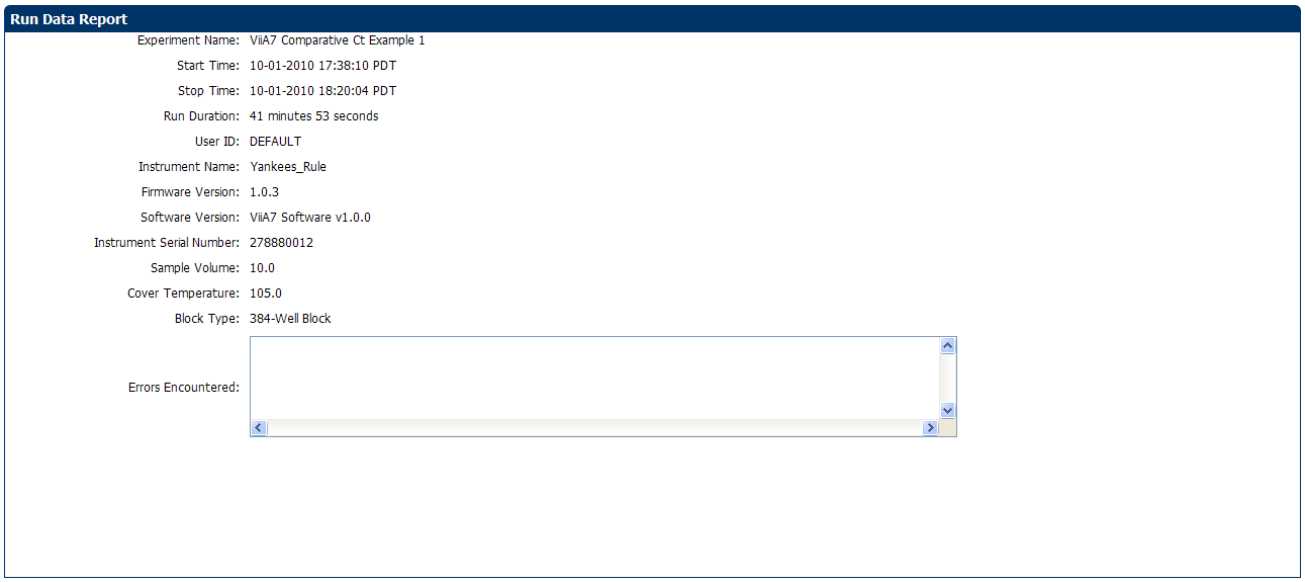
The figure below shows the Run Method screen as it appears in the example experiment.



View Run Data

Click **View Run Data** from the Run Experiment Menu.

Your View Run Data screen should like this:

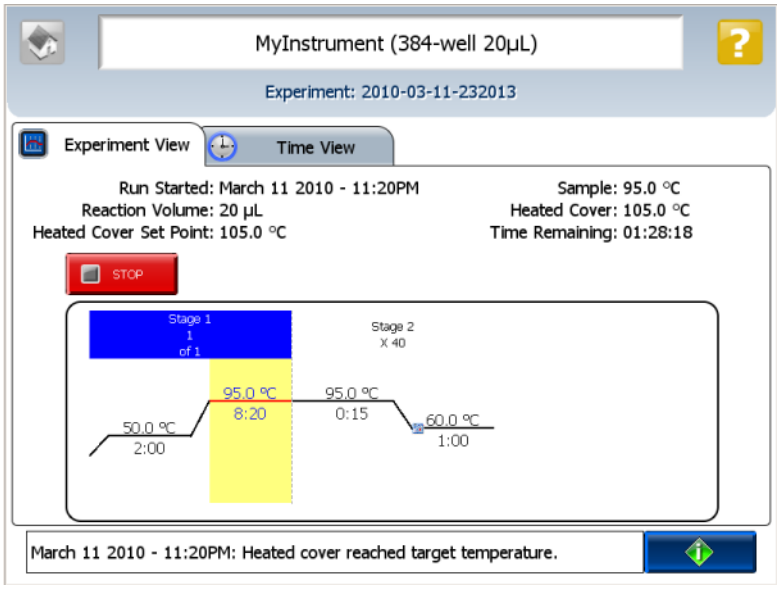


From the ViiA™ 7 Instrument touchscreen

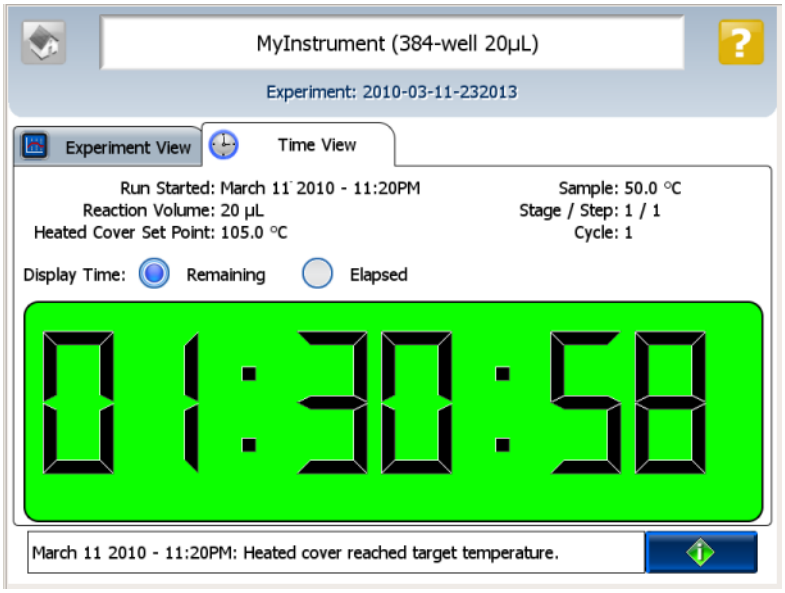
You can also view the progress of the run from the touchscreen of the ViiA™ 7 Instrument.

The Run Method screen on the ViiA™ 7 Instrument touchscreen looks like this:

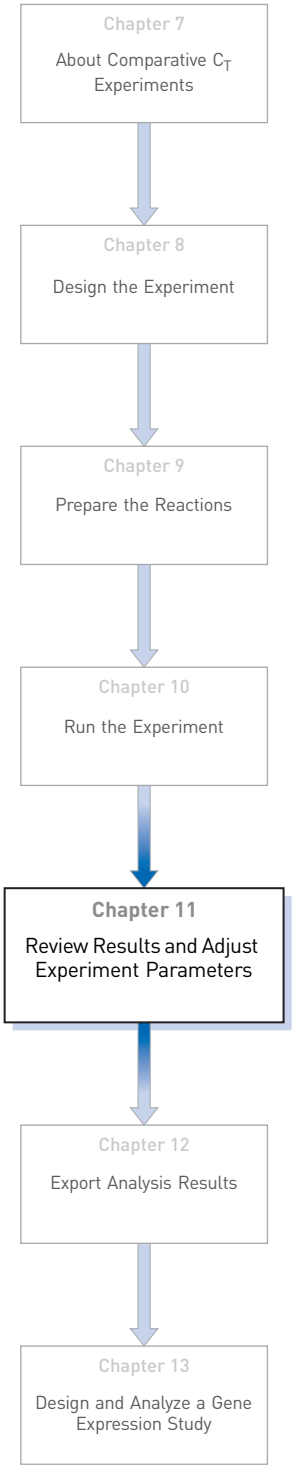
Experiment view



Time View



11 Review Results and Adjust Experiment Parameters



In Section 11.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 11.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

- **Section 11.1 Review Results 103**
- Analyze the example experiment. 103
- Assess the gene expression profile using the Gene Expression Plot. 103
- Identify well problems using the Well Table 105
- Assess amplification results using the Amplification Plot 107
- Confirm accurate dye signal using the Multicomponent Plot 114
- Determine signal accuracy using the Raw Data Plot 116
- View the endogenous control profile using the QC Plot 118
- Review the flags in the QC Summary 119
- For more information. 121
- **Section 11.2 Adjust parameters for re-analysis of your own experiments . . 123**
- Adjust analysis settings 123
- Improve C_T precision by omitting wells 127

11.1

Section 11.1 Review Results

Analyze the example experiment

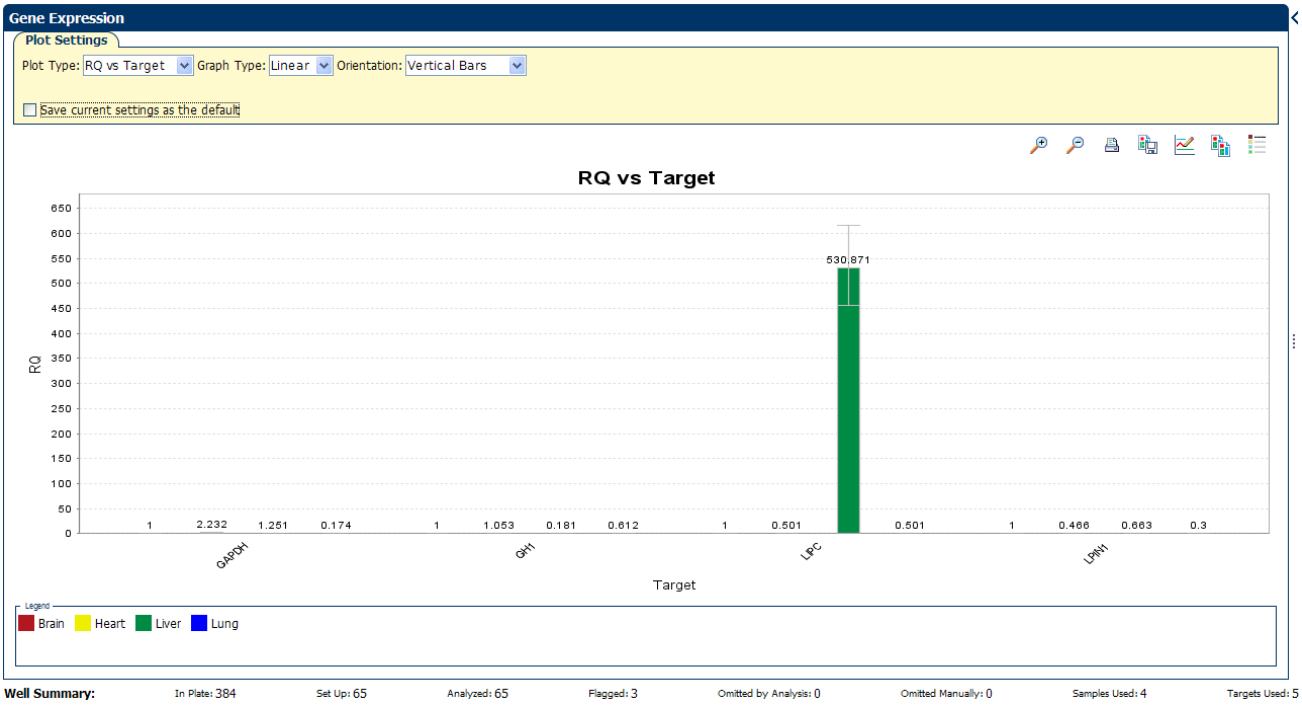
1. Open the example experiment file that you ran in Chapter 10.
2. Click **Analyze**. The software analyzes the data using the default analysis settings.

   Note: You can also access the experiment to analyze from the Home screen.

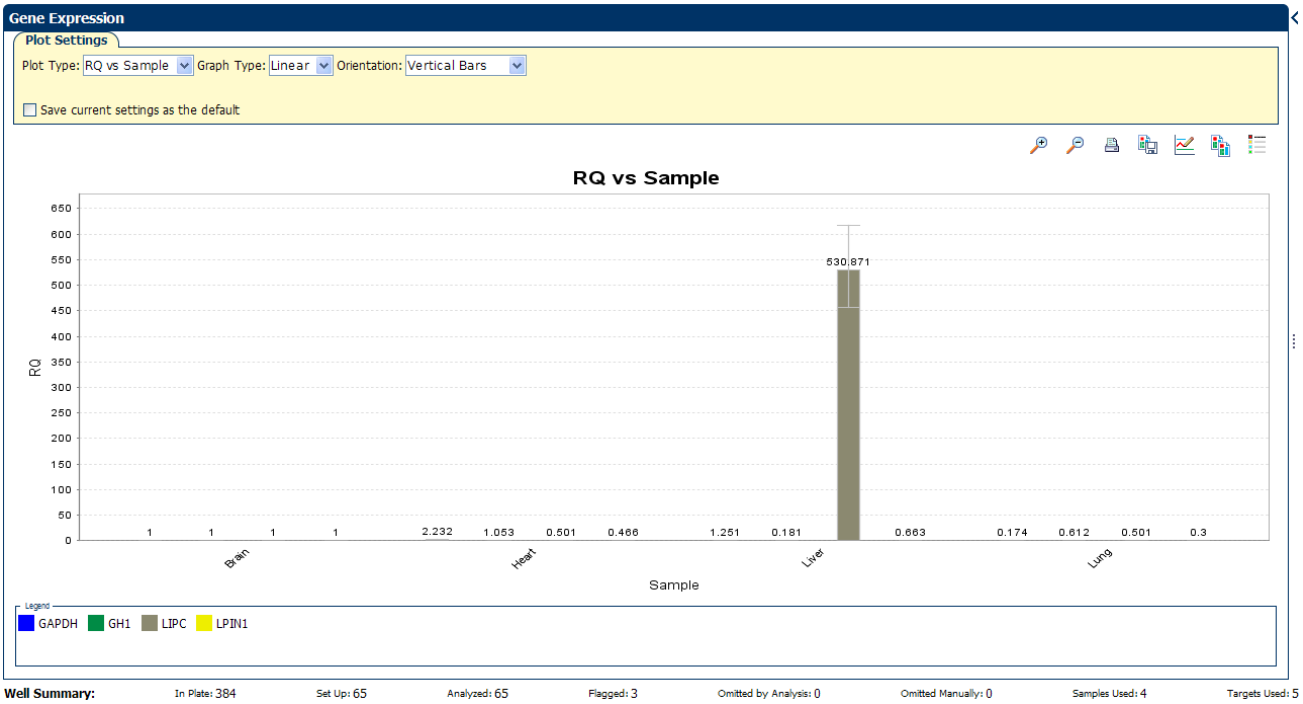
Assess the gene expression profile using the Gene Expression Plot

The Gene Expression Plot screen displays the results of relative quantification calculations in the gene expression profile. There are two plots available:

- **RQ vs Target** – Groups the relative quantification (RQ) values by target. Each sample is plotted for each target. You can view the plot as the linear, log10, Ln, and log2 graph types. The Gene Expression plot when viewed as a linear graph type looks like this:



- **RQ vs Sample** – Groups the relative quantification (RQ) values by sample. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.



Example experiment values

Review each target in the Gene Expression Plot screen for the expression level (or fold change) of the target sample relative to the reference sample.

View the Gene Expression Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Gene Expression**.

   Note: If no data are displayed, click **Analyze**.

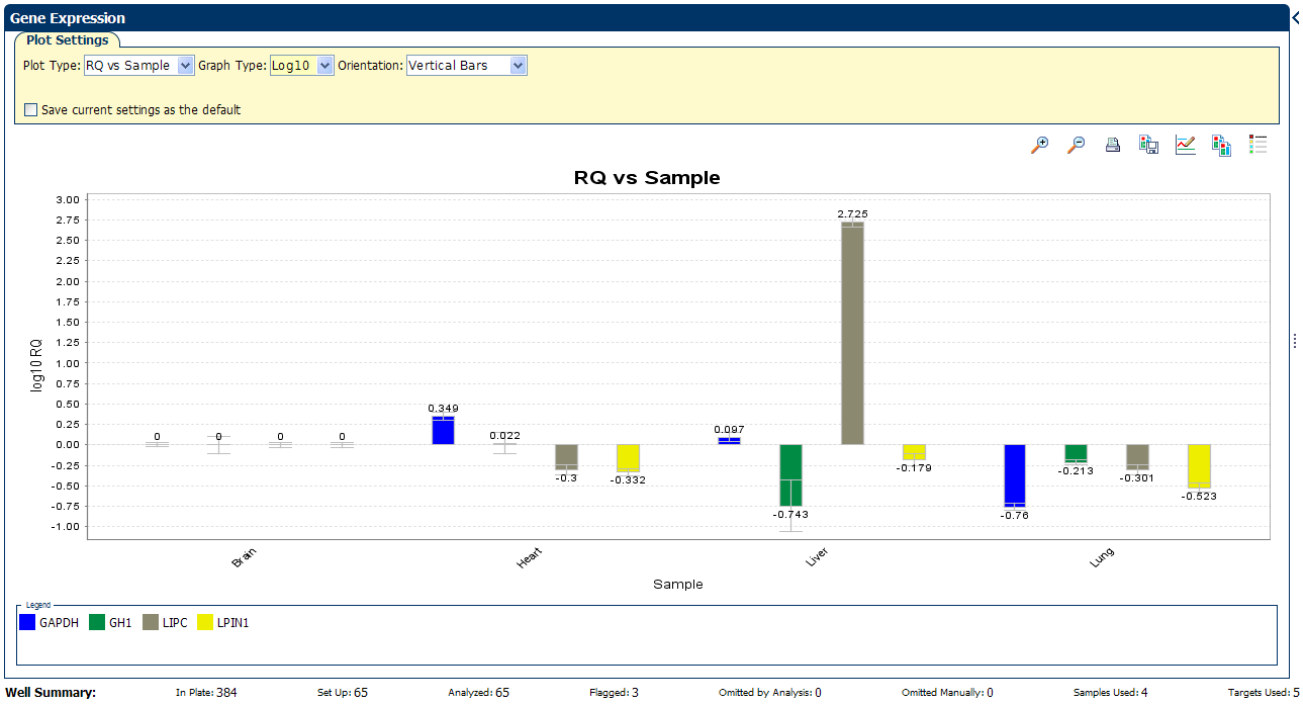
2. In the Gene Expression Plot screen, select:

Menu	Selection
Plot Type	RQ vs Sample (default)
Graph Type	Log10
Orientation	Vertical Bars

3. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

In the example experiment, as shown below, the expression level of each target gene in liver, heart, and lung is displayed relative to its respective expression level in the reference sample (brain). Since the reference sample is compared to itself, the relative expression level is 1. When the result is displayed in the Log10 graph type, the expression level of the reference sample appears as 0 in the graph (log10 of 1 = 0).



Assessing the gene expression plot your own experiments

When you analyze your own Comparative C_T experiment, look for differences in gene expression (as a fold change) relative to the reference sample.

Identify well problems using the Well Table

The Well Table displays data for each well in the reaction plate, including:

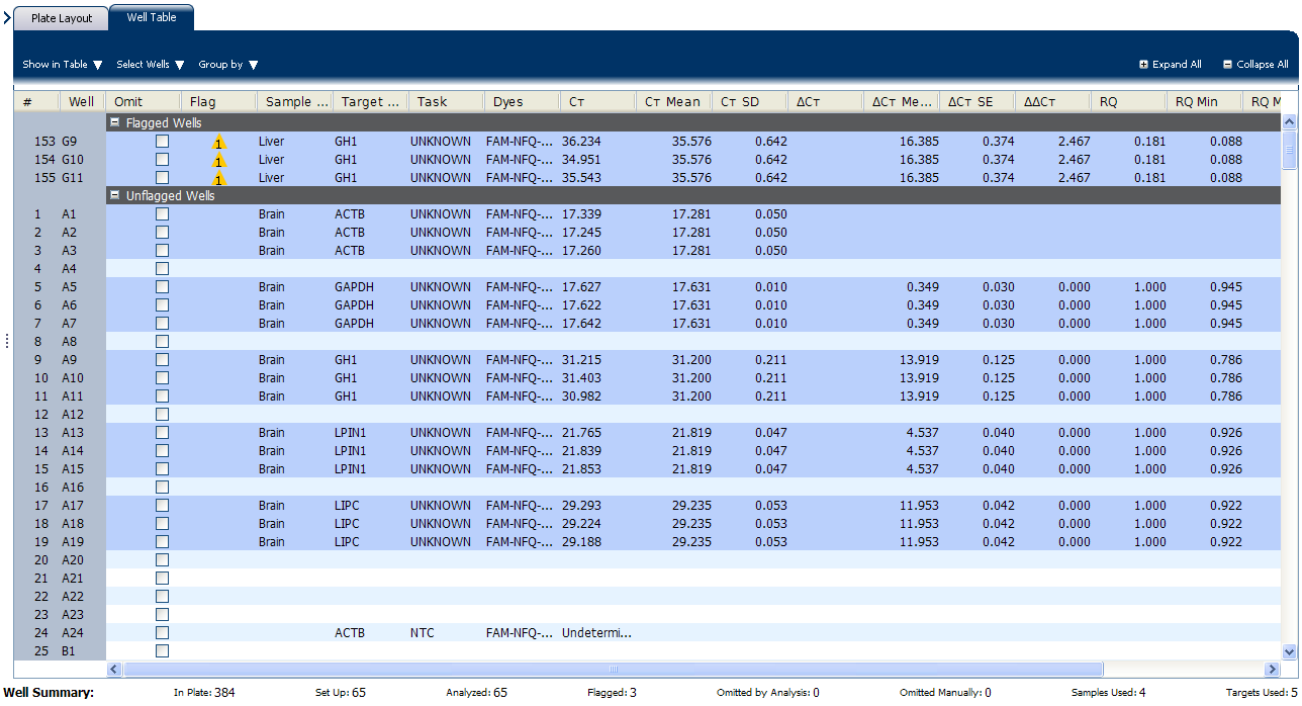
- The sample name, target name, task, and dyes
- The calculated threshold cycle (C_T), normalized fluorescence (Rn), and quantity values
- Flags

Example experiment values and flags

Review the Well Table to evaluate the C_T precision of the replicate groups.

View the well table

1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**, then click the **Well Table** tab.
2. From the Group By drop-down menu, select **Replicate**.
3. Look at the C_T SD column to evaluate the C_T precision of the replicate groups. In the example experiment, there are three outliers. You will omit these wells in the troubleshooting section ("Improve C_T precision by omitting wells" on page 127).



#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	CT	CT Mean	CT SD	ΔCT	ΔCT Me...	ΔCT SE	ΔΔCT	RQ	RQ Min	RQ M
Flagged Wells																	
153	G9	☐	1	Liver	GH1	UNKNOWN	FAM-NFQ-...	36.234	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
154	G10	☐	1	Liver	GH1	UNKNOWN	FAM-NFQ-...	34.951	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
155	G11	☐	1	Liver	GH1	UNKNOWN	FAM-NFQ-...	35.543	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
Unflagged Wells																	
1	A1	☐		Brain	ACTB	UNKNOWN	FAM-NFQ-...	17.339	17.281	0.050							
2	A2	☐		Brain	ACTB	UNKNOWN	FAM-NFQ-...	17.245	17.281	0.050							
3	A3	☐		Brain	ACTB	UNKNOWN	FAM-NFQ-...	17.260	17.281	0.050							
4	A4	☐															
5	A5	☐		Brain	GAPDH	UNKNOWN	FAM-NFQ-...	17.627	17.631	0.010		0.349	0.030	0.000	1.000	0.945	
6	A6	☐		Brain	GAPDH	UNKNOWN	FAM-NFQ-...	17.622	17.631	0.010		0.349	0.030	0.000	1.000	0.945	
7	A7	☐		Brain	GAPDH	UNKNOWN	FAM-NFQ-...	17.642	17.631	0.010		0.349	0.030	0.000	1.000	0.945	
8	A8	☐															
9	A9	☐		Brain	GH1	UNKNOWN	FAM-NFQ-...	31.215	31.200	0.211		13.919	0.125	0.000	1.000	0.786	
10	A10	☐		Brain	GH1	UNKNOWN	FAM-NFQ-...	31.403	31.200	0.211		13.919	0.125	0.000	1.000	0.786	
11	A11	☐		Brain	GH1	UNKNOWN	FAM-NFQ-...	30.982	31.200	0.211		13.919	0.125	0.000	1.000	0.786	
12	A12	☐															
13	A13	☐		Brain	LPIN1	UNKNOWN	FAM-NFQ-...	21.765	21.819	0.047		4.537	0.040	0.000	1.000	0.926	
14	A14	☐		Brain	LPIN1	UNKNOWN	FAM-NFQ-...	21.839	21.819	0.047		4.537	0.040	0.000	1.000	0.926	
15	A15	☐		Brain	LPIN1	UNKNOWN	FAM-NFQ-...	21.853	21.819	0.047		4.537	0.040	0.000	1.000	0.926	
16	A16	☐															
17	A17	☐		Brain	LIPC	UNKNOWN	FAM-NFQ-...	29.293	29.235	0.053		11.953	0.042	0.000	1.000	0.922	
18	A18	☐		Brain	LIPC	UNKNOWN	FAM-NFQ-...	29.224	29.235	0.053		11.953	0.042	0.000	1.000	0.922	
19	A19	☐		Brain	LIPC	UNKNOWN	FAM-NFQ-...	29.188	29.235	0.053		11.953	0.042	0.000	1.000	0.922	
20	A20	☐															
21	A21	☐															
22	A22	☐															
23	A23	☐															
24	A24	☐			ACTB	NTC	FAM-NFQ-...	Undetermi...									
25	B1	☐															

Note: To show/hide columns in the Well Table, select/deselect the column name from the Show in Table drop-down menu.

Assessing the well table in your own experiments

When you analyze your own Comparative C_T experiment, look for standard deviation in the replicate groups (C_T SD values). If needed, omit outliers (see "Improve C_T precision by omitting wells" on page 127).

Assess amplification results using the Amplification Plot

Amplification plots available for viewing

The Amplification Plot screen displays amplification of all samples in the selected wells. There are three plots available:

- **ΔRn vs Cycle** – ΔRn is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. This plot displays ΔRn as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle** – Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays Rn as a function of cycle number. You can use this plot to identify and examine irregular amplification.
- **C_T vs Well** – C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. You can use this plot to locate outlying amplification (outliers).

Each plot can be viewed as a linear or log10 graph type.

Purpose

The purpose of viewing the amplification plot for the example experiment is to identify:

- Correct baseline and threshold values
- Outliers

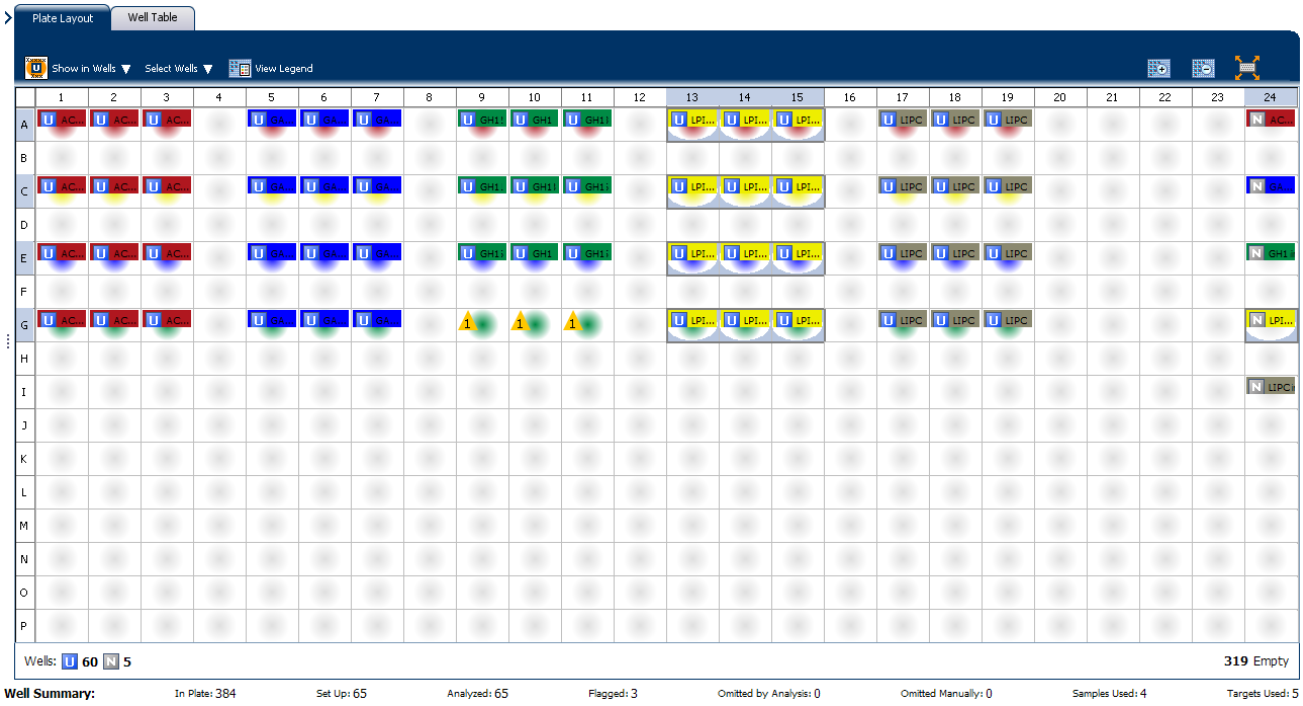
View the Amplification Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the LP1N1 wells in the Amplification Plot screen:
   a. Click the **Plate Layout** tab.
   b. From the Select Wells drop-down menus, select **Target**, then **LP1N1**.

The Plate Layout screen should look like this:



3. In the Amplification Plot screen:, enter:

Menu	Selection
Plot Type	ΔRn vs Cycle (default)
Plot Color	Well (default)
(This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.)	Check (default)

4. View the baseline values:
   a. From the Graph Type drop-down menu, select **Linear**.
   b. Select the **Baseline** check box to show the start cycle and end cycle.

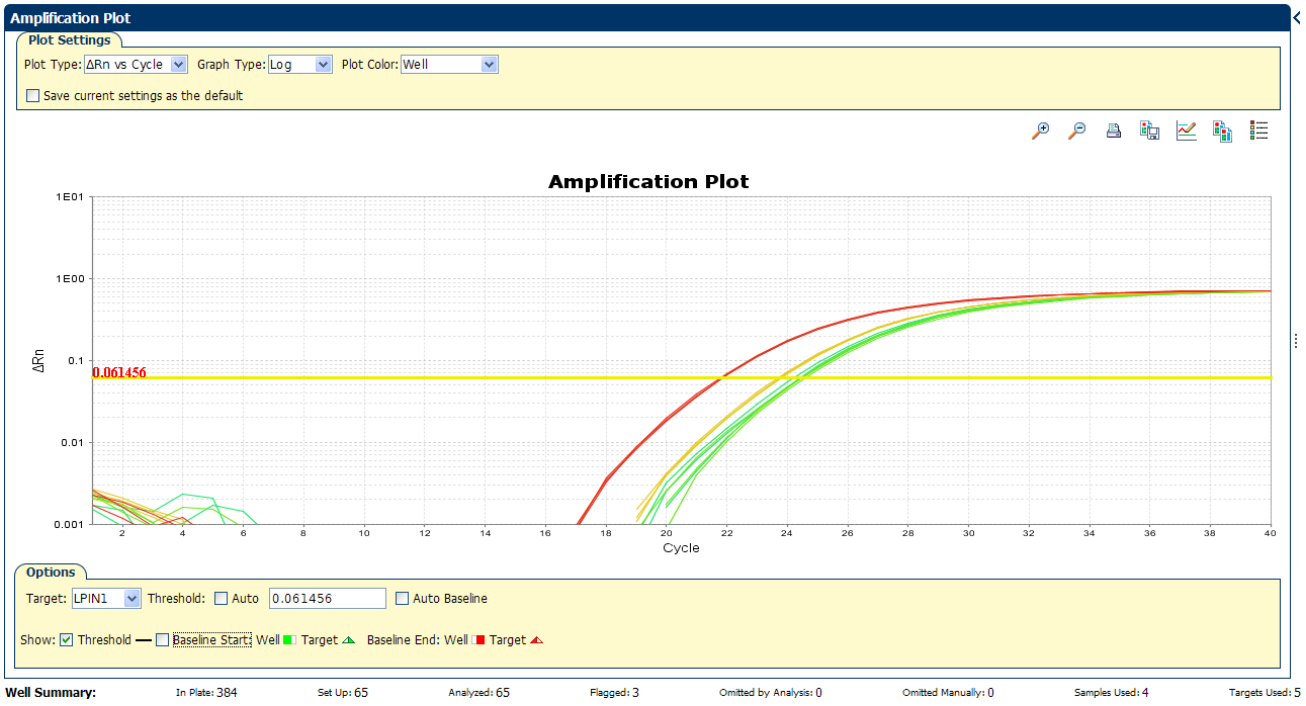
c. Verify that the baseline is set correctly: The end cycle should be set a few cycles before the cycle number where significant fluorescent signal is detected. In the example experiment, the baseline is set correctly.

5. View the threshold values:

Menu	Select
Graph Type	Log
Target	LP1N1

a. Select the **Threshold** check box to show the threshold.

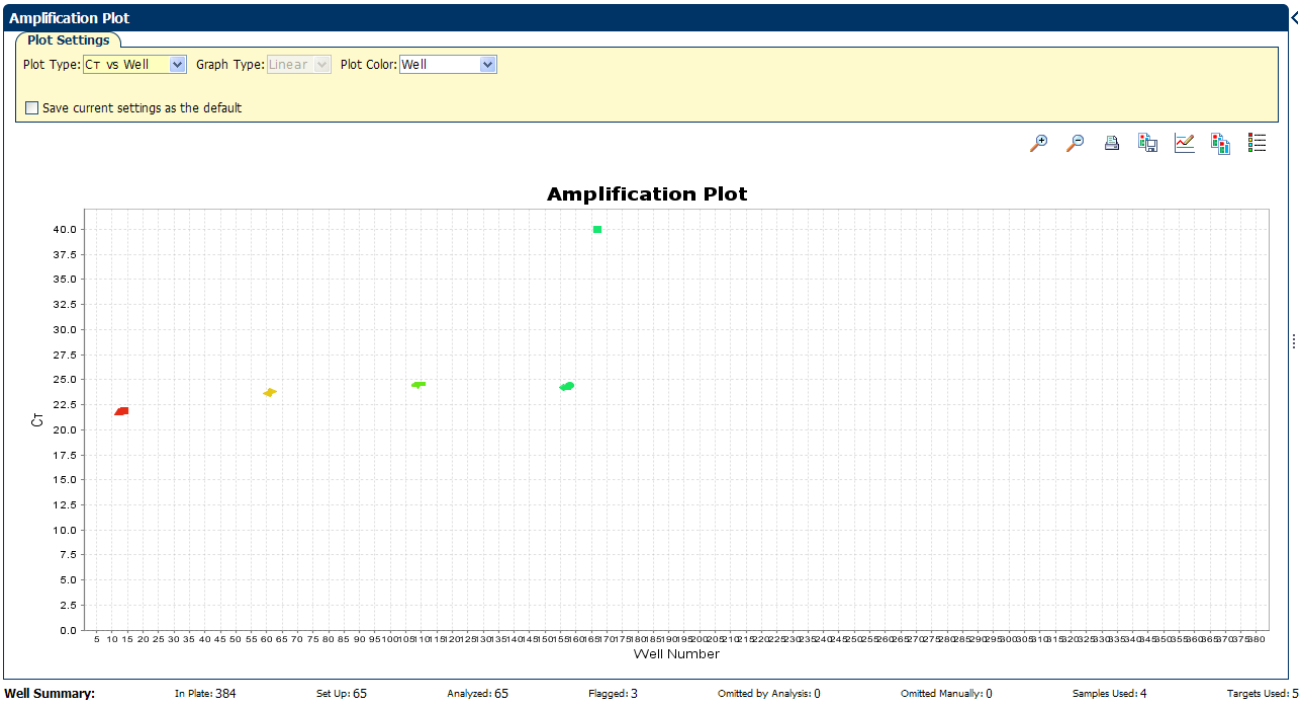
b. Verify that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.



6. Locate any outliers:

   a. From the Plot Type drop-down menu, select **C_T vs Well**.

   b. Look for outliers from the amplification plot. In the example experiment, there are no outliers for LP1N1.



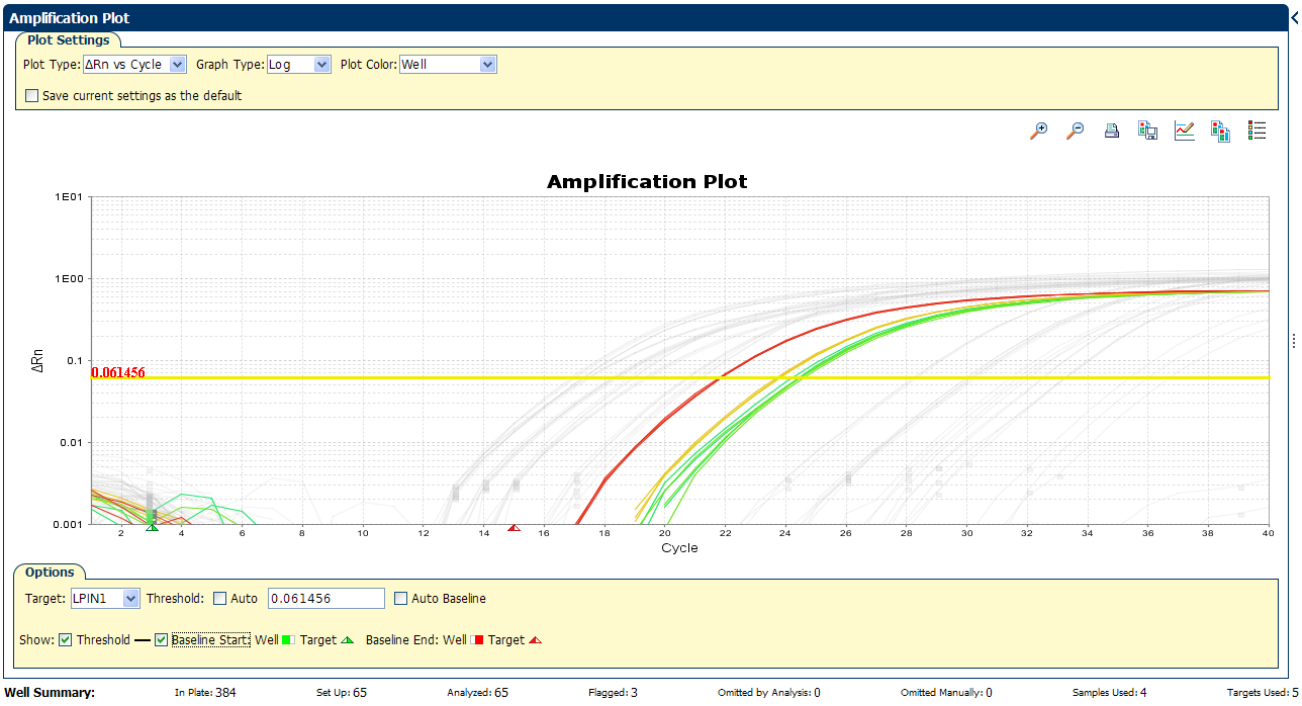
7. Repeat steps 2 through 6 for the GH1, TGFB1, LIPC, GAPDH, 18S, and ACTB wells. In the example experiment, there is one outlier for 18S. You will omit these wells in the troubleshooting section ("Improve C_T precision by omitting wells" on page 127).

Tips for analyzing your own experiments

When you analyze your own Comparative C_T experiment, look for:

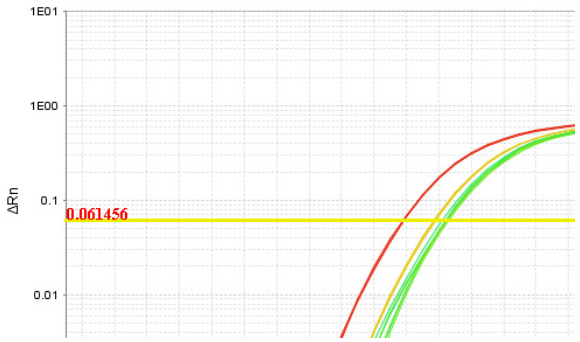
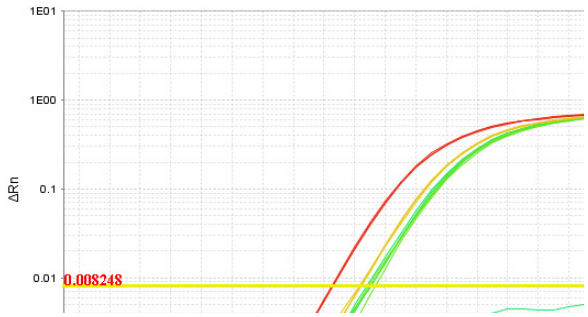
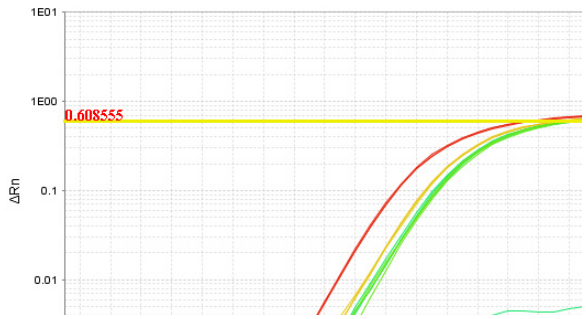
- **Outliers**
- **A typical amplification plot** – The ViiA™ 7 Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear phase
  - Exponential (geometric phase)
  - Baseline

  A typical amplification plot should look like this:

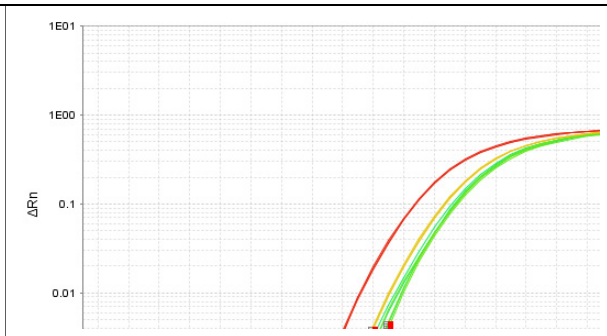
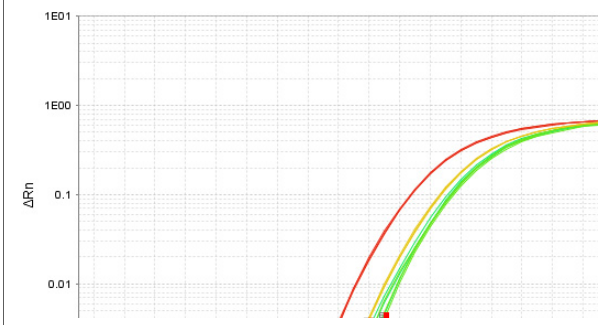
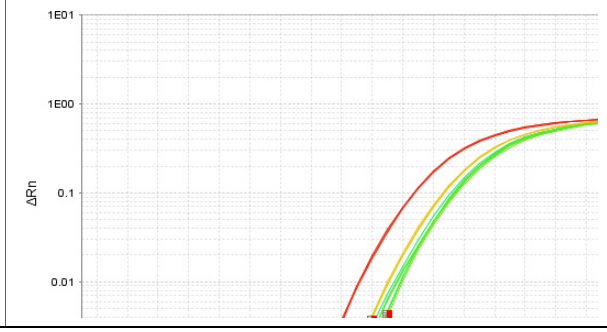


IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the ViiA™ 7 Software. Therefore, Applied Biosystems recommends that you examine the Amplification Plot screen and review the assigned baseline and threshold values for each well after analysis completes.

- **Correct threshold values**

Threshold Set Correctly The threshold is set in the exponential phase of the amplification curve. Threshold settings above or below the optimum increase the standard deviation of the replicate groups.	
Threshold Set Too Low The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the exponential phase of the curve.	
Threshold Set Too High The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar down into the exponential phase of the curve.	

- **Correct baseline values**

Baseline Set Correctly The amplification curve begins after the maximum baseline.	
Baseline Set Too Low The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.	
Baseline Set Too High The amplification curve begins before the maximum baseline. Decrease the End Cycle value.	

- View the analyzed data using the relative threshold settings

  The ViiA™ 7 Software provides the Relative Threshold method to view the analyzed data. The relative threshold algorithm lets you compare the data per well and per target. These options allow analysis of a single gene across samples or, alternatively, a single sample across genes with no dependency on targets, thereby reducing variability.

  To view the analyzed data using the relative threshold settings, see "Adjust analysis settings" on page 123.

If your experiment does not meet the guidelines above, troubleshoot as follows:

- Omit wells (see "Improve C_T precision by omitting wells" on page 127).

  Or

- Manually adjust the baseline and/or threshold (see "Adjust analysis settings" on page 123).

Confirm accurate dye signal using the Multicomponent Plot

The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the Comparative C_T example experiment, you review the Multicomponent Plot screen for:

- ROX™ dye (passive reference)
- FAM™ dye (reporter)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

View the Multicomponent Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Multicomponent Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the unknown and standard wells one at a time in the Multicomponent Plot screen:
   a. Click the **Plate Layout** tab.
   b. Select one well in the plate layout; the well is shown in the Multicomponent Plot screen.

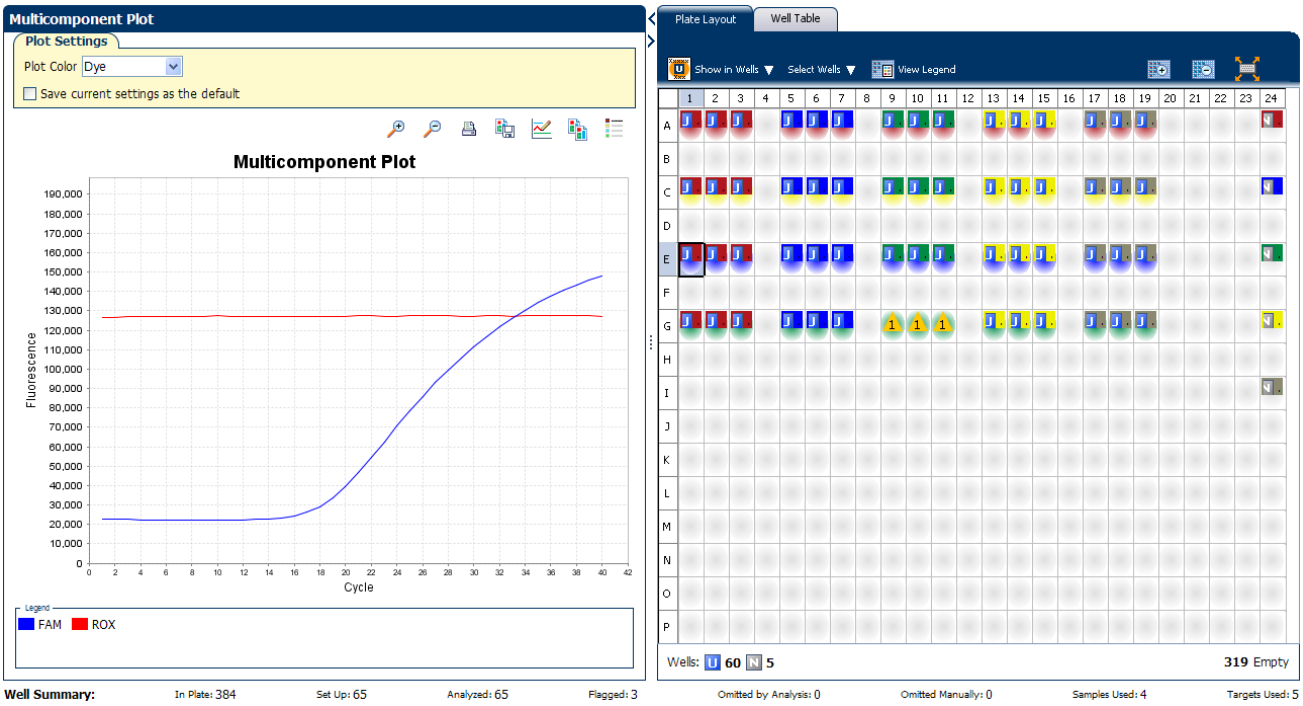
      Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

3. From the Plot Color drop-down menu, select **Dye**.

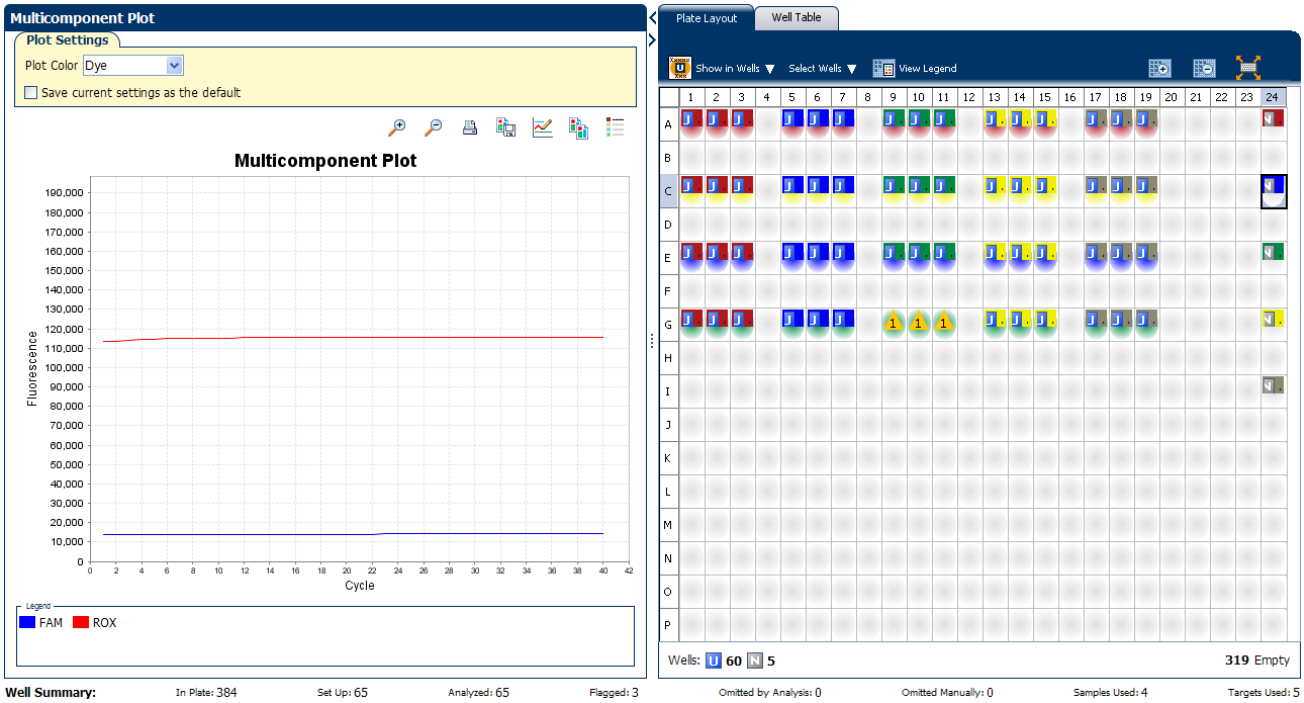
4. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

5. Check the FAM dye signals. In the example experiment, the FAM dye signal increases throughout the PCR process, indicating normal amplification.



6. Select the negative control wells one at time and check for amplification. In the example experiment, there is no amplification in the negative control wells.



Tips for confirming dye accuracy in your own experiment

When you analyze your own Comparative C_T experiment, look for:

- **Passive reference** – The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye** – The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- **Irregularities in the signal** – There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative control wells** – There should not be any amplification in the negative control wells.

Determine signal accuracy using the Raw Data Plot

The Raw Data Plot screen displays the raw fluorescence signal (not normalized) for each optical filter for the selected wells during each cycle of the real-time PCR.

About the example experiment

In the Comparative C_T example experiment, you review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the Raw Data Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Raw Data Plot**.

   Note: If no data are displayed, click **Analyze**.

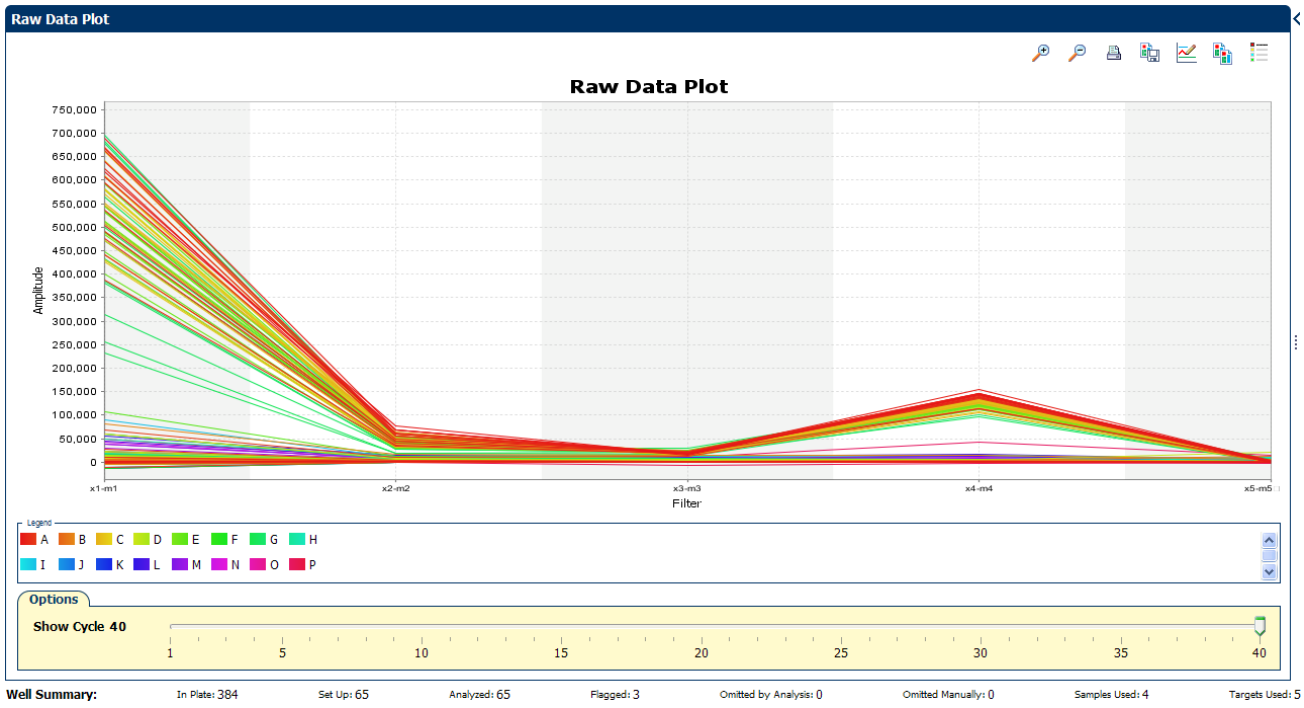
2. Display all 48 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the Plate Layout tab.

3. Click **Show a legend for the plot** (default).

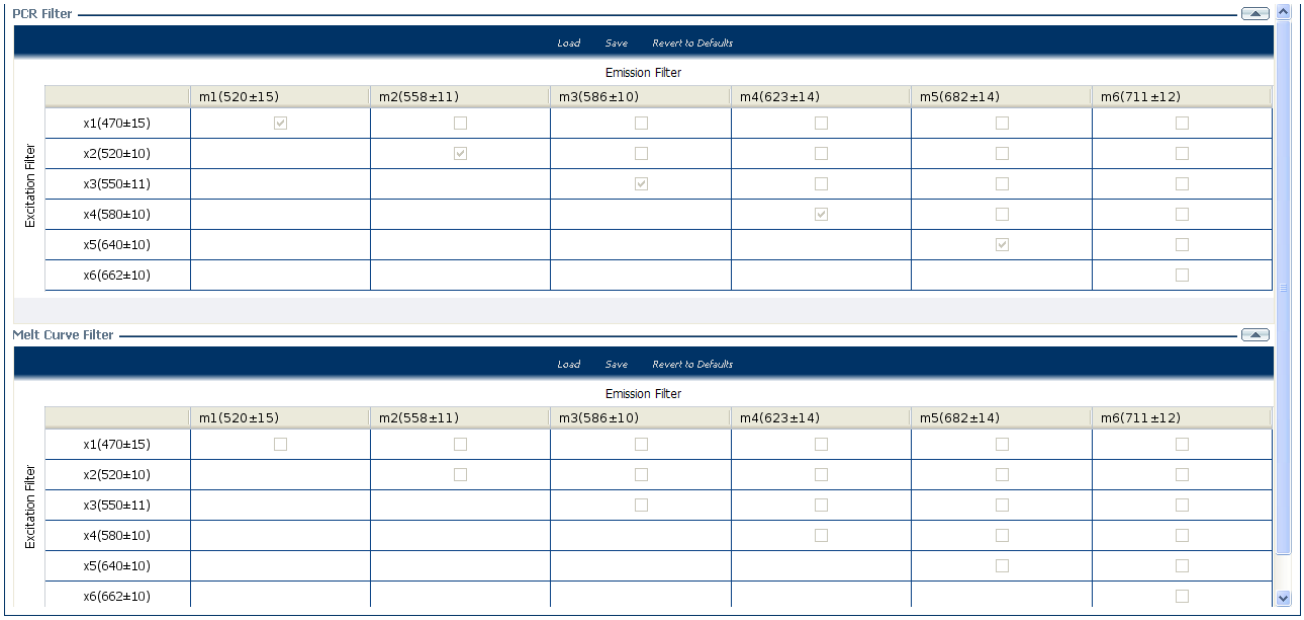
   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

   Note: The legend displays the color code for each row of the reaction plate (see the legend in the Raw Data Plot shown below).

4. Click and drag the Show Cycle pointer from cycle 1 to cycle 40. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.



The filters used for the example experiment are:



Tips for determining signal accuracy in your own experiment

When you analyze your own Comparative C_T experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

View the endogenous control profile using the QC Plot

In the Comparative C_T experiment, the QC Plot screen displays the Endogenous Control Profile plot for all the targets in the experiment. The QC Plot serves as a tool to help you choose the best endogenous control for that experiment. The endogenous control profile plot is a visual display of the C_T level of the endogenous control across the sample. You can view up to four endogenous controls at a time. The sample is plotted on the X-axis, and C_T is plotted on the Y-axis. The expression is viewed as a color and shape combination in the plot. Endogenous controls are also known as reference genes.

Example experiment settings

In the example experiment, you can view the endogenous control profile of ACTB, GAPDH, GH1, and LPIN1 the QC Plot screen.

View the QC Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **QC Plot**.

   Note: If no data are displayed, click **Analyze**.

2. In the QC Plot screen, click **Target Table**.
   a. In the Candidate Control column, select the check box of the target of the endogenous control profile to plot. In the example experiment, the endogenous controls selected are ACTB, GAPDH, GH1, and LPIN1.
   b. Select a color for each target, from the Color drop-down menu.
   c. Select a shape for each target, from the Shape drop-down menu.

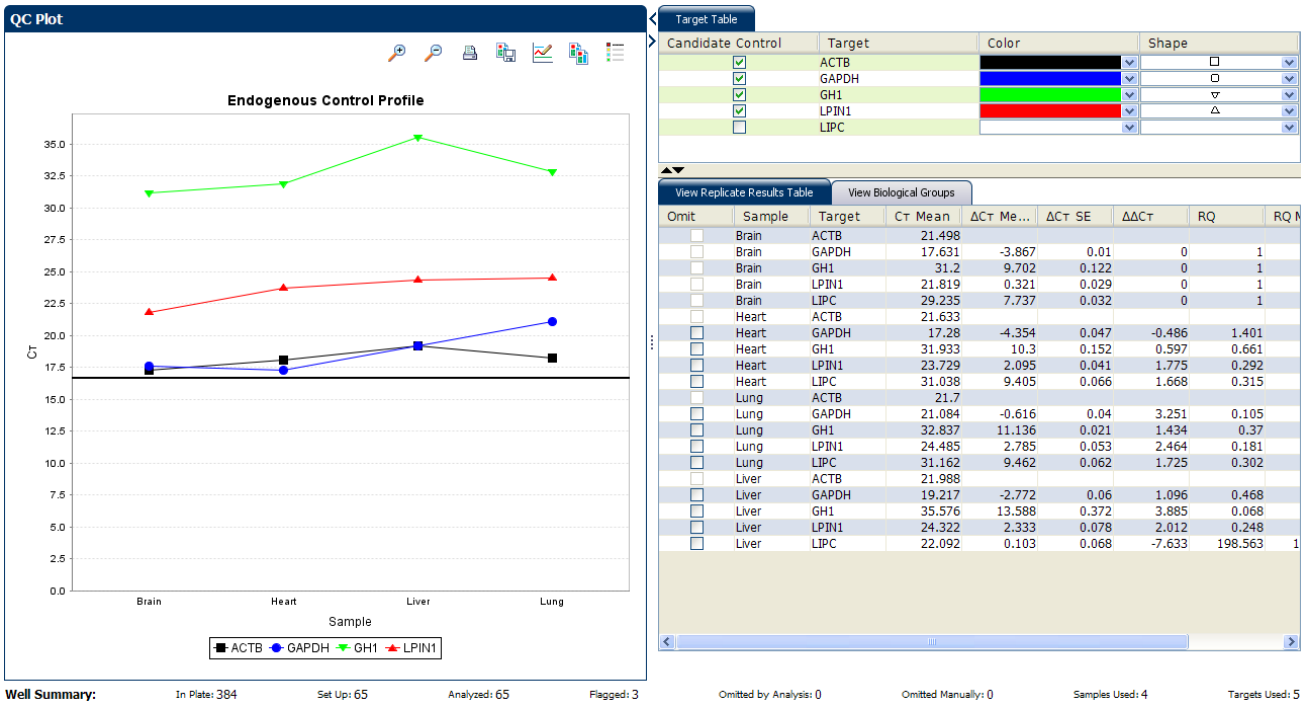
3. Click the **View Replicate Results Table**.

4. Select the check box of the samples to plot. In the example experiment, all the four samples, Brain, Heart, Liver, and Lung are selected.

5. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

The QC Plot in the Comparative C_T example experiment looks like this:



Note: This example experiment does not define Biological Groups.

Review the flags in the QC Summary

The QC Summary screen displays a list of the ViiA™ 7 Software flags, including the flag frequency and location for the open experiment.

Review the QC Summary screen in the Comparative C_T example experiment for any flags triggered by the experiment data. Wells G9, G10, and G11 have data that triggered the HIGHSD flag.

View the QC Summary

1. From the Experiment Menu pane, select **Analysis ▸ QC Summary**.

   Note: If no data are displayed, click **Analyze**.

2. Review the Flags Summary.

   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment. If the frequency is > 0, the flag appears somewhere in the experiment; the well position is listed in the Wells column.

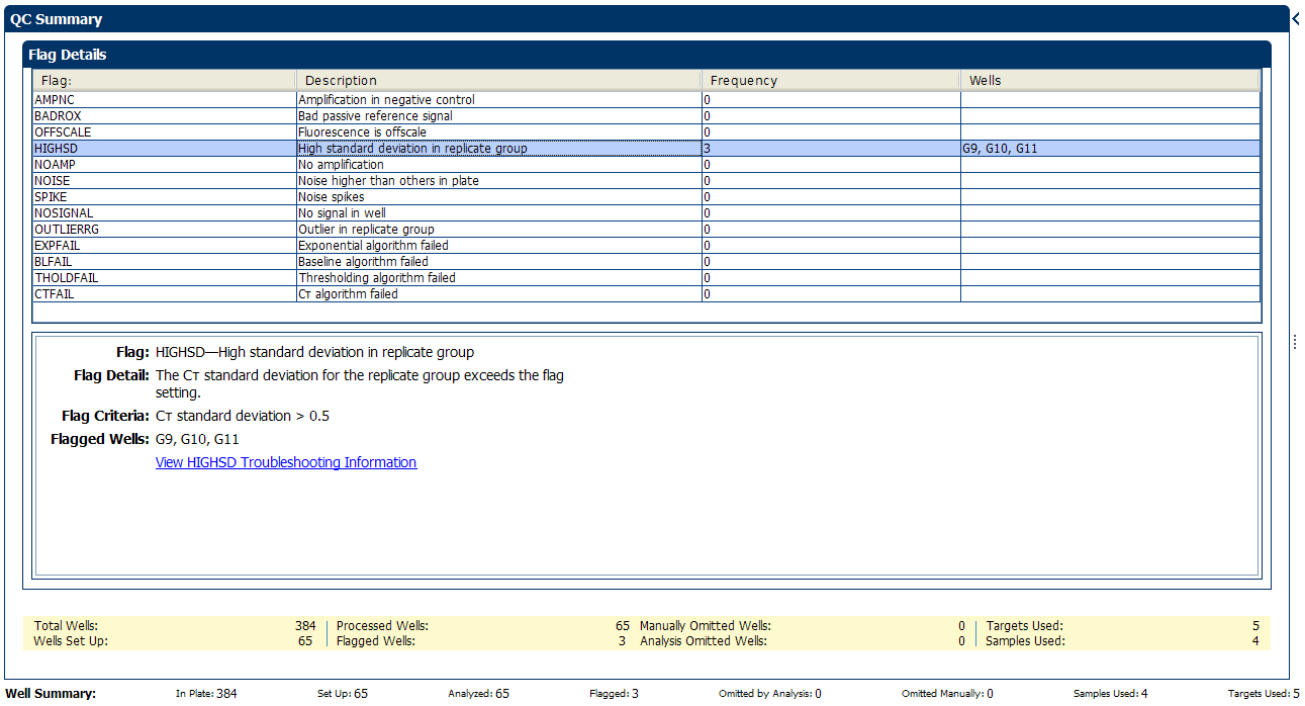
   In the example experiment, there are seven flagged wells.

3. In the Flag Details table, click each flag with a frequency >0 to display detailed information about the flag. In the example experiment. The HIGHSD flag appears three times, in the wells G9, G10, and G11, indicating high standard deviation in the replicate group.

   Note: The HIGHSD flag appears because the C_T values exceed the expected range due to low expression of the GH1 gene in the Liver sample.

4. *(Optional)* For those flags with frequency >0, click the troubleshooting link to view information on correcting the flag.

   The QC Summary for the example experiment looks like this:



Possible flags

The flags listed below may be triggered by the experiment data.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed

Flag	Description
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
Secondary analysis flags	
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the Frequency column for each flag).

For more information

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with ViiA™7 System Experiments*.	4441434

11.2

Section 11.2 Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

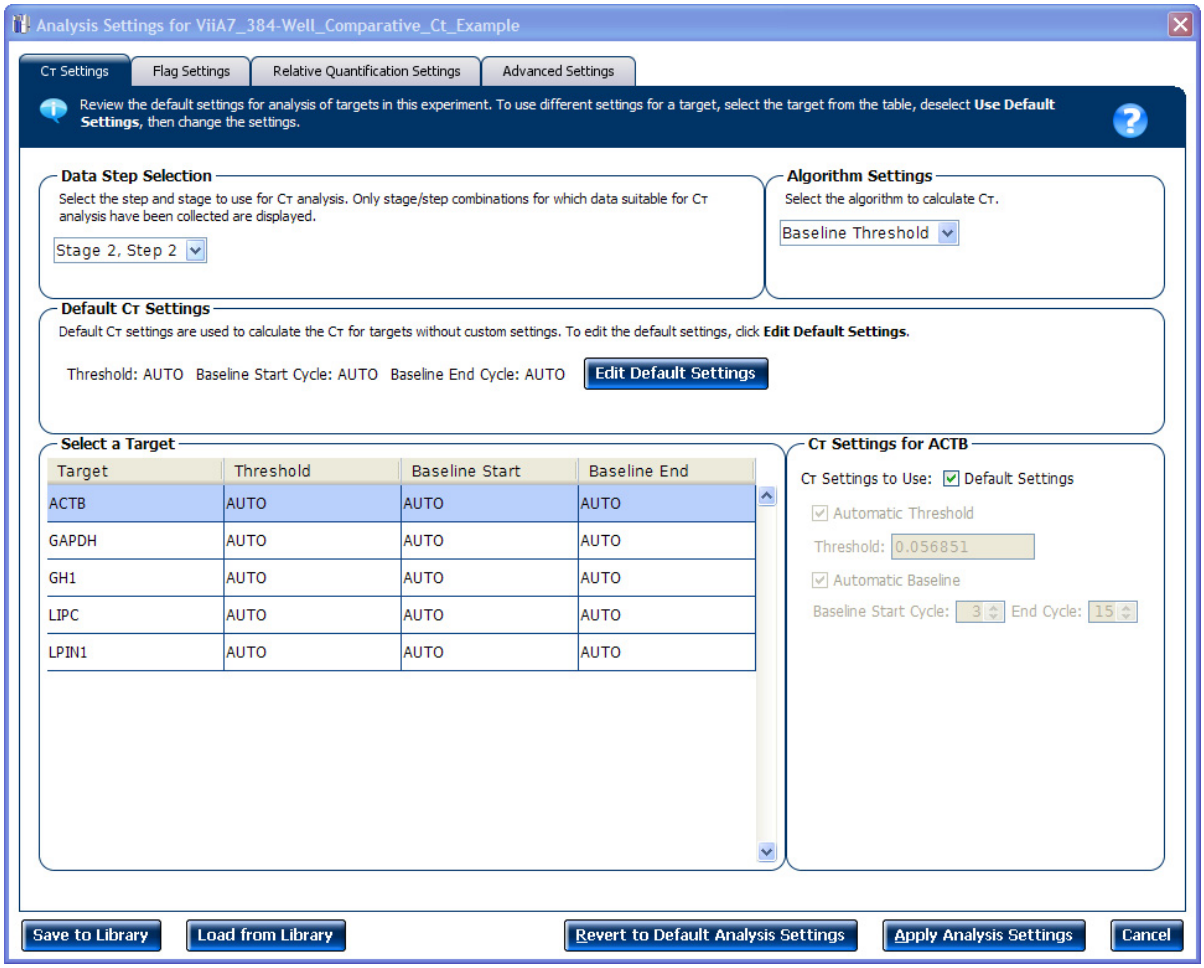
The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, and advanced options.

If the default analysis settings in the ViiA™ 7 Software are not suitable for your own experiment, you can change the settings in the Analysis Settings dialog box, then reanalyze your experiment.

View the analysis settings

1. From the Experiment Menu pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the Analysis Settings dialog box.
   In the example experiment, the default analysis settings are used for each tab:
   - C_T Settings
   - Flag Settings
   - Relative Quantification Settings
   - Advanced Settings

The Analysis Settings dialog box for a Comparative C_T experiment looks like this:



3. View and, if necessary, change the analysis settings (see "Adjust analysis settings" below).

   Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see About the Analysis Settings Library in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*.

4. Click **Apply Analysis Settings** to apply the current analysis settings.

   Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

C_T Settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method**.**

- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for gene quantification.

The Relative Threshold algorithm lets you compare the data on a per-well or per-target basis. This setting is ideal for analyzing a single gene across samples or a single sample across genes with no dependence on targets, thereby reducing variability. Any settings for baseline or threshold do not affect the analysis when you use the Relative Threshold algorithm.

- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the targets that do not have custom settings. To edit the default settings, click **Edit Default Settings**.

- **C_T Settings for Target**

  When you manually set the threshold and baseline, Applied Biosystems recommends:

Setting	Recommendation
Threshold	Enter a value for the threshold so that the threshold is: • Above the background. • Below the plateau and linear regions of the amplification curve. • Within the exponential phase of the amplification curve.
Baseline	Select the Start Cycle and End Cycle values so that the baseline ends before significant fluorescent signal is detected.

Note: This setting is applicable only to the Baseline Threshold algorithm.

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag Settings

Use the Flag Settings tab to:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the ViiA™ 7 Software.

To adjust the flag settings

1. In the Use column, select the check boxes for flags to apply during analysis.
2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

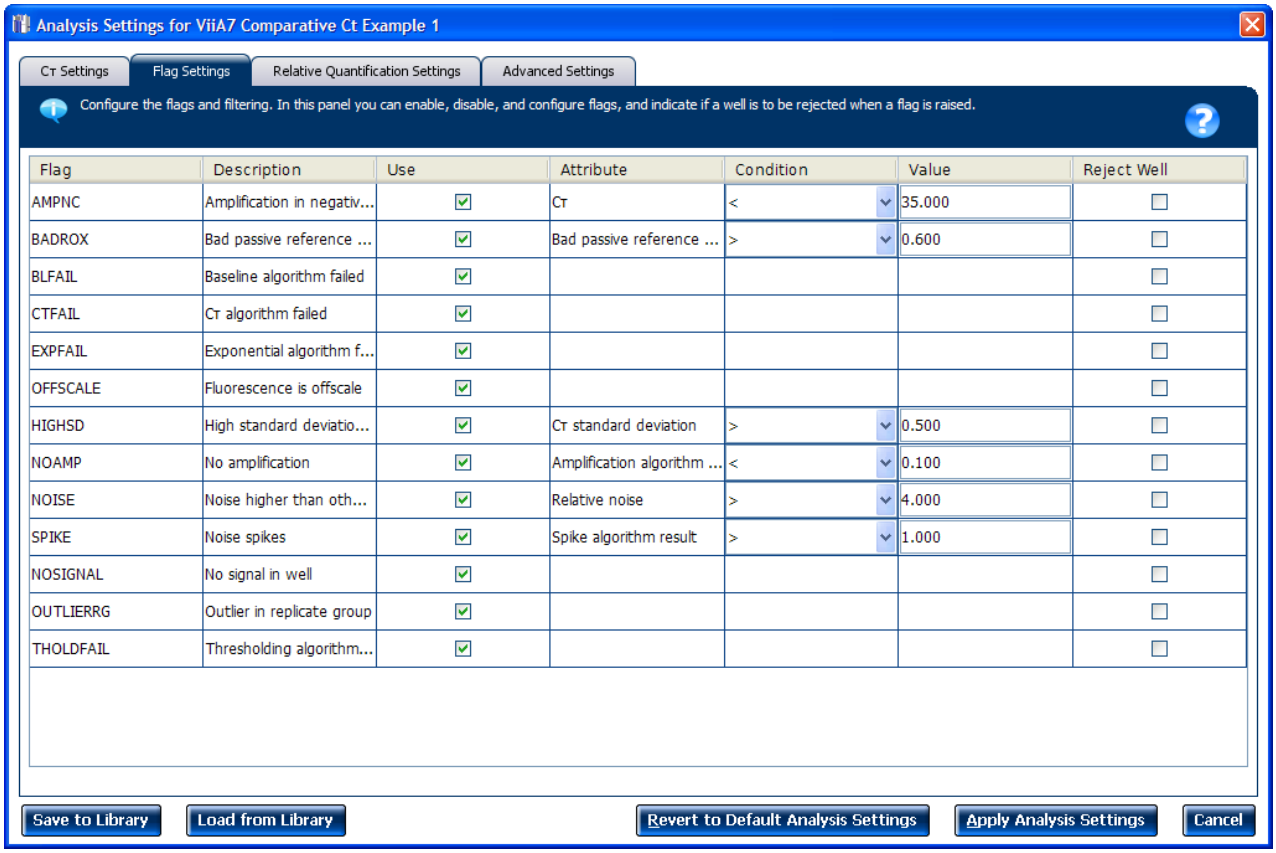
   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations may change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.

4. Click **Apply Analysis Settings** in the Analysis Settings dialog box. If the run status is complete, the data are reanalyzed.

   The Flag Settings tab looks like this:



Relative Quantification Settings

Use the Relative Quantification Settings tab to:

- Change the type of analysis, singleplex or multiplex.
- Change the reference sample and/or endogenous control.
- Reject Outliers with ΔC_Tvalues less than or equal to the entered value.

  Note: The Outlier Rejection settings apply only to multiplex reactions.

- Select the algorithm to use to determine the relative quantification minimum and maximum values (error bars):
  - **Confidence Level** - Select to calculate the RQ minimum and maximum values based on the selected confidence level. Select the confidence level to use.
  - **Standard Deviations** - Select to calculate the RQ minimum and maximum values based on the selected number of standard deviations. Select the number of standard deviations to use.

Advanced Settings

Use the Advanced Settings tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold setting.

To use custom baseline settings for a well-target combination:

1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** check box.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, then enter the baseline start cycle and baseline end cycle.

Improve C_T precision by omitting wells

Experimental error may cause some wells to be amplified insufficiently or not at all. These wells typically produce C_T values that differ significantly from the average for the associated replicate wells. If included in the calculations, these outliers can result in erroneous measurements; to ensure C_T precision, omit the outliers from the analysis.

In the Comparative C_T example experiment, there are seven outliers. To remove these wells from analysis.

1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.
2. In the Amplification Plot screen, select **C_T vs. Well** from the Plot Type drop-down menu.
3. Select the **Well Table** tab.
4. In the Well Table, identify outliers:
   a. From the Group By drop-down menu, select **Replicate**.

b. Look for outliers in the replicate group (make sure they are flagged). In the example experiment, wells G9, G10, and G11 have outliers.

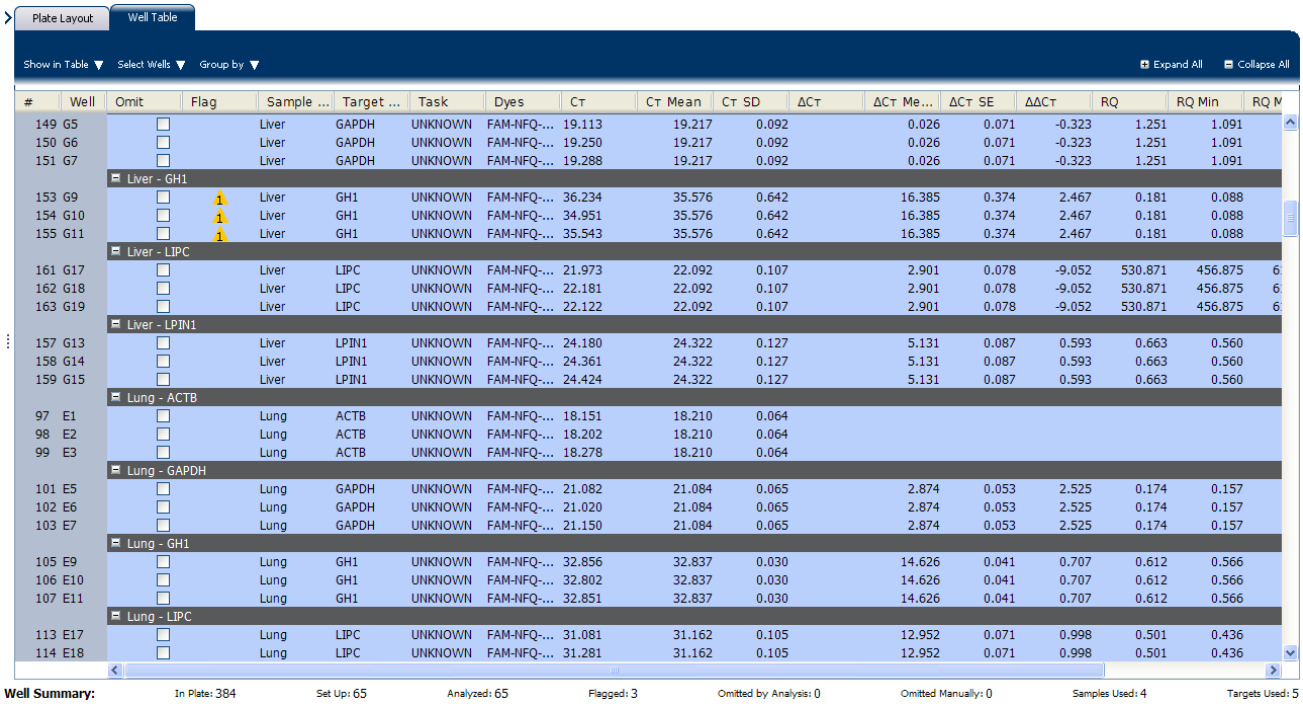


Plate Layout | Well Table

Show in Table ▼ Select Wells ▼ Group by ▼ Expand All Collapse All

#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	CT	CT Mean	CT SD	ΔCT	ΔCT Me...	ΔCT SE	ΔΔCT	RQ	RQ Min	RQ M
149	G5	☐		Liver	GAPDH	UNKNOWN	FAM-NFQ-...	19.113	19.217	0.092		0.026	0.071	-0.323	1.251	1.091	
150	G6	☐		Liver	GAPDH	UNKNOWN	FAM-NFQ-...	19.250	19.217	0.092		0.026	0.071	-0.323	1.251	1.091	
151	G7	☐		Liver	GAPDH	UNKNOWN	FAM-NFQ-...	19.288	19.217	0.092		0.026	0.071	-0.323	1.251	1.091	
		Liver - GH1															
153	G9	☐	1	Liver	GH1	UNKNOWN	FAM-NFQ-...	36.234	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
154	G10	☐	1	Liver	GH1	UNKNOWN	FAM-NFQ-...	34.951	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
155	G11	☐	1	Liver	GH1	UNKNOWN	FAM-NFQ-...	35.543	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
		Liver - LIPC															
161	G17	☐		Liver	LIPC	UNKNOWN	FAM-NFQ-...	21.973	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6
162	G18	☐		Liver	LIPC	UNKNOWN	FAM-NFQ-...	22.181	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6
163	G19	☐		Liver	LIPC	UNKNOWN	FAM-NFQ-...	22.122	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6
		Liver - LPIN1															
157	G13	☐		Liver	LPIN1	UNKNOWN	FAM-NFQ-...	24.180	24.322	0.127		5.131	0.087	0.593	0.663	0.560	
158	G14	☐		Liver	LPIN1	UNKNOWN	FAM-NFQ-...	24.361	24.322	0.127		5.131	0.087	0.593	0.663	0.560	
159	G15	☐		Liver	LPIN1	UNKNOWN	FAM-NFQ-...	24.424	24.322	0.127		5.131	0.087	0.593	0.663	0.560	
		Lung - ACTB															
97	E1	☐		Lung	ACTB	UNKNOWN	FAM-NFQ-...	18.151	18.210	0.064							
98	E2	☐		Lung	ACTB	UNKNOWN	FAM-NFQ-...	18.202	18.210	0.064							
99	E3	☐		Lung	ACTB	UNKNOWN	FAM-NFQ-...	18.278	18.210	0.064							
		Lung - GAPDH															
101	E5	☐		Lung	GAPDH	UNKNOWN	FAM-NFQ-...	21.082	21.084	0.065		2.874	0.053	2.525	0.174	0.157	
102	E6	☐		Lung	GAPDH	UNKNOWN	FAM-NFQ-...	21.020	21.084	0.065		2.874	0.053	2.525	0.174	0.157	
103	E7	☐		Lung	GAPDH	UNKNOWN	FAM-NFQ-...	21.150	21.084	0.065		2.874	0.053	2.525	0.174	0.157	
		Lung - GH1															
105	E9	☐		Lung	GH1	UNKNOWN	FAM-NFQ-...	32.856	32.837	0.030		14.626	0.041	0.707	0.612	0.566	
106	E10	☐		Lung	GH1	UNKNOWN	FAM-NFQ-...	32.802	32.837	0.030		14.626	0.041	0.707	0.612	0.566	
107	E11	☐		Lung	GH1	UNKNOWN	FAM-NFQ-...	32.851	32.837	0.030		14.626	0.041	0.707	0.612	0.566	
		Lung - LIPC															
113	E17	☐		Lung	LIPC	UNKNOWN	FAM-NFQ-...	31.081	31.162	0.105		12.952	0.071	0.998	0.501	0.436	
114	E18	☐		Lung	LIPC	UNKNOWN	FAM-NFQ-...	31.281	31.162	0.105		12.952	0.071	0.998	0.501	0.436	

Well Summary: In Plate: 384 Set Up: 65 Analyzed: 65 Flagged: 3 Omitted by Analysis: 0 Omitted Manually: 0 Samples Used: 4 Targets Used: 5

c. Select the **Omit** check box next to outlying well(s).

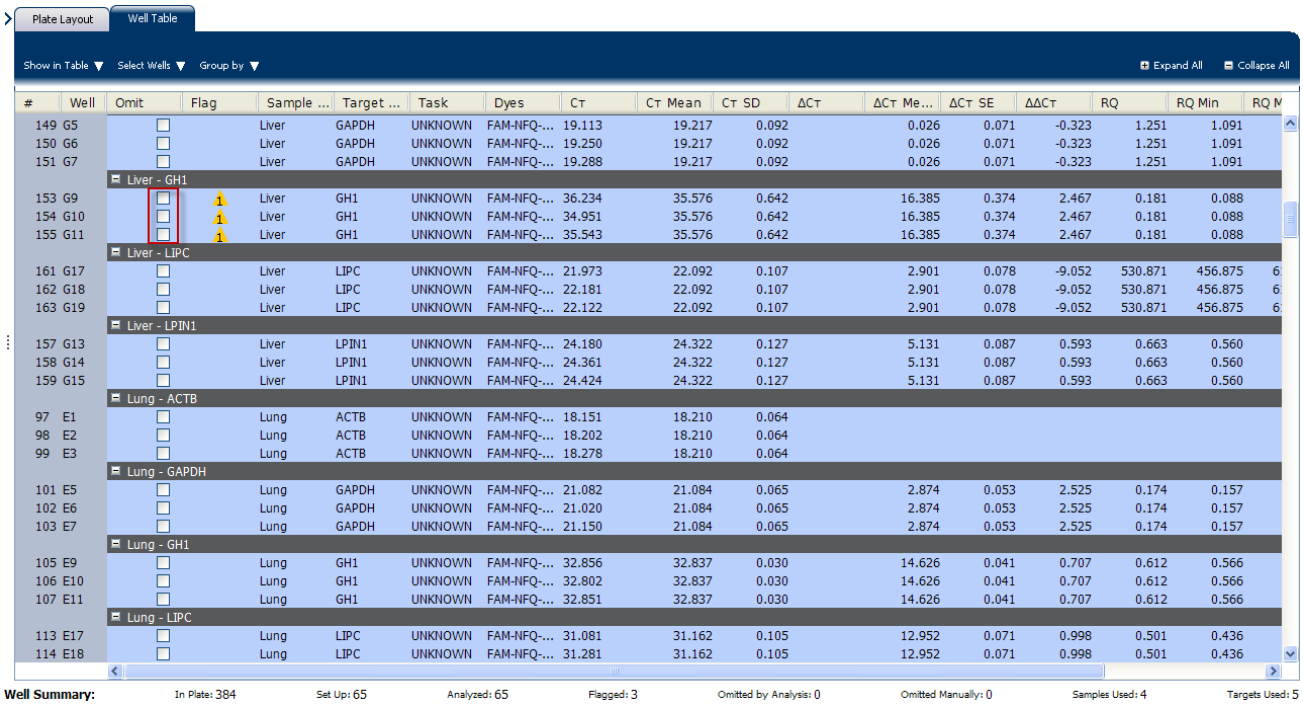


Plate Layout | Well Table

Show in Table ▼ Select Wells ▼ Group by ▼ Expand All Collapse All

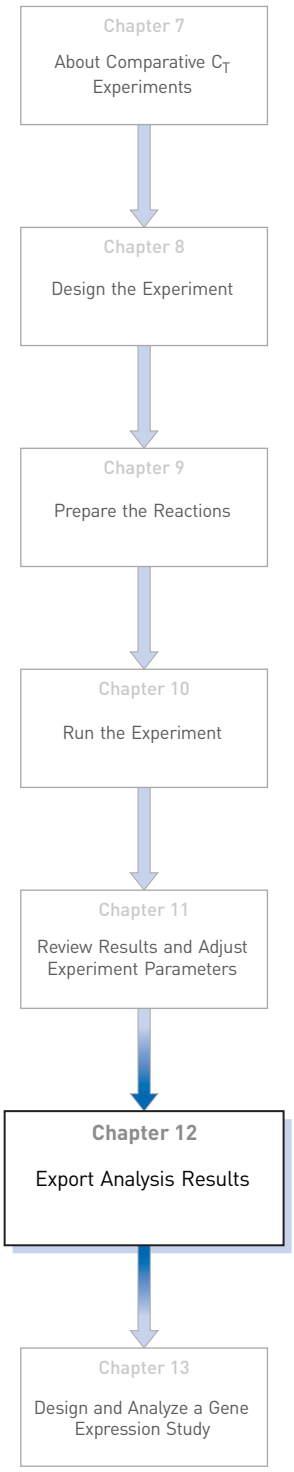
#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	CT	CT Mean	CT SD	ΔCT	ΔCT Me...	ΔCT SE	ΔΔCT	RQ	RQ Min	RQ M
149	G5	☐		Liver	GAPDH	UNKNOWN	FAM-NFQ-...	19.113	19.217	0.092		0.026	0.071	-0.323	1.251	1.091	
150	G6	☐		Liver	GAPDH	UNKNOWN	FAM-NFQ-...	19.250	19.217	0.092		0.026	0.071	-0.323	1.251	1.091	
151	G7	☐		Liver	GAPDH	UNKNOWN	FAM-NFQ-...	19.288	19.217	0.092		0.026	0.071	-0.323	1.251	1.091	
		Liver - GH1															
153	G9	☐	1	Liver	GH1	UNKNOWN	FAM-NFQ-...	36.234	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
154	G10	☐	1	Liver	GH1	UNKNOWN	FAM-NFQ-...	34.951	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
155	G11	☐	1	Liver	GH1	UNKNOWN	FAM-NFQ-...	35.543	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
		Liver - LIPC															
161	G17	☐		Liver	LIPC	UNKNOWN	FAM-NFQ-...	21.973	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6
162	G18	☐		Liver	LIPC	UNKNOWN	FAM-NFQ-...	22.181	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6
163	G19	☐		Liver	LIPC	UNKNOWN	FAM-NFQ-...	22.122	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6
		Liver - LPIN1															
157	G13	☐		Liver	LPIN1	UNKNOWN	FAM-NFQ-...	24.180	24.322	0.127		5.131	0.087	0.593	0.663	0.560	
158	G14	☐		Liver	LPIN1	UNKNOWN	FAM-NFQ-...	24.361	24.322	0.127		5.131	0.087	0.593	0.663	0.560	
159	G15	☐		Liver	LPIN1	UNKNOWN	FAM-NFQ-...	24.424	24.322	0.127		5.131	0.087	0.593	0.663	0.560	
		Lung - ACTB															
97	E1	☐		Lung	ACTB	UNKNOWN	FAM-NFQ-...	18.151	18.210	0.064							
98	E2	☐		Lung	ACTB	UNKNOWN	FAM-NFQ-...	18.202	18.210	0.064							
99	E3	☐		Lung	ACTB	UNKNOWN	FAM-NFQ-...	18.278	18.210	0.064							
		Lung - GAPDH															
101	E5	☐		Lung	GAPDH	UNKNOWN	FAM-NFQ-...	21.082	21.084	0.065		2.874	0.053	2.525	0.174	0.157	
102	E6	☐		Lung	GAPDH	UNKNOWN	FAM-NFQ-...	21.020	21.084	0.065		2.874	0.053	2.525	0.174	0.157	
103	E7	☐		Lung	GAPDH	UNKNOWN	FAM-NFQ-...	21.150	21.084	0.065		2.874	0.053	2.525	0.174	0.157	
		Lung - GH1															
105	E9	☐		Lung	GH1	UNKNOWN	FAM-NFQ-...	32.856	32.837	0.030		14.626	0.041	0.707	0.612	0.566	
106	E10	☐		Lung	GH1	UNKNOWN	FAM-NFQ-...	32.802	32.837	0.030		14.626	0.041	0.707	0.612	0.566	
107	E11	☐		Lung	GH1	UNKNOWN	FAM-NFQ-...	32.851	32.837	0.030		14.626	0.041	0.707	0.612	0.566	
		Lung - LIPC															
113	E17	☐		Lung	LIPC	UNKNOWN	FAM-NFQ-...	31.081	31.162	0.105		12.952	0.071	0.998	0.501	0.436	
114	E18	☐		Lung	LIPC	UNKNOWN	FAM-NFQ-...	31.281	31.162	0.105		12.952	0.071	0.998	0.501	0.436	

Well Summary: In Plate: 384 Set Up: 65 Analyzed: 65 Flagged: 3 Omitted by Analysis: 0 Omitted Manually: 0 Samples Used: 4 Targets Used: 5

5. Click **Analyze** to reanalyze the experiment data with the outlying well(s) removed from the analysis.

   Note: You can also omit undesirable wells in an experiment from the Plate Layout screen. To omit a well from the Plate Layout screen, right-click the well and select **Omit**.

12 Export Analysis Results



1. Open the Comparative C_T example experiment file that you analyzed in Chapter 11.

2. In the Experiment Menu, click **Export.**

   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.

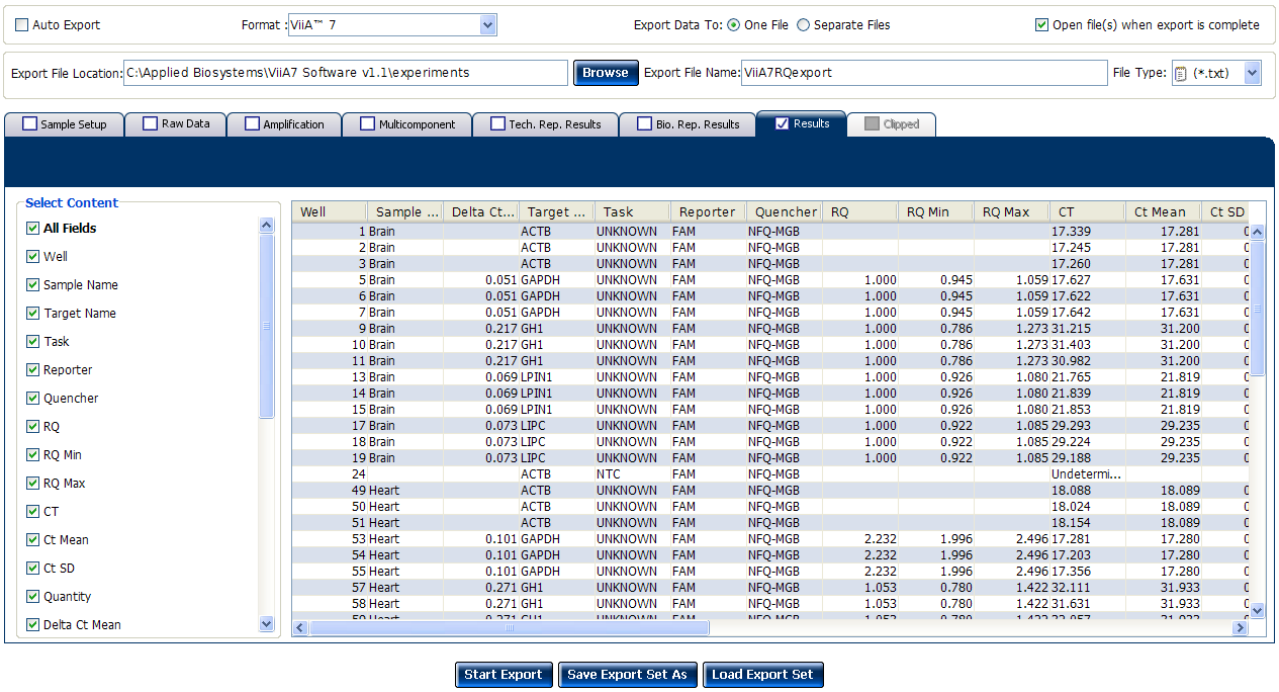
3. Select **ViiA™7 Format**.

   Note: Select **7900** Format if you want to export the Clipped Data.

4. Complete the Export dialog box as shown below:

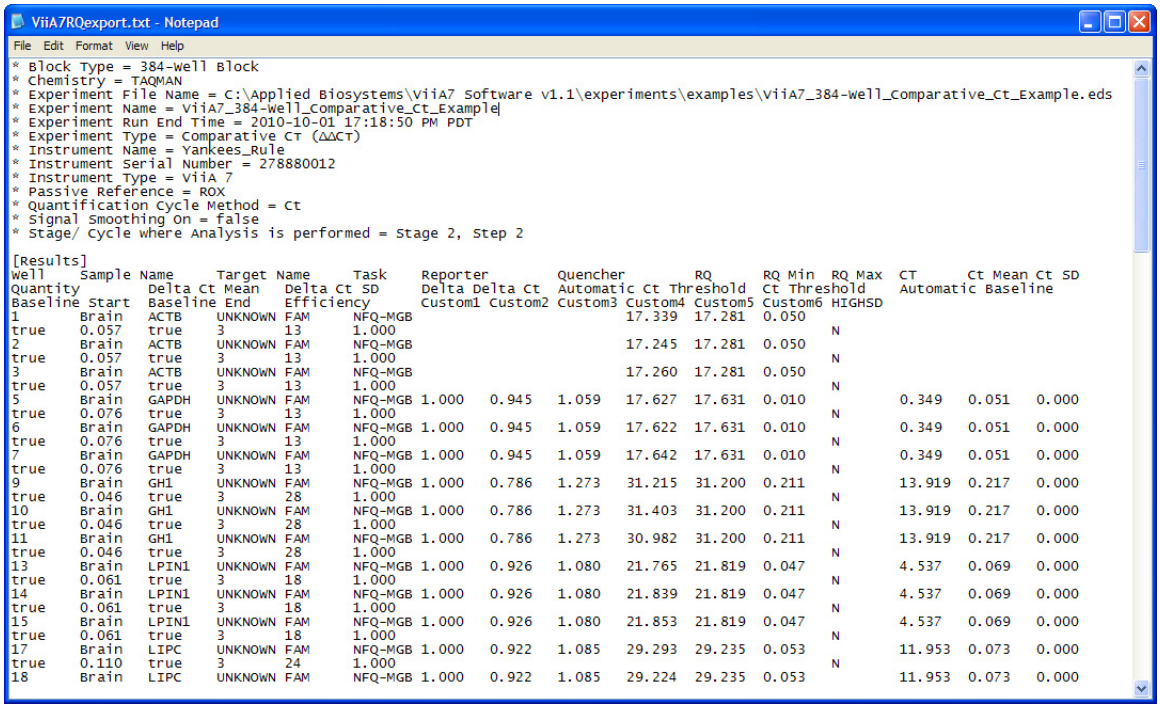
Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	ViiA7RQexport
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\ViiA7 Software v1.1\experiments

Your Export screen should look like this:

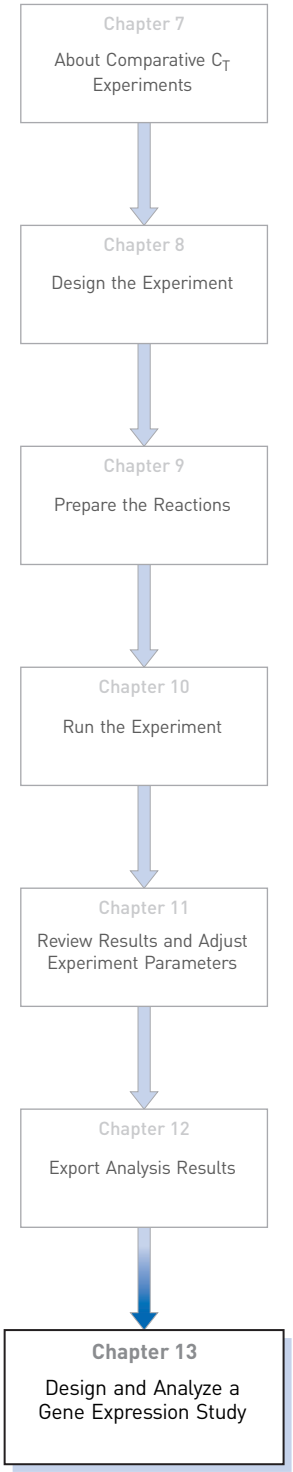


Well	Sample ...	Delta Ct...	Target ...	Task	Reporter	Quencher	RQ	RQ Min	RQ Max	CT	Ct Mean	Ct SD
1	Brain		ACTB	UNKNOWN	FAM	NFQ-MGB				17.339	17.281	0
2	Brain		ACTB	UNKNOWN	FAM	NFQ-MGB				17.245	17.281	0
3	Brain		ACTB	UNKNOWN	FAM	NFQ-MGB				17.260	17.281	0
5	Brain	0.051	GAPDH	UNKNOWN	FAM	NFQ-MGB	1.000	0.945	1.059	17.627	17.631	0
6	Brain	0.051	GAPDH	UNKNOWN	FAM	NFQ-MGB	1.000	0.945	1.059	17.622	17.631	0
7	Brain	0.051	GAPDH	UNKNOWN	FAM	NFQ-MGB	1.000	0.945	1.059	17.642	17.631	0
9	Brain	0.217	GH1	UNKNOWN	FAM	NFQ-MGB	1.000	0.786	1.273	31.215	31.200	0
10	Brain	0.217	GH1	UNKNOWN	FAM	NFQ-MGB	1.000	0.786	1.273	31.403	31.200	0
11	Brain	0.217	GH1	UNKNOWN	FAM	NFQ-MGB	1.000	0.786	1.273	30.982	31.200	0
13	Brain	0.069	LPIN1	UNKNOWN	FAM	NFQ-MGB	1.000	0.926	1.080	21.765	21.819	0
14	Brain	0.069	LPIN1	UNKNOWN	FAM	NFQ-MGB	1.000	0.926	1.080	21.839	21.819	0
15	Brain	0.069	LPIN1	UNKNOWN	FAM	NFQ-MGB	1.000	0.926	1.080	21.853	21.819	0
17	Brain	0.073	LIPC	UNKNOWN	FAM	NFQ-MGB	1.000	0.922	1.085	29.293	29.235	0
18	Brain	0.073	LIPC	UNKNOWN	FAM	NFQ-MGB	1.000	0.922	1.085	29.224	29.235	0
19	Brain	0.073	LIPC	UNKNOWN	FAM	NFQ-MGB	1.000	0.922	1.085	29.188	29.235	0
24			ACTB	NTC	FAM	NFQ-MGB				Undetermi...		
49	Heart		ACTB	UNKNOWN	FAM	NFQ-MGB				18.088	18.089	0
50	Heart		ACTB	UNKNOWN	FAM	NFQ-MGB				18.024	18.089	0
51	Heart		ACTB	UNKNOWN	FAM	NFQ-MGB				18.154	18.089	0
53	Heart	0.101	GAPDH	UNKNOWN	FAM	NFQ-MGB	2.232	1.996	2.496	17.281	17.280	0
54	Heart	0.101	GAPDH	UNKNOWN	FAM	NFQ-MGB	2.232	1.996	2.496	17.203	17.280	0
55	Heart	0.101	GAPDH	UNKNOWN	FAM	NFQ-MGB	2.232	1.996	2.496	17.356	17.280	0
57	Heart	0.271	GH1	UNKNOWN	FAM	NFQ-MGB	1.053	0.780	1.422	32.111	31.933	0
58	Heart	0.271	GH1	UNKNOWN	FAM	NFQ-MGB	1.053	0.780	1.422	31.631	31.933	0

Your exported file when opened in Notepad should look like this:



13 Design and Analyze a Gene Expression Study



The ViiA™ 7 Software can combine the analysis of experiments that use the Standard Curve, the Relative Standard Curve, or the Comparative C_T ($\Delta\Delta C_T$) quantification methods into a Gene Expression study. A study provides a wider range for analyzing and comparing target behavior across multiple experiments. This chapter explains how to design and analyze multiple Comparative C_T experiments as a study.

Note: You can import different types of quantification experiments into a single Gene Expression study, but make sure the run method is identical for all the experiments in that study.

Note: When you design a Gene Expression study, make sure that each experiment in that Gene Expression study has a unique name. Absence of a unique name, leads to failure of the run.

This chapter covers:

- About Gene Expression studies 134
- About the example study 134
- Design a study. 135
- Analyze the example study. 140
- View the analysis settings 143
- Assess amplification results using the Amplification Plot 147
- Assess the gene expression profile using the Gene Expression Plot. 150
- Confirm accurate dye signal using the Multicomponent Plot 158
- View the QC Plots. 160
- View the QC Summary 168
- Compare analysis settings. 170
- Export the study 175
- For more information. 178

About Gene Expression studies

In a Gene Expression study, you can...	You cannot...
• Specify the endogenous control(s) and reference sample for the study. • Set individual efficiency values for each target. • Select the control type when applicable. • Set baseline and threshold values and confidence levels, or set the number of standard deviations for Comparative $_{CT}$ Min./Max. • Omit wells individually or together through their association with replicate groups (technical or biological).	• Create, add, or modify samples. • Create, add, or modify targets. • Change assay tasks. You can perform these operations in individual experiments.

About the example study

In the Comparative C_T example study:

- Two reaction plates (experiments) are used.
- Experiments that you add to the study are two Comparative C_T experiments that have already been analyzed.
- The cDNA was prepared from total RNA that was isolated from the following 4 samples:
  - Heart
  - Liver
  - Brain
  - Lung
- Five targets (assays) are used:
  - LIPC: Hs00165106_m1
  - GAPDH: Hs99999905_m1
  - ACTB: Hs99999903_m1
  - LP1N1: Hs00299515_m1
  - GH1: Hs00236859_m1
- The reference sample is Brain.
- The endogenous control is ACTB.
- Each experiment in the study was designed for singleplex PCR, where the target and endogenous control assays are run in separate wells.
- For each experiment in the study, reactions were set up for 2-step RT-PCR:
  - The cDNA was reverse-transcribed from total RNA samples using Invitrogen VILO Kit.
  - The reactions were prepared using TaqMan® Fast Universal PCR Master Mix (2X).

Design a study

Create a new study

To create a new study in the ViiA™ 7 Software, from the Home screen, click **New Gene Expression Study**.

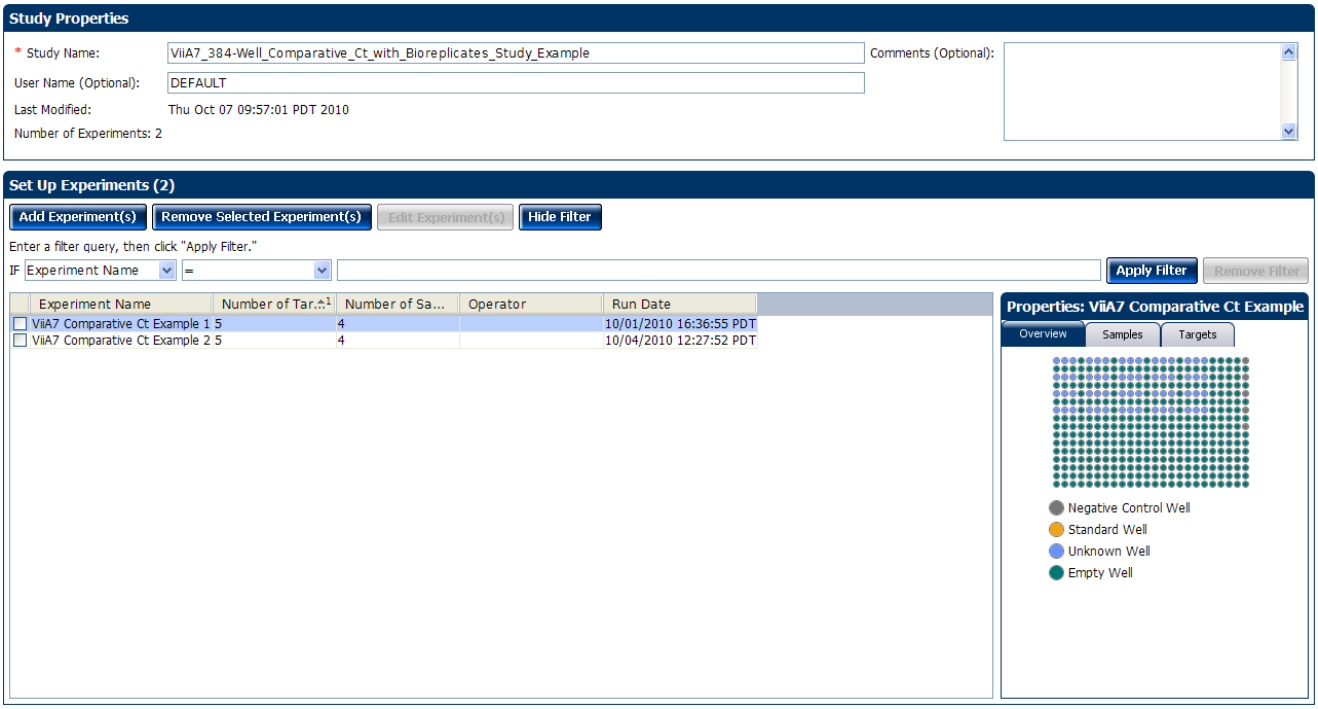
Set up the study in the Study Properties screen

1. In the Study Menu pane, select **Setup ▸ Study Properties**
2. In the Study Properties pane, click the **Study Name** field, then enter **ViiA7_384-Well_Comparative_Ct_with_Bioreplicates_Study_Example**.
3. Click the **Comments** field, then enter **Example Comparative C_T Study for the Comparative Ct Experiments**.
4. In the Setup Experiments pane, click **Add Experiment(s)**.
5. In the Open dialog box, browse to the **ViiA7 Comparative Ct Example 1.eds and ViiA7 Comparative Ct Example 2.eds** files at:

<drive>:\Applied Biosystems\ViiA7 Software v1.1\experiments\examples

The ViiA™ 7 Software displays the details of the experiment in the Properties pane.

Your Study Properties screen should look like this:



Filter the experiments in the study

To narrow your search for an experiment, define and apply a filter:

1. In the left-most drop-down menu, select an experiment attribute to query.
2. In the center drop-down menu, select an operator for the query.
3. In the right-most field, enter the condition to look for, then click **Apply Filter**.

After you apply a filter, click **Hide Filter/Show Filter** to hide or show the filter tool, or click **Remove Filter** to remove the filter.

Define Replicates

In the Define Replicates screen, create biological replicate groups and use them to associate samples for the analysis. Biological replicates allow you to assess the representative nature of your results as they relate to the population being studied. Including biological replicates can give insight into any natural variation that is in the population.

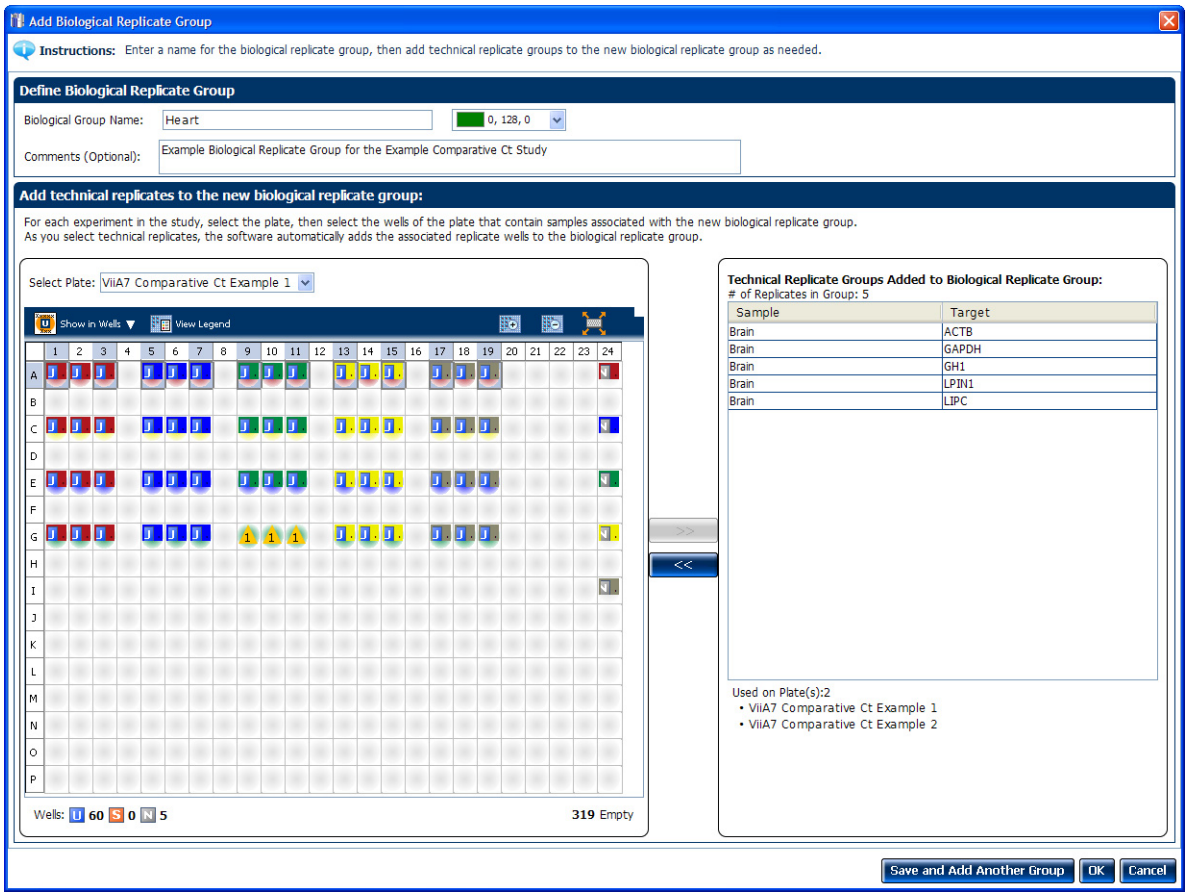
Example study settings

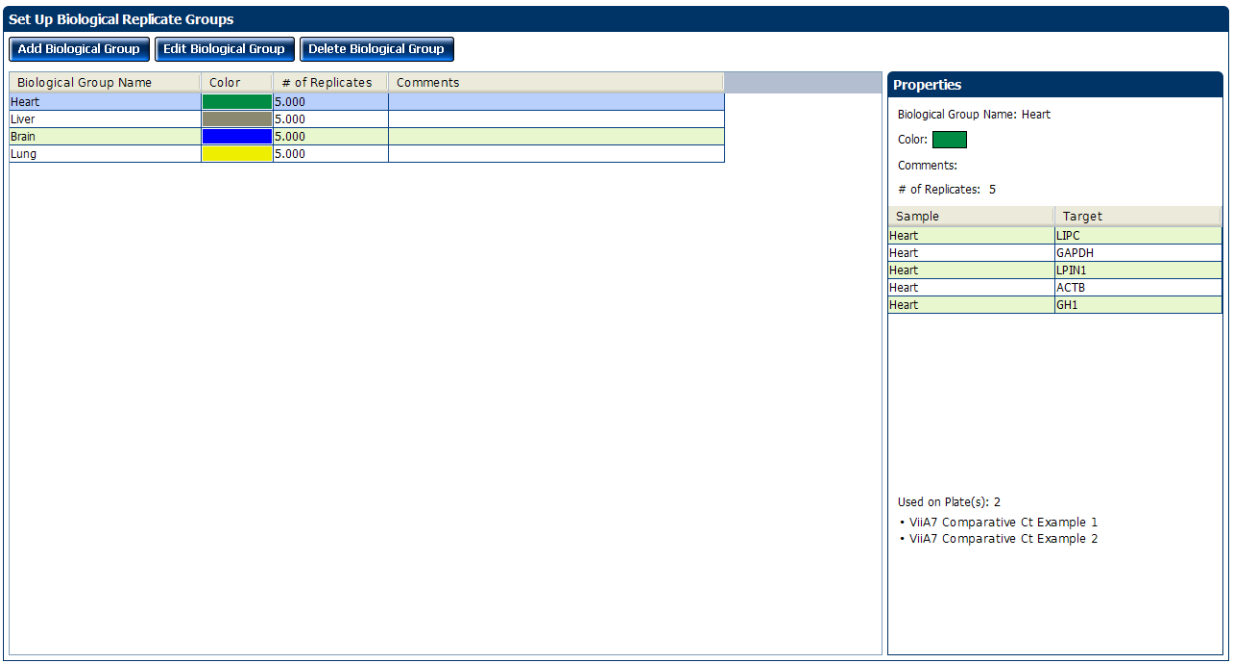
The Gene Expression example study contains four biological replicate groups. These are lung, heart, brain, and liver. Each biological groups consists eight replicates.

To define replicates

1. In the Study Menu pane, select **Setup** ▸ **Define Replicates**.
2. Click **Add Biological Group** to open the Add Biological Replicate Group dialog box.
3. Define the biological replicate group:
   a. Click the **Biological Group Name** field, then enter **Heart.**
   b. In the Color field, select the color.
   c. Click the **Comments** field, then enter **Example Biological Replicate Group for the Example Comparative Ct Study**.
4. Add technical replicates:
   a. From the Select Plate drop-down menu, select **ViiA7 Comparative Ct Example 1.eds**.
   b. In the plate layout, select wells **A1, A3, A5, A7, A9, A11, A13, A15, A17, and A19,** then click to add the technical replicate wells that are associated with the selected well to the biological group.
   c. Click **OK**.
   d. Perform steps a through c to add the remaining technical replicate wells associated with the selected well to the biological group.

5. On the Set Up Biological Replicates screen, select the **Heart** biological group that you just added to the study. The ViiA™ 7 Software displays the details of the biological group in the Properties pane.





6. Click **Analyze**, then close the study:
   a. Select **File** ▸ **Close**.
   b. At the prompt, click **Yes** to save the changes.
   c. In the Save Study as dialog box, click **Save** to accept the default file name and location. The example study is saved and closed, and you are returned to the Home screen.

Edit a Biological Replicate Group

1. Open the study of interest.
2. In the Study Menu pane, select **Setup** ▸ **Define Replicates**.
3. Click **Edit Biological Group** to open the Edit Biological Replicate Group dialog box.
4. Edit the group information:
   a. From the Biological Group Name drop-down menu, select the group to edit.
   b. To change the group name, click **Edit**, enter a new name, then click **OK**.
   c. To change the color, select a color from the drop-down menu.
   d. (Optional) Enter comments.
5. From the Select Plate drop-down menu, select the experiment of interest.
6. Add samples to the biological group:
   a. In the Plate Layout, select the well(s) of the plate that contain samples to add to the biological group.
   b. Click to add the samples that are associated with the selected wells to the biological group.
7. Remove samples from the biological group:
   a. In the Technical Replicate Groups Added to Biological Replicate Group pane, select a sample. You can select only one sample at a time.
   b. Click to remove the sample from the biological group.
8. Repeat steps 5 through 7 for the other experiments in the study.
9. Click **OK** to save the changes and return to the study; click **Cancel** to exit the dialog box without saving the changes.

Remove Biological Replicate Groups

1. Open the study of interest.
2. In the Study Menu pane, select **Setup** ▸ **Define Replicates**.

3. In the Define Replicates screen, select the group to remove, then click **Delete Biological Group**.

4. Click **Yes** to remove the group from the study; click **No** to keep the group in the study.

Tips for designing your own study

- Enter a study name that is descriptive and easy to remember. You can enter up to 100 characters in the Study Name field. The study name is used as the default file name.

  You can only use the alpha-numeric, period (.), hyphen (-), underscore (_) and spaces ( ) characters in the Experiment Name field.
- *(Optional)* Enter comments to describe the study. You can enter up to 1000 characters in the Comments field.
- Use the default user name, or enter a new user name, to identify the owner of the study. You can enter up to 100 characters in the User Name field.

  Note: If security is enabled, the User Name field is automatically populated with the name you selected at log in.

- You can add an unlimited number of experiments (reaction plates) to the study. Click **Add Experiment (s)** or **Remove Selected Experiment(s)** to add or remove experiments to or from a study. Note the following:
  - Each experiment in the study must:
    - Have one or more common endogenous control(s). The endogenous control(s) must be present on each reaction plate within the study.
    - Have identical thermal cycling parameters (the same number of steps and cycles). The ViiA™ 7 Software cannot combine in the same study experiments that use Fast and Standard thermal cycling conditions.
    - Have the same passive reference.
  - As the default, the ViiA™ 7 Software assigns the endogenous control and reference sample for a study based on the analysis settings of the first experiment that you added to the study.
  - If experiments that contain biological replicate groups are added to a study, the ViiA™ 7 Software automatically merges the matching biological groups.
  - The ViiA™ 7 Software automatically analyzes a study after you add more than one experiment to it. Consequently, to ensure that the software uses the correct settings, Applied Biosystems recommends that you review the analysis settings of your study after adding multiple experiments.
- When adding experiments to the study, **Ctrl-click** multiple experiments in the Open dialog box to add them to the study.
- Select an experiment that has been added to the study to view its properties in the Properties pane.
- Filter the experiments added to the study to simplify the list for easier review. See "Filter the experiments in the study" on page 136.
- Enter a biological replicate group name that is descriptive and easy to remember. You can enter up to 100 characters in the Biological Group Name field. You can only use the alpha-numeric, full-stop (.), hyphen (-), underscore (_) and space ( ) characters in the Experiment Name field.

- (Optional) Enter comments to describe the biological replicate group. You can enter up to 1000 characters in the Comments field.
- You can add an unlimited number of technical replicates to a biological group.

IMPORTANT! A sample cannot belong to more than one biological group.

  - Click-drag over the desired wells, or **Ctrl-click** or **Shift-click** in the plate layout to select multiple wells.
  - Click the upper-left corner of the plate layout to select all wells.
- You can change the name of a biological replicate group, change its color identification and description, and add or remove technical replicates. See "Edit a Biological Replicate Group" on page 138.
- You can remove an existing biological replicate group. See "Remove Biological Replicate Groups" on page 138.

IMPORTANT! After you remove a biological replicate group from a study, you cannot restore it.

Analyze the example study

This section explains how to use the ViiA™ 7 Software to analyze the Gene Expression example study. It also describes Applied Biosystems recommended best practices as you perform the analysis.

Note: The Comparative C_T Study Example.edm file demonstrates the use of biological replicate groups. An additional example study that does not use biological replicate groups is provided with the ViiA™ 7 Software.

Access the example study

For the Gene Expression example study, open the data file that is installed with the ViiA™ 7 Software. The data file was created with the same design parameters that are provided in "About Gene Expression studies" on page 134.

You can find the data file for the example study on your computer at:

<*drive*>:\Applied Biosystems\ViiA7 Software v1.1\experiments\examples\ViiA7_384-Well_Comparative_Ct_with_Bioreplicates_Study_Example.edm

where:

<*drive*> is the computer hard drive on which the ViiA™ 7 Software is installed. The default installation drive for the software is the D drive.

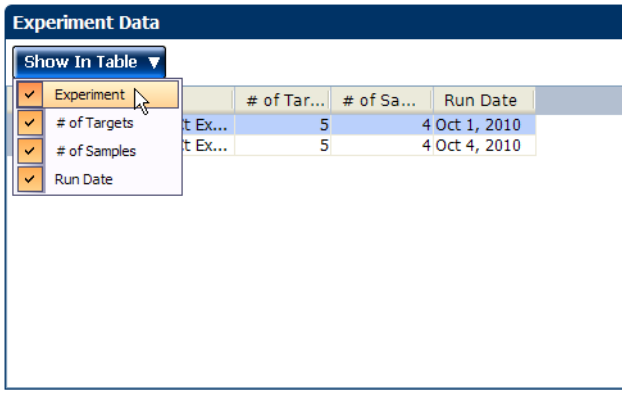
View the experiment data and well results

The Experiment Data and Well Results Data panes appear in the Amplification Plot, Multicomponent Plot, and Multiple Plots View screens.

The Gene Expression screen displays the Replicate Results Data and the Well Results Data panes.

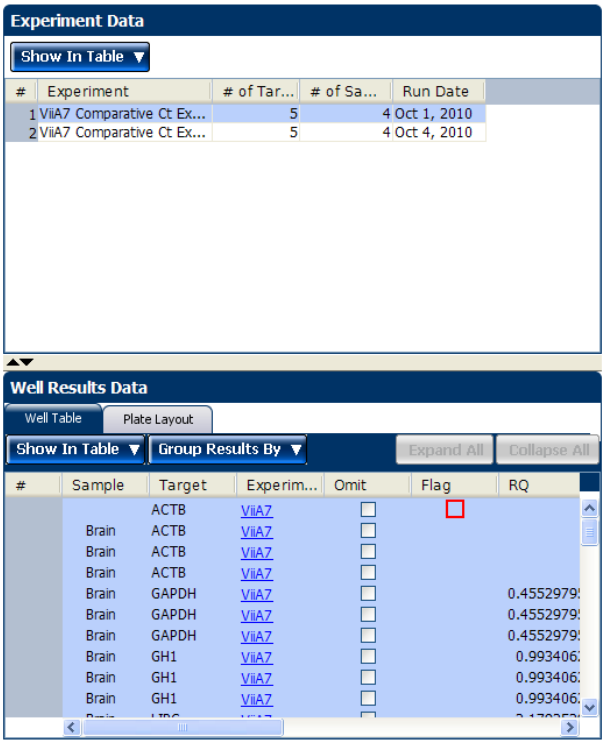
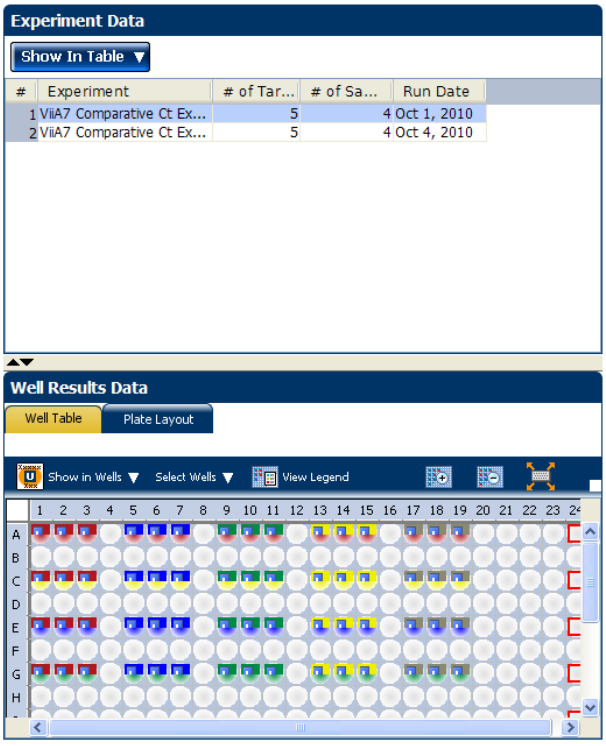
Only the Well Results Data pane appears in the QC Plots View screen.

- To display or hide columns in the Experiment Data pane– from the Show In Table drop-down menu, select or deselect one or more options: **Experiments**, **# of Targets**, **# of Samples**, **Run Dat**e.



- To display a subset of the study data in the plots– Select one or more rows in the Experiment Data pane or the Well Results Data pane, then select the **Hide unselected data from plot** check box appearing in the plot pane to display data only from the selected rows.

The Experiments Data pane lists each reaction plate (experiment) that is added to a study. The data that are displayed in the Well Results Data pane depend on which tab you select in the Experiment Data pane:

Tab	Description	Illustration
Well Table tab	When you select one or more experiments in the Experiment Data pane, the well table displays the wells that make up the selected experiment(s).	
Plate Layout tab	When you select one experiment in the Experiment Data pane, the plate layout displays the plate layout for the selected experiment. If you select more than one experiment in the Experiment Data pane, only the plate layout for the first experiment is displayed in the Plate Layout tab.	

View the analysis settings

The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, and relative quantification. If the default analysis settings in the ViiA™ 7 Software are not suitable for your study, you can change the settings in the Analysis Settings dialog box, then reanalyze your study.

Note: In the Comparative C_T example study, the default analysis settings are used without changes.

To adjust the analysis settings

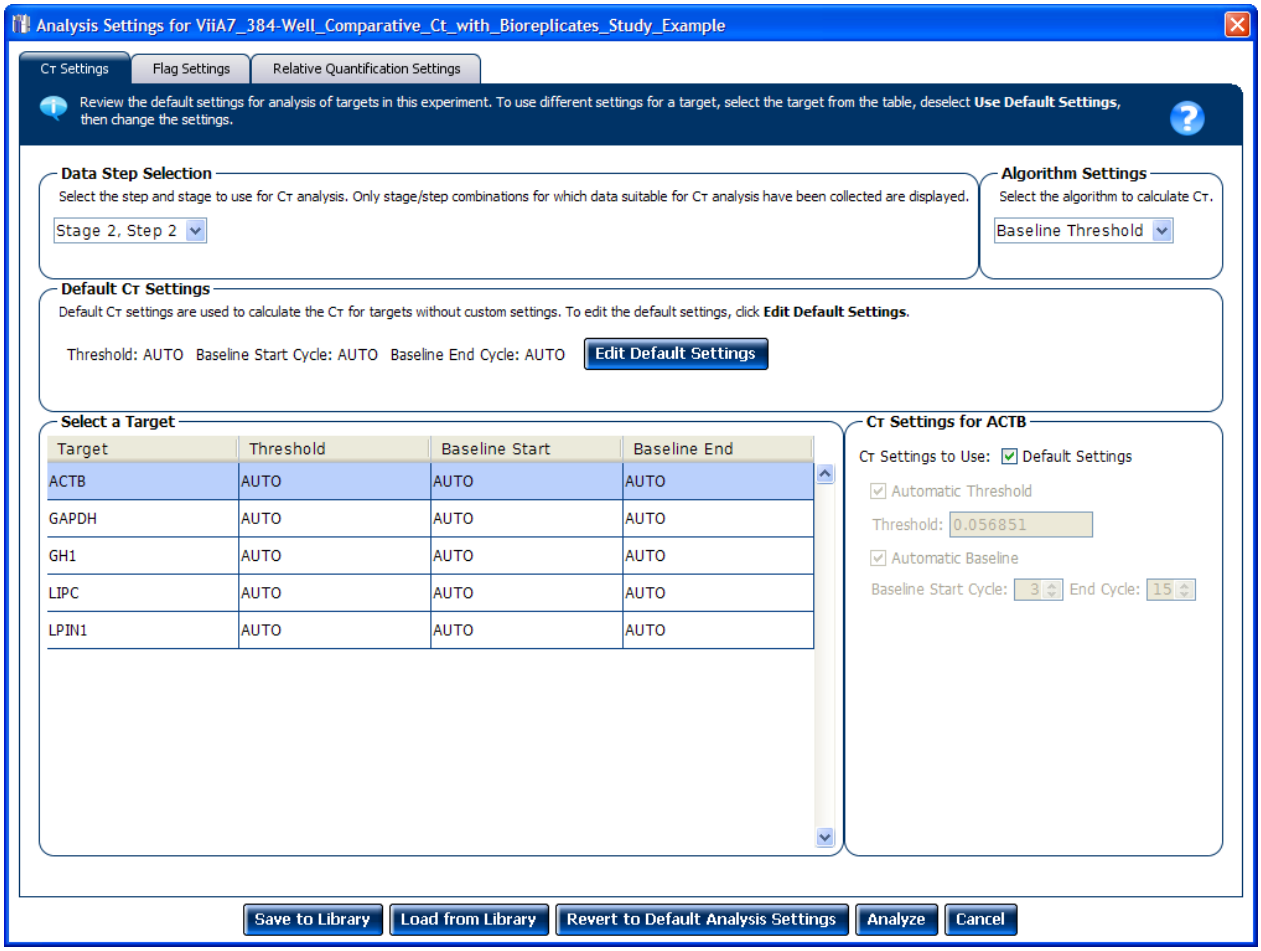
1. From the Study Menu pane, select **Analysis**.
2. Click **Analysis Settings** to open the Analysis Settings dialog box.
3. Select the **Relative Quantification Settings** tab, then view the default reference sample and endogenous control. In the example study, the default reference sample is brain and the default endogenous control is 18S.
4. Select the **C_T Settings** tab, then the **Flag Settings** tab. In the example study, the default analysis settings are used in each tab.

   Note: When you use the Relative Threshold option, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the Frequency column for each flag
5. View and, if necessary, change the analysis settings. For more information on the changes to analysis settings, refer to "Tips for analyzing your own study" on page 144.

   Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see About the Analysis Settings Library in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*.
6. Click **Apply Analysis Settings** to apply the current analysis settings.

   Note: You can go back to the default analysis settings by clicking **Revert to Default Analysis Settings**.
7. Click **Analyze**.

The Analysis Settings dialog box for a Comparative C_T Study looks like this:



Tips for analyzing your own study

Unless you have already determined the optimal settings for your study, use the default analysis settings in the ViiA™ 7 Software. If the default settings are not suitable for your study, change the settings as described below.

C_T Settings

- **Data Step Selection**

  Use this feature to select multiple locations of analysis, in case several are chosen.

- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

  The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for gene quantification.

  The Relative Threshold algorithm lets you compare the data on a per-well or per-target basis. This setting is ideal for analyzing a single gene across samples or a single sample across genes with no dependence on targets, thereby reducing variability. Any settings for baseline or threshold do not affect the analysis when you use the Relative Threshold algorithm.

- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the targets that do not have custom settings. To edit the default settings, click **Edit Default Settings**.

- **C_T Settings for Target**

  When you manually set the threshold and baseline, Applied Biosystems recommends:

Setting	Recommendation
Threshold	Enter a value for the threshold so that the threshold is: • Above the background. • Below the plateau and linear regions of the amplification curve. • Within the exponential phase of the amplification curve.
Baseline	Select the Start Cycle and End Cycle values so that the baseline ends before significant fluorescent signal is detected.

Note: This setting is applicable only to the Baseline-Threshold algorithm.

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag Settings

Use the Flag settings tab to:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the ViiA™ 7 Software.

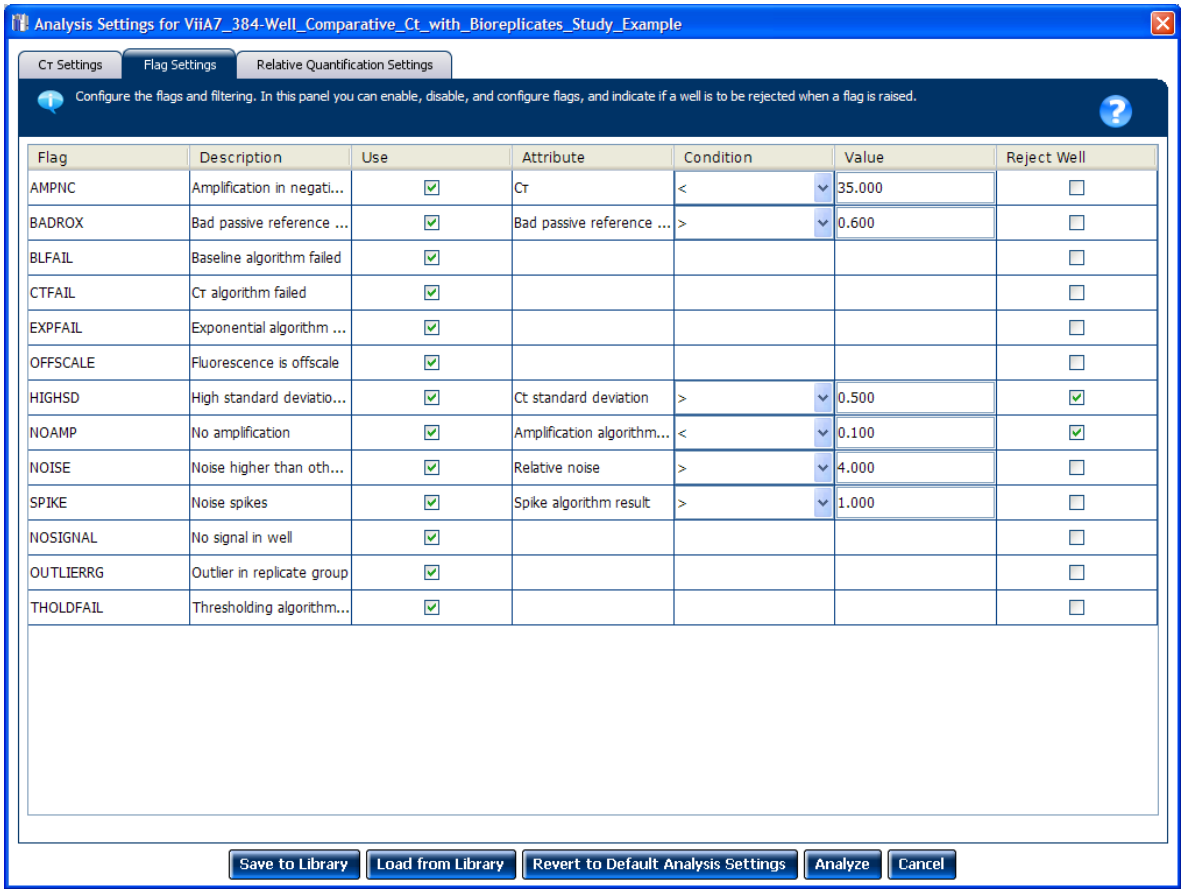
To adjust the flag settings

1. In the Use column, select the check boxes for flags to apply during analysis.
2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.
3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations may change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.
4. Click **Apply Analysis Settings** in the Analysis Settings dialog box. If the run status is complete, the data are reanalyzed.

The Flag Settings tab looks like this:



Relative Quantification Settings

Use the Relative Quantification Settings tab to:

- Change the reference sample and/or endogenous control. As the default, the ViiA™ 7 Software assigns the endogenous control and reference sample for a study based on the analysis settings of the first experiment added to it. You can add multiple endogenous controls to a study.
- Correct the amplification efficiency. You can enter a percentage value between 1% and 150% for each target. When you set an assay to have a value that differs from 100% efficiency, the software uses the relative standard curve algorithm.
- (For multiplex reactions) Specify the ΔC_T value at which to reject replicates (outlier rejection).
- Select the algorithm to use to determine the relative quantification minimum and maximum values (error bars):
  - **Confidence Level** - Select to calculate the RQ minimum and maximum values based on the selected confidence level. Select the confidence level to use.
  - **Standard Deviations** - Select to calculate the RQ minimum and maximum values based on the selected number of standard deviations. Select the number of standard deviations to use.

Assess amplification results using the Amplification Plot

In the comparative C_T example study, you review each target in the Amplification Plot screen for correct baseline and threshold values.

1. From the Study Menu pane, select **Analysis** ▸ **Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.

2. In the Experiment Data pane, select all of the experiments (click and drag to select all rows in the table).

3. In the Amplification Plot pane, set the parameters for the plot:
   a. From the Plot Type drop-down menu, select **ΔRn vs Cycle**.
   b. From the Plot Color drop-down menu, select **Well**.
   c. Click **Show a legend for the plot**.

      Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

   d. From the Target drop-down menu, select **ACTB** to highlight all ACTB wells in the study.

4. View the baseline values:
   a. From the Graph Type drop-down menu, select **Linear**.
   b. Select the **Show Baseline** check box to show the start cycle and end cycle.

c. Verify that the baseline is set correctly: The end cycle should be set a few cycles before the cycle number where significant fluorescence is detected. In the example study, the baseline is set correctly.

Note: The data shown in the screenshot below is of the example experiment. Results vary depending on the experiment setup.

5. View the threshold values:
   a. From the Graph Type drop-down menu, select **Log**.
   b. Deselect the **Show Baseline** check box, then select the **Show Threshold** check box to show the threshold.

c. Verify that the threshold is set correctly. In the example study, the threshold is in the exponential phase.

6. Repeat steps 3 through 5 for the remaining targets.

Tips for assessing amplification in your own study

Ensure that your study meets the following requirements:

- **A typical amplification plot** – See the amplification plot examples in the Chapter 5 and Chapter 11.
- **Correct baseline and threshold values** – See the threshold examples and the baseline examples in Chapter 5 and Chapter 11.

If your study does not meet these requirements, you can:

- Manually adjust the baseline and/or threshold. See "View the analysis settings" on page 143.
- Omit individual wells from the analysis. See "Omit wells from the analysis" on page 175.

Assess the gene expression profile using the Gene Expression Plot

The Gene Expression Plot screen displays the results of the relative quantification calculations in the gene expression profile. Three plots are available:

- **RQ vs Target** – Groups the relative quantification (RQ) values by target. Each sample is plotted for each target. You can view the plot as the following graph types: linear, log10, Ln, log2.
- **RQ vs Sample** – (displayed only when the Technical Replicates tab is selected in the Replicate Results Data pane) – Groups the relative quantification (RQ) values by sample. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.
- **RQ vs BioGroup** – (displayed only when the Biological Replicates tab is selected in the Replicate Results Data pane) – Groups the relative quantification (RQ) values by biological replicate group. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.

About the example study

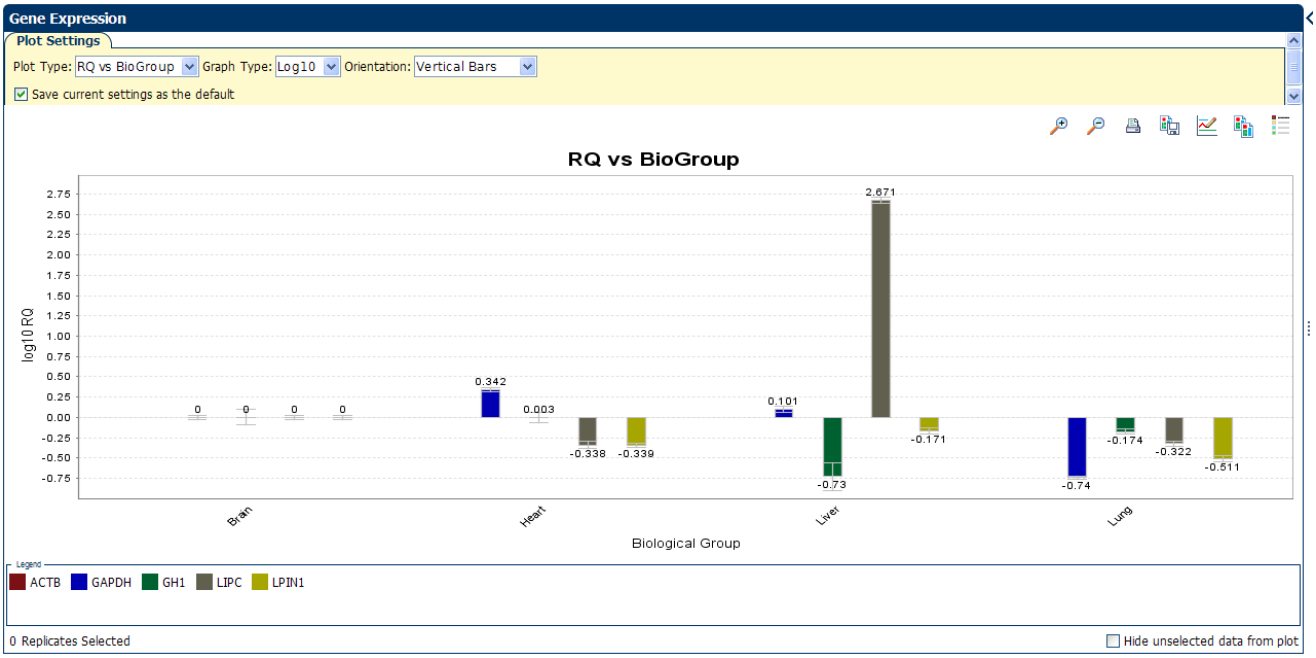
In the Comparative C_T example study, you review each target in the Gene Expression Plot screen for the expression level (or fold change) of the target sample relative to the reference biological replicate group.

View the Gene Expression Plot

1. From the Study Menu pane, select **Analysis** ▸ **Gene Expression**.
2. In the Gene Expression Plot pane, set the parameters for the plot:
   a. In the Plot Type drop-down menu, select **RQ vs BioGroup**.
   b. In the Graph Type drop-down menu, select **Log10**.
   c. In the Orientation drop-down menu, select **Vertical Bars**.
   d. Click **Show a legend for the plot**.

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

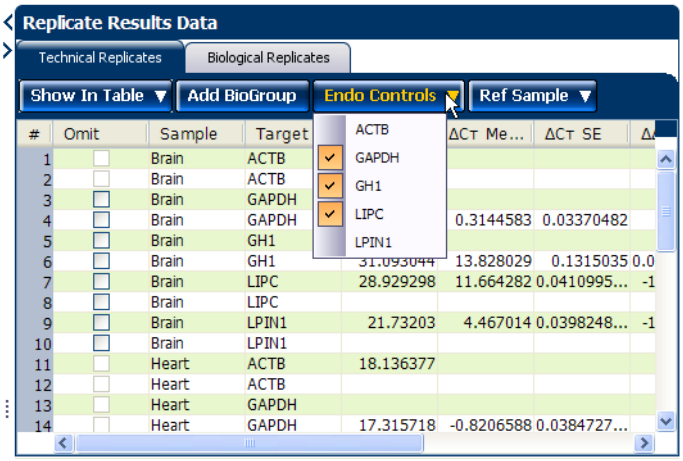
In the example study, the expression levels of multiple targets in the biological replicate group are displayed relative to the expression levels of the same targets in the reference biological replicate group (universal). Because the reference sample is compared to itself, the relative expression level is 1. When the result is displayed in the Log10 graph type, the expression level of the reference sample appears as 0 in the graph (log10 of 1 = 0).

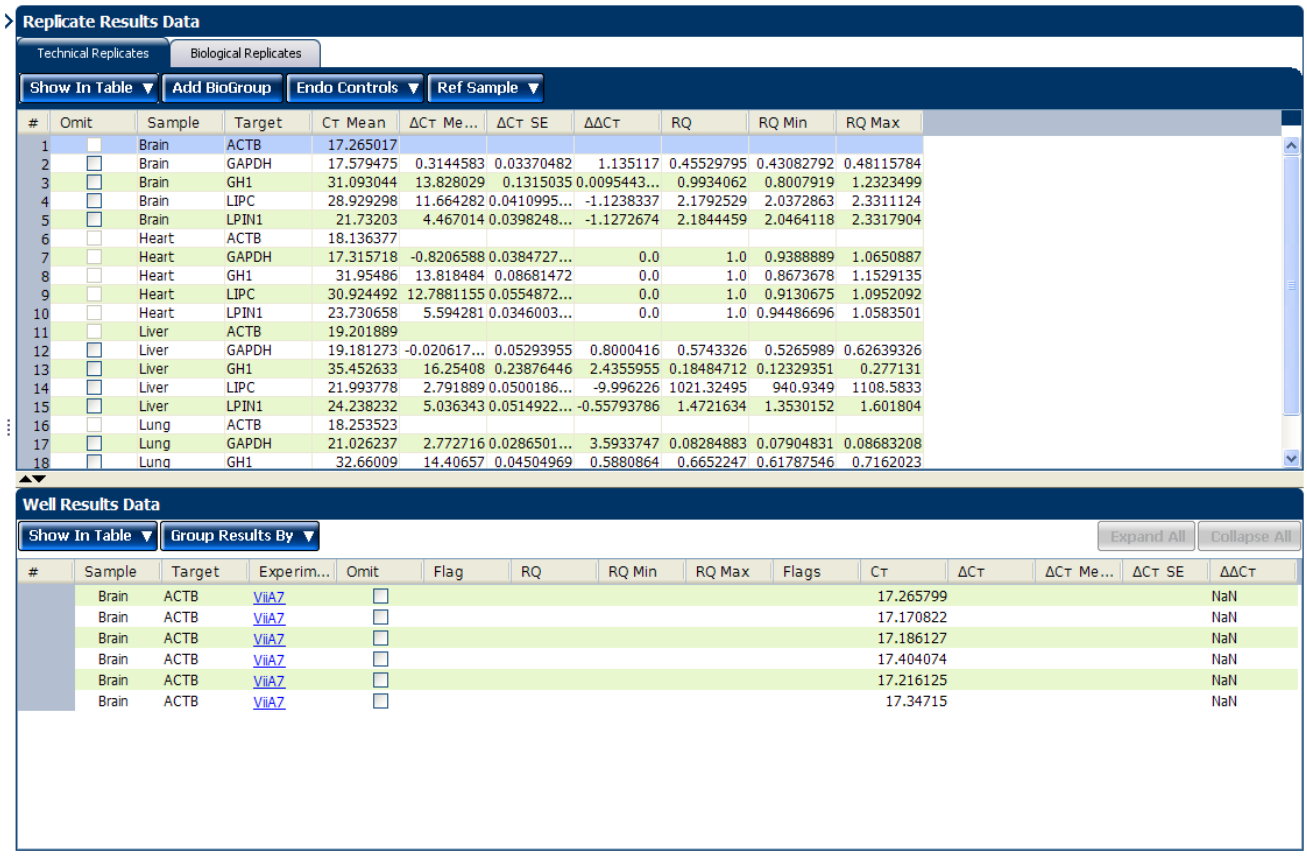


3. Select multiple endogenous controls:
   a. In the Replicate Results Data pane, select the **Technical Replicates** tab.

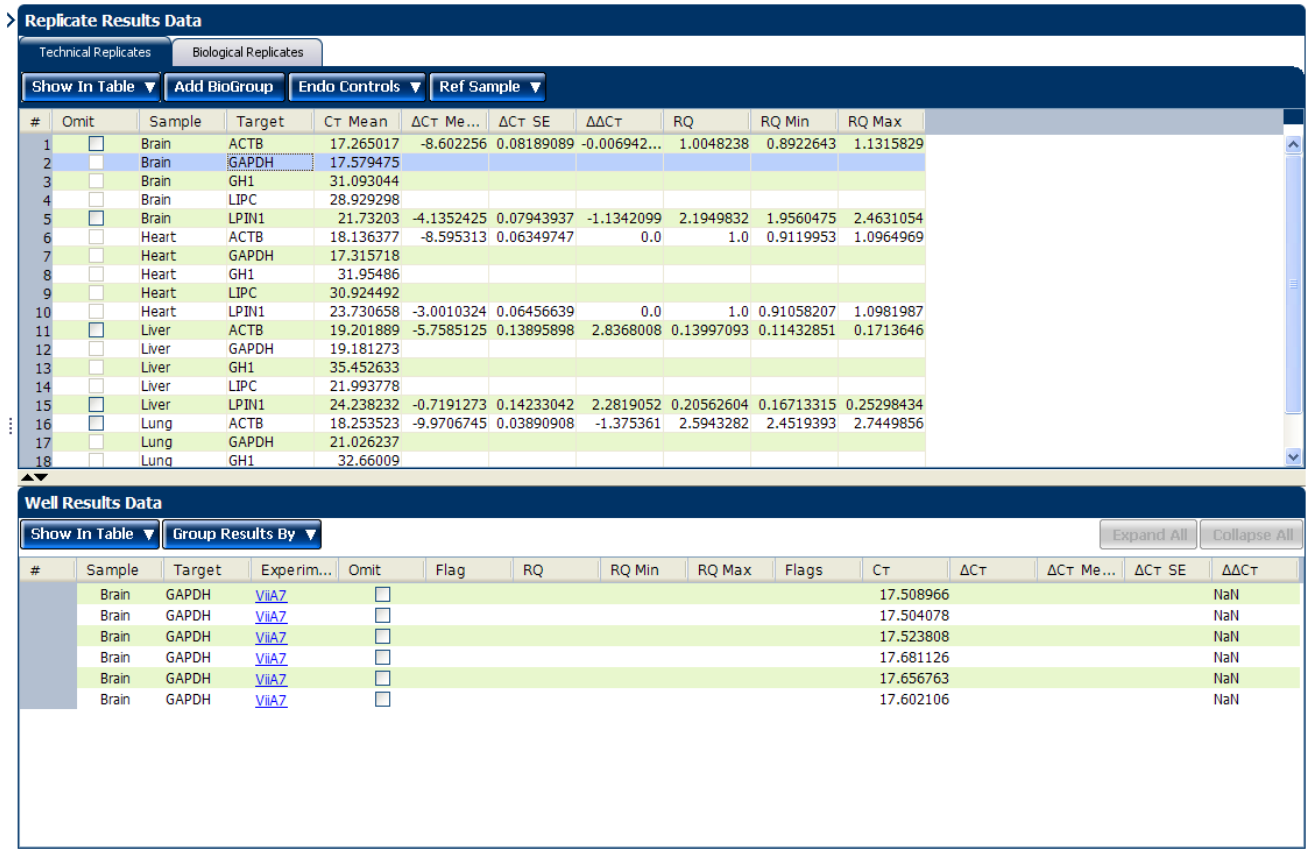
b. From the Endo Controls drop-down menu, select **GAPDH**, **GH1**, and **LIPC**, then deselect **ACTB**.

Note: You can also select the endogenous controls in the Analysis Settings dialog box. See "View the analysis settings" on page 143.





4. Click **Analyze**. In the example study shown below, all samples for the endogenous controls **GAPDH**, **ACTB**, and **LIPC** have no values (with the exception of the C_T Mean value), and the RQ values for the remaining samples change. For example, in the example study, the RQ value for the brain sample changes from 1.0 (with 18S as the endogenous control) to none with the new endogenous controls.



Replicate Results Data

Technical Replicates | Biological Replicates

Show In Table ▼ | Add BioGroup | Endo Controls ▼ | Ref Sample ▼

#	Omit	Sample	Target	CT Mean	ΔCT Me...	ΔCT SE	ΔΔCT	RQ	RQ Min	RQ Max
1	☐	Brain	ACTB	17.265017	-8.602256	0.08189089	-0.006942...	1.0048238	0.8922643	1.1315829
2	☐	Brain	GAPDH	17.579475						
3	☐	Brain	GH1	31.093044						
4	☐	Brain	LIPC	28.929298						
5	☐	Brain	LPIN1	21.73203	-4.1352425	0.07943937	-1.1342099	2.1949832	1.9560475	2.4631054
6	☐	Heart	ACTB	18.136377	-8.595313	0.06349747	0.0	1.0	0.9119953	1.0964969
7	☐	Heart	GAPDH	17.315718						
8	☐	Heart	GH1	31.95486						
9	☐	Heart	LIPC	30.924492						
10	☐	Heart	LPIN1	23.730658	-3.0010324	0.06456639	0.0	1.0	0.91058207	1.0981987
11	☐	Liver	ACTB	19.201889	-5.7585125	0.13895898	2.8368008	0.13997093	0.11432851	0.1713646
12	☐	Liver	GAPDH	19.181273						
13	☐	Liver	GH1	35.452633						
14	☐	Liver	LIPC	21.993778						
15	☐	Liver	LPIN1	24.238232	-0.7191273	0.14233042	2.2819052	0.20562604	0.16713315	0.25298434
16	☐	Lung	ACTB	18.253523	-9.9706745	0.03890908	-1.375361	2.5943282	2.4519393	2.7449856
17	☐	Lung	GAPDH	21.026237						
18	☐	Lung	GH1	32.66009						

Well Results Data

Show In Table ▼ | Group Results By ▼ | Expand All | Collapse All

#	Sample	Target	Experim...	Omit	Flag	RQ	RQ Min	RQ Max	Flags	CT	ΔCT	ΔCT Me...	ΔCT SE	ΔΔCT
	Brain	GAPDH	ViiA7	☐						17.508966				NaN
	Brain	GAPDH	ViiA7	☐						17.504078				NaN
	Brain	GAPDH	ViiA7	☐						17.523808				NaN
	Brain	GAPDH	ViiA7	☐						17.681126				NaN
	Brain	GAPDH	ViiA7	☐						17.656763				NaN
	Brain	GAPDH	ViiA7	☐						17.602106				NaN

Tips for assessing the gene profile in your own study

Look for:

- Differences in gene expression (as a fold change) relative to the reference sample.
- Standard deviation in the replicate groups (C_T SD values).

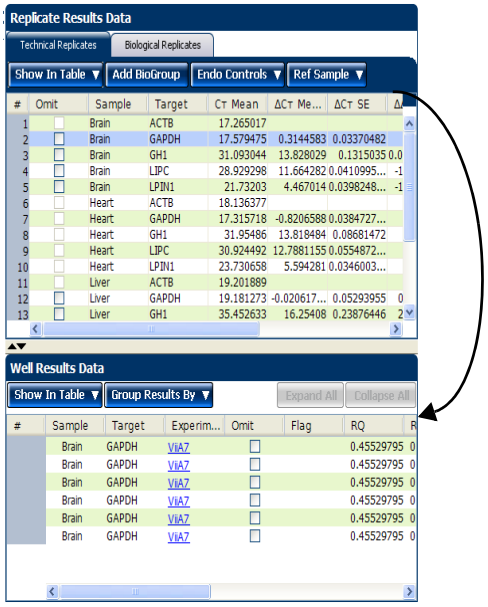
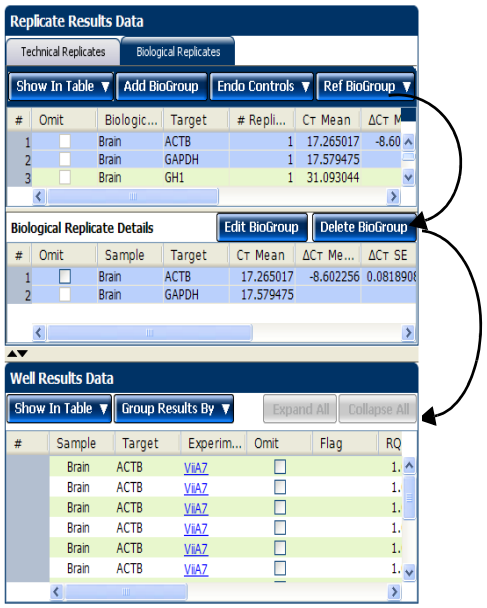
If needed, you can omit outliers. See "Omit replicates from the analysis" on page 158.

Note: To display a subset of the study data in the Gene Expression Plot pane, select one or more rows in the Technical Replicates tab or the Biological Replicates tab, then select **Hide unselected data from plot** to display data only from the selected rows.

View the replicate results data and the well results data

The Replicate Results Data pane lists each reaction plate (experiment) that is added to a study. The results of the study are arranged by technical or biological replicate association.

The data that are displayed in the Well Results Data pane depend on which tab you select in the Replicate Results Data pane:

Technical Replicates Tab	Biological Replicates Tab
This tab arranges the results of the relative quantification analysis by technical replicate group. The ViiA™ 7 Software displays the results for each sample/target combination as a row in the table. You can view the members of a technical replicate group by selecting the appropriate row in the table. When a row is selected, the Well Results Data pane displays the wells that make up the selected technical replicate group. 	This tab arranges the results of the relative quantification analysis by biological replicate group. The ViiA™ 7 Software displays the results for each biological group as a row in the table (each row displays a biological sample with its target). You can view the members of a biological replicate group by selecting the appropriate row from the table. When a row is selected: • The Biological Replicate Details table displays the technical replicate groups that make up the selected biological replicate group. • The Well Results Data pane displays the individual members of the technical replicate groups that make up the selected biological replicate group. 

Column descriptions

The table below provides definitions for the column headings that appear in the tables in the Technical Replicates and Biological Replicates tabs.

Note: To show or hide columns in a table, select or deselect the column name from the Show In Table drop-down menu.

Column	Description
Δ_{CT}	The calculated Δ_{CT} value for the replicate group associated with the test sample. **Note:** The ΔC_T value is calculated only for multiplex experiments and is calculated at the well level (that is, the individual technical replicate level) by subtracting the target C_T value from the endogenous control $_{CT}$ value.
ΔC_T Mean	The arithmetic average of the technical replicate C_T values for the sample replicate group. **Note:** The ΔC_T Mean value is calculated at the reaction plate level and represents the mean difference between the target $_{CT}$ values and the endogenous control C_T values for all the technical replicates for that sample that are present on the plate.
ΔC_T SE	The Standard Error of the mean associated with the reported Mean ΔC_T value. **Note:** The Δ_{CT} SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level $_{CT}$ value variation between the target and the endogenous control. **Note:** If you select the Standard Deviation option in the RQ Min/Max calculations on the Relative Quantification Settings in the Analysis Settings dialog box, Δ_{CT} SD, that is the Standard Deviation values are calculated by the ViiA™ 7 Software.
$\Delta\Delta C_T$	The calculated $\Delta\Delta_{CT}$ value for the replicate group associated with the reference sample.
# Replicates	The number of biological replicate groups in the study.
Biological Group	The name of the biological replicate group.
C_T	Threshold cycle; the PCR cycle number at which the fluorescence meets the threshold in the amplification plot.
C_T Mean	The arithmetic average of the technical replicate $_{CT}$ values.
Experiment	The name of the experiment file (for example, heart.eds).
Flag	The number of QC flags that the well generated as listed in the ▲ symbol.
Omit (Replicate Results Data pane)	Indicates the omission status of the members of the associated technical or biological replicate group(s): • A check mark (✓) indicates that all replicates have been removed from the analysis. • A hyphen (**–**) indicates that one or more replicates have been removed from the analysis.
Omit (Well Results Data pane)	Indicates the omission status of the well. A check mark (✓) indicates that the well has been removed from the analysis.
RQ	The calculated relative level of gene expression for the replicate group that is associated with the test sample.

Column	Description
RQ Max	The maximum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box. **Note:** The maximum includes the variability associated with the endogenous control and targets in only the test samples.
RQ Min	The minimum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box. **Note:** The minimum includes the variability associated with the endogenous control and targets in only the test samples.
Sample	The sample associated with the data displayed in the row.
Target	The target assay associated with the data displayed in the row.
Well	The location of the well in the reaction plate.

About the example study

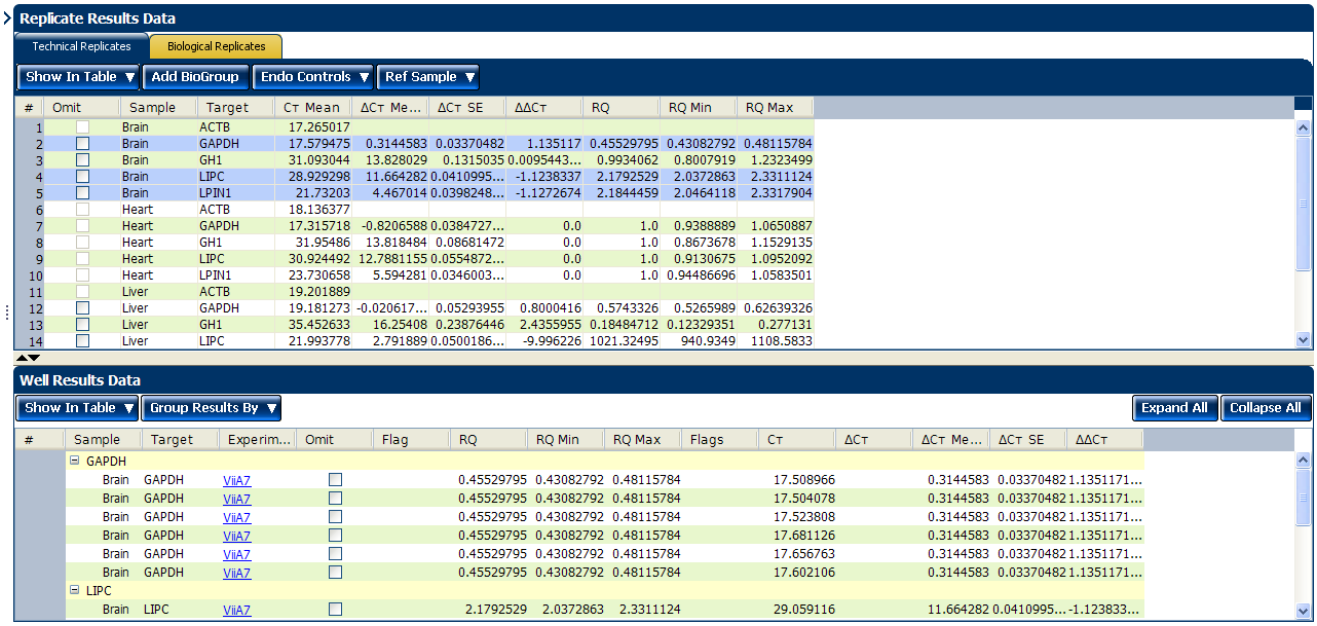
In the Comparative C_T example study, you review the Replicate Results Data pane and the Well Results Data pane to evaluate the C_T precision of the replicate groups and view related RQ information.

View the results data

1. From the Study Menu pane, select **Analysis ▸ Gene Expression**.
2. Click at the top left of the Replicate Results Data pane.
3. View the technical replicates:
   a. Click the **Technical Replicates** tab. The table displays the results by technical replicate group.
   b. In the Technical Replicates table, select the following groups:
      - Brain/GAPDH (row2)
      - Brain/LIPC (row 4)
      - Brain/LPIN1 (row 5)

      The Well Results Data pane displays all wells that make up the selected groups.
4. View the values in the Well Results Data pane:
   a. From the Group Results By drop-down menu, select **Target**.

b. View the C_T, ΔC_T Mean, and ΔC_T SE values to evaluate the C_T precision of the replicate groups. In the example study, the low ΔC_T SE values indicate these replicates have good C_T precision.



Tips for viewing replicate results in your own study

- Select the **Technical Replicates** tab or the **Biological Replicates** tab to organize and view the sample data according to the associated technical replicate group or biological replicate group.
- View all wells for a technical or biological replicate group by selecting the appropriate row in the table. When a row is selected, the Well Results Data pane displays the wells that make up the group. **Ctrl-click** to select multiple rows.
- Change the endogenous control by clicking **Endo Control**, then selecting a new target.
- Change the reference sample by clicking **Ref Sample**, then selecting a new sample.
- Add biological replicate groups by clicking **Add BioGroup**.
- Omit biological or technical replicates from the analysis. See "Omit replicates from the analysis" on page 158.

  Note: The Comparative C_T Study Example.edm file demonstrates the use of biological replicate groups. An additional example study that does not use biological replicate groups is provided with the ViiA™ 7 Software. You can find this study file on your computer at:
  <drive>:\Applied Biosystems\ViiA7 Software v1.1\experiments\examples\ViiA7 Gene Expression Study Example.edm.

Omit replicates from the analysis

To omit a technical or biological replicate from the analysis:

1. From the Study Menu pane, select **Analysis ▸ Gene Expression**.
2. Select the **Technical Replicates** or **Biological Replicates** tab according to the type of replicate that you want to omit.
3. In the replicate table, scroll to the biological or technical replicate of interest, then select the corresponding check box in the Omit column.
4. Click **Analyze** when you finish omitting wells.

IMPORTANT! You cannot omit *all* technical replicates that belong to a reference sample or a reference biological group, or that serve as the endogenous control for a study.

Note: You can also omit the biological replicates in the Biological Replicate Details table at the bottom of the Biological Replicates tab.

Confirm accurate dye signal using the Multicomponent Plot

The Multicomponent Plot screen displays the complete spectral contribution of each dye over the duration of the PCR run in a selected well of any experiment that is added to the study.

About the example study

In the comparative C_T example study, you review the Multicomponent Plot screen for:

- ROX™ dye (passive reference)
- FAM™ dye (reporter)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

View the Multicomponent Plot

1. From the Study Menu pane, select **Analysis ▸ Multicomponent Plot**.
2. In the Experiment Data pane, select the **ViiA7 Comparative Ct Example 1.eds** experiment.
3. Display the unknown wells one at a time in the Multicomponent Plot pane:
   a. Click the **Plate Layout** tab.
   b. Select one well in the plate layout; the well is shown in the Multicomponent Plot pane.

Note: If you select multiple wells, the Multicomponent Plot pane displays the data for all selected wells simultaneously.

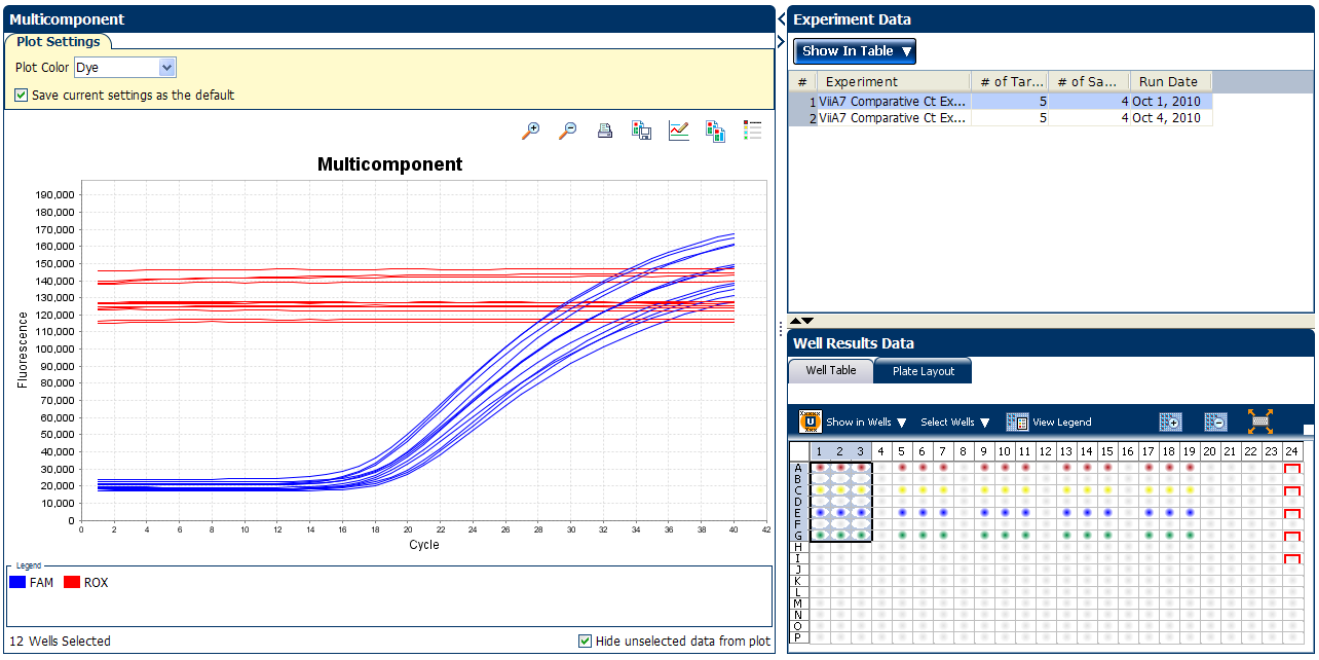
4. In the Multicomponent Plot pane, set the parameters for the plot:
   a. From the Plot Color drop-down menu, select **Dye**.
   b. Click **Show a legend for the plot**.

      Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

5. Check the FAM dye signal. In the example study, the FAM dye signal increases throughout the PCR process, which indicates normal amplification.

6. Check the ROX dye signal. In the example study, the ROX dye signal remains constant throughout the PCR process, which indicates typical data.

7. Repeat steps 2 through 6 for the remaining experiments in the study.



Tips for confirming dye-signal accuracy in your own studies

- **Passive reference** – The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye** – The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- **Irregularities in the fluorescence** – There should not be any spikes, dips, and/or sudden changes in the fluorescence.
- **Negative control wells** – There should not be any amplification in the negative control wells.

If your study does not meet the guidelines above, you can omit individual wells from the analysis. See "Omit wells from the analysis" on page 175.

View the QC Plots

The QC Plots screen displays the endogenous control and replicate analysis results for each reaction plate (experiment) added to the study. The following plots are derived from the experiments added to a study:

- Endogenous Control Profile
- Box Plot
- Technical Replicates Correlation
- Biological Replicates Correlation

View the Endogenous Control Profile plot

The QC Plots screen displays the Endogenous Control Profile plot for the endogenous controls used in the experiments added to a gene expression study. The endogenous control profile plot displays how much of the endogenous control is expressed in a sample. The sample is plotted on the X-axis, and the C_T is plotted on the Y-axis. The expression is viewed as a color and shape combination in the plot.

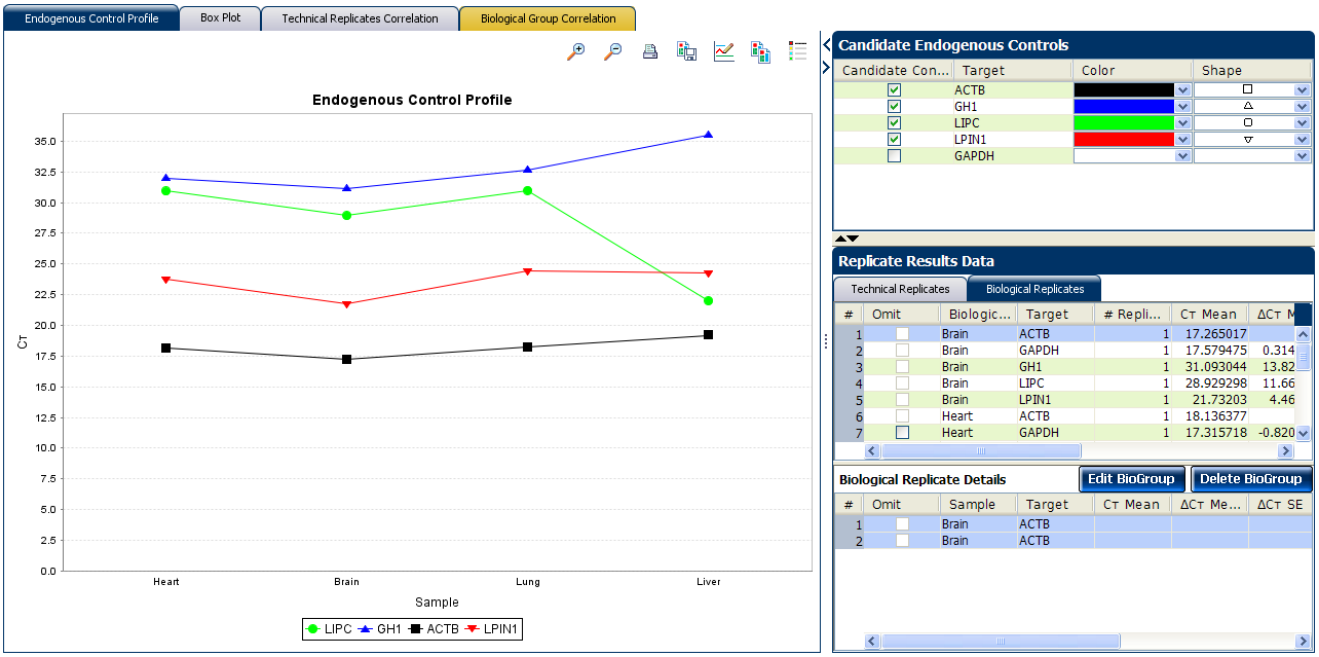
In the example study, you view four potential endogenous controls expressed in four samples. The potential endogenous controls are:

- ACTB
- GH1
- LIPC
- LPIN1

The samples are brain, heart, lung, and liver.

1. From the Study Menu pane, select **Analysis ▸ QC Plots**.
2. In the QC Plots pane, click **Endogenous Control Profile**.
3. In the **Candidate Endogenous Controls** pane, select the check boxes of those Targets whose profile you want to view in the plot pane. In the example study, the **Target** ACTB is chosen to be the endogenous control because it is expressed at similar levels in three out of four of the given samples.
4. In the Replicate Results Data pane, view results by replicate group.
   a. Click the **Technical Replicates** tab. The table displays the results by technical replicate group.

b. Click the **Biological Replicates** tab. The table displays the results by biological replicate group.



View the box plots

The Box Plot displays the C_T distribution of a particular **Target** in samples. You can see the individual C_T values/ raw data with this plot.

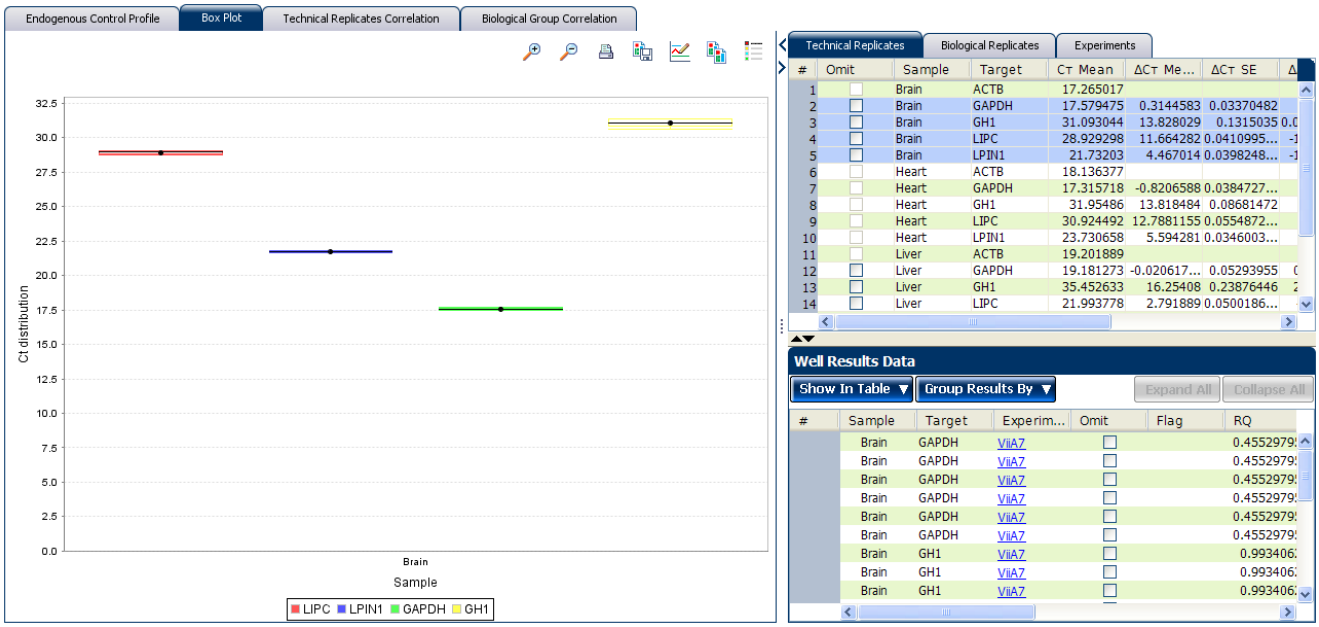
In the example study, you view the box plots of five targets in four different samples:

- LPIN1
- GAPDH
- LIPC
- GH1
- ACTB

The samples are Heart, Liver, Lung, and Brain.

1. In the QC Plots pane, click **Box Plots** to access the Replicate Results Data pane.
2. In the Replicate Results Data pane, click the **Technical Replicates** tab. The table displays the results by technical replicate group.
3. Click the **Biological Replicates** tab. The table displays the results by biological replicate group.
4. Click the Experiments tab to select the experiment whose Box plot to view.

5. View the values in the Well Results Data pane. View the C_T, ΔC_T Mean, and ΔC_T SE values to evaluate the C_T precision of the replicate groups. In the example study, the low ΔC_T SE values indicate these replicates have good C_T precision.



View the Technical Replicates Correlation plot

The Technical Replicates Correlation plot displays the correlation between the target genes in one or more samples.

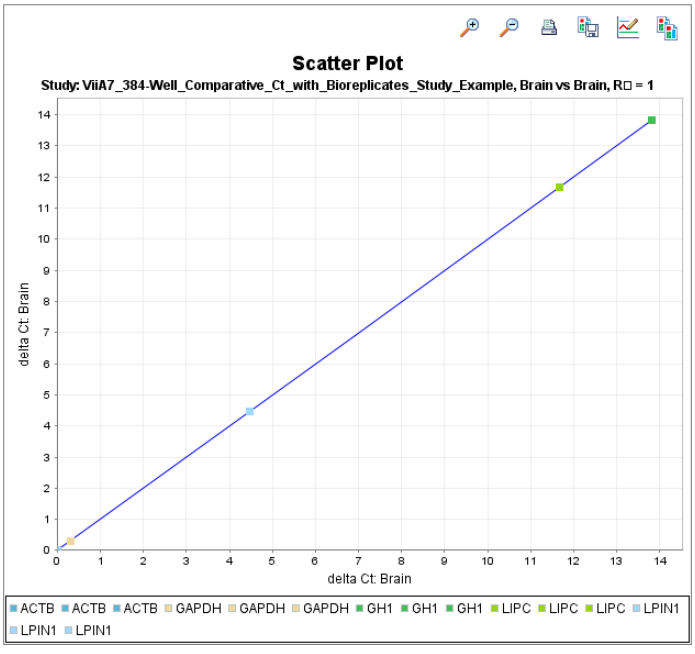
The Technical Correlation Group plot is made of two components, the scatter plot and the heat map.

Scatter Plot

The scatter plot shows the distribution of ΔC_T of targets for different samples.

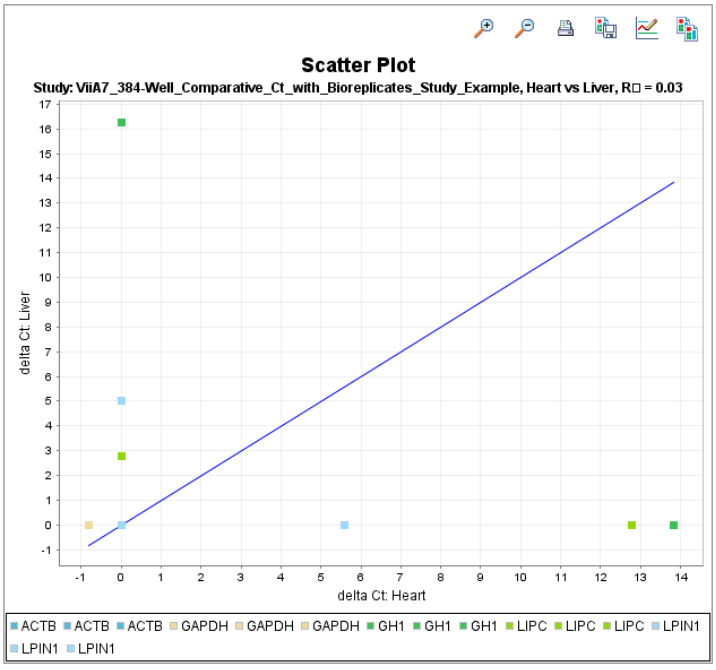
If a correlation (represented by R^2 or the coefficient of determination) exists among the targets in the samples, the targets appear on or along the line of reference. If the correlation is weak or not present, the targets appear scattered in the plot, away from the line of reference.

The line of reference is fixed in the ViiA™ 7 Software.



If:

- R^2 =/≈ 1, then the correlation is strong
- R^2 < 1, then the correlation is weak
- R^2 = 0, then there is no correlation



Heat maps

The heat map shows the variation of coefficient determination for different scatter plots. Each cell of the heat map represents a different scatter plot, and therefore a different value for R^2. The cells inclined to red represent a lower R^2 value; the cells inclined to green represent a higher R^2 value.

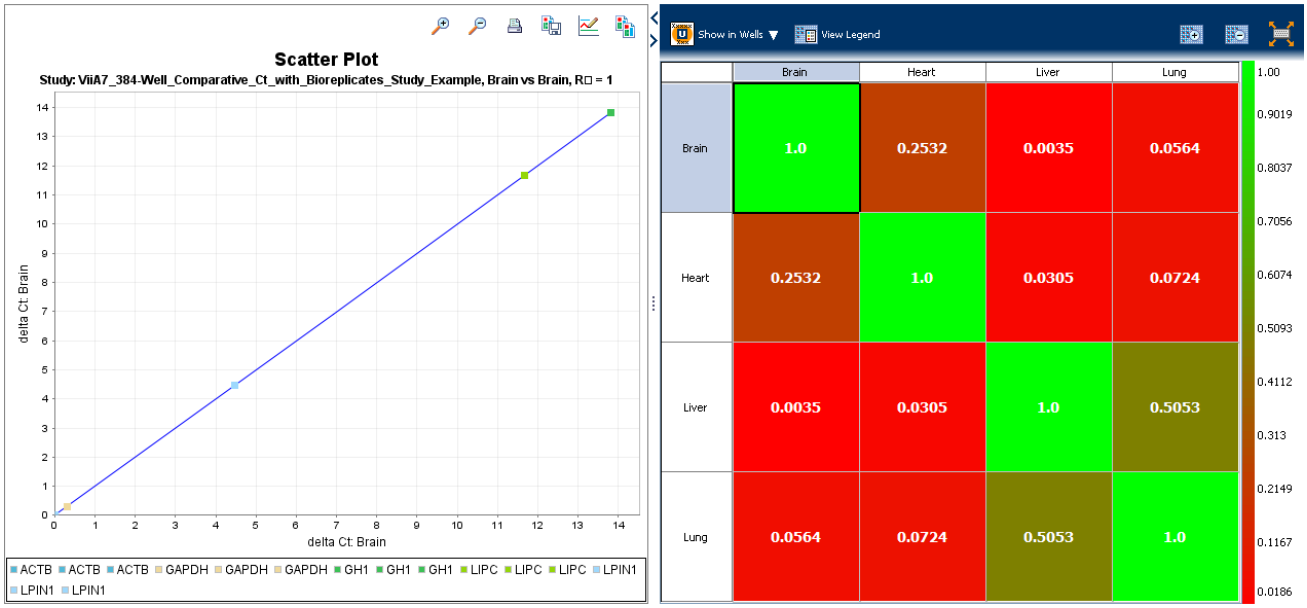
In the example study, you view the scatter plots and heat maps of five targets in four different samples:

- LPIN1
- GAPDH
- LIPC
- GH1
- ACTB

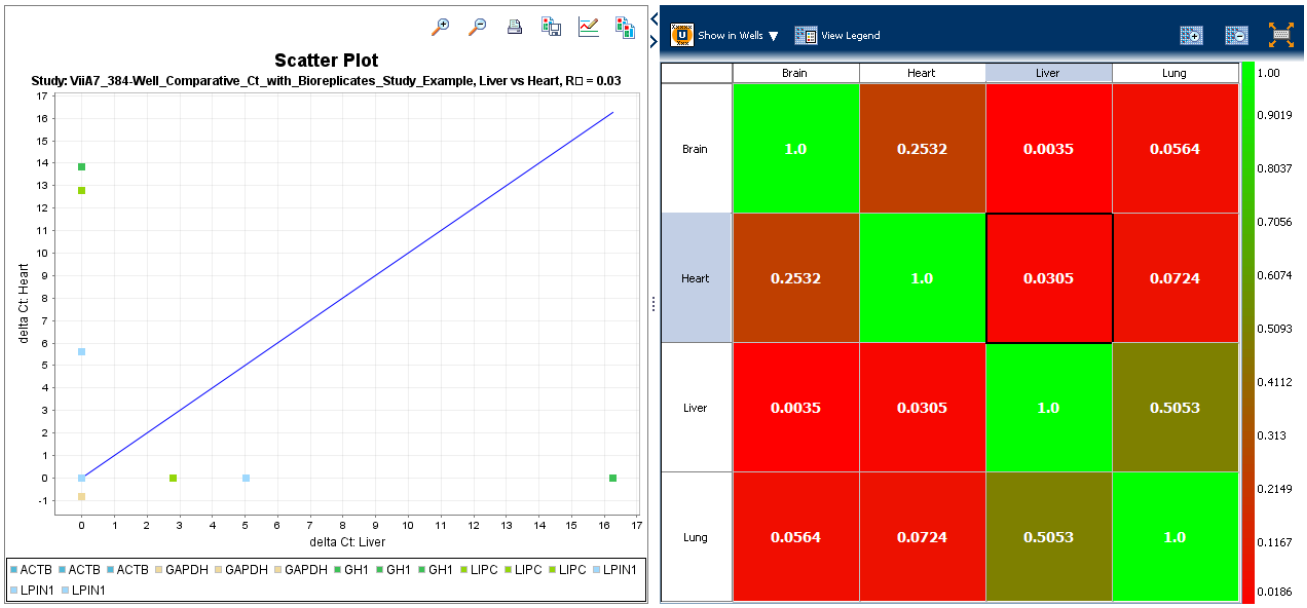
The samples are Brain, Heart, Kidney and Lung.

1. In the QC Plots pane, click **Technical Replicates Correlation** to access the Heat Map.

2. In the Heat Map, click the cell with a correlation value, R^2 =/≈ 1. The corresponding scatter plot pane displays the scatter plot of the targets in that technical replicate group along the line of reference.



3. Click the cell with a correlation value, R^2 =/≈ 0. The corresponding scatter plot pane displays the scatter plot of the targets in that technical replicate group away from the line of reference.



View the Biological Group Correlation plot

The Biological Group Correlation plot displays the correlation between the target genes in one or more biological group samples. Biological groups provide a broader set of samples, with the same targets.

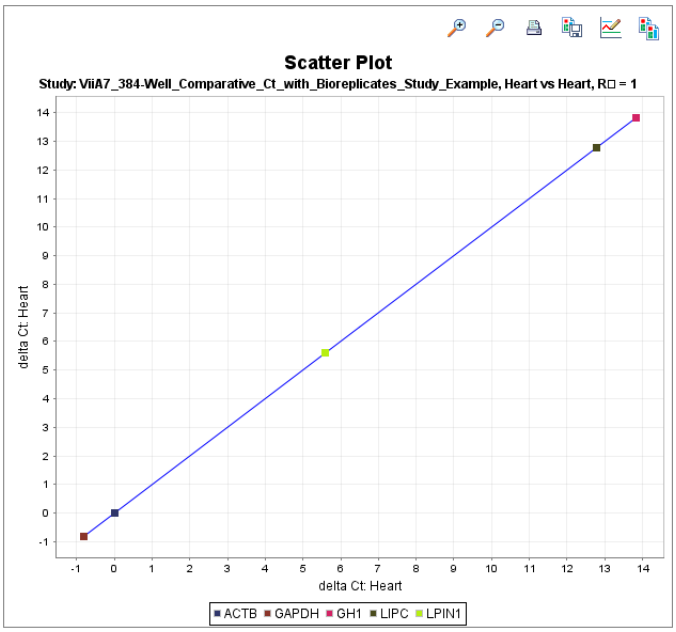
Note: If the experiments in your study do not use biological replicate groups, see "Define Replicates" on page 136 to create a new biological group.

The Biological Group Correlation plot is also made of two components, the scatter plot and the heat map.

Scatter Plot

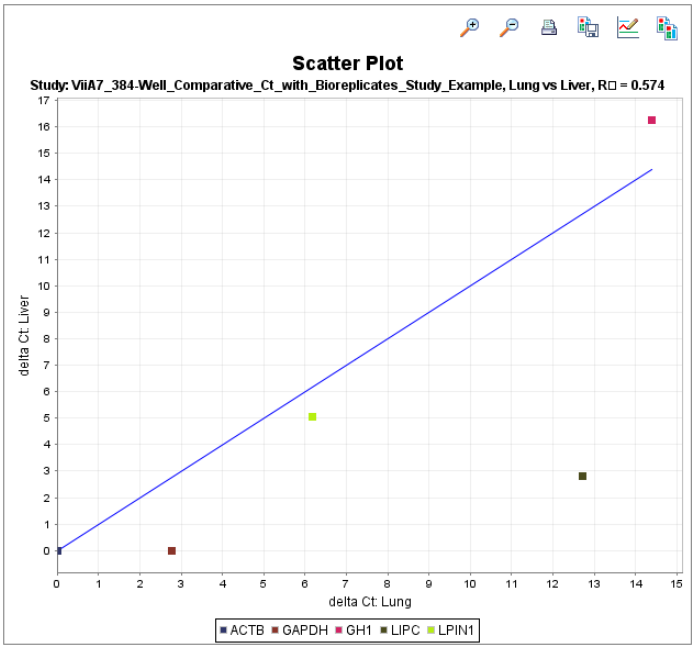
The scatter plot shows the distribution of ΔC_T of targets for different biological groups. If a correlation (represented by R^2 or the coefficient of determination) exists among the targets in the biological groups, the targets appear on or along the line of reference. A weak correlation or no correlation is represented by the targets being scattered in the plot and away from the line of reference.

Note: The line of reference is fixed in the ViiA™ 7 Software.



If:

- R^2 =/≈ 1, then the correlation is strong
- R^2 < 1, then the correlation is weak
- R^2 = 0, then there is no correlation



Heat maps

The heat map shows the variation of coefficient determination for different scatter plots. Each cell of the heat map represents a different scatter plot, and therefore a different value for R^2. The dull green cells represent a lower R^2 value; the bright green cells represent a higher R^2 value.

In the example study, you view the scatter plots and heat maps of eight targets across four biological groups:

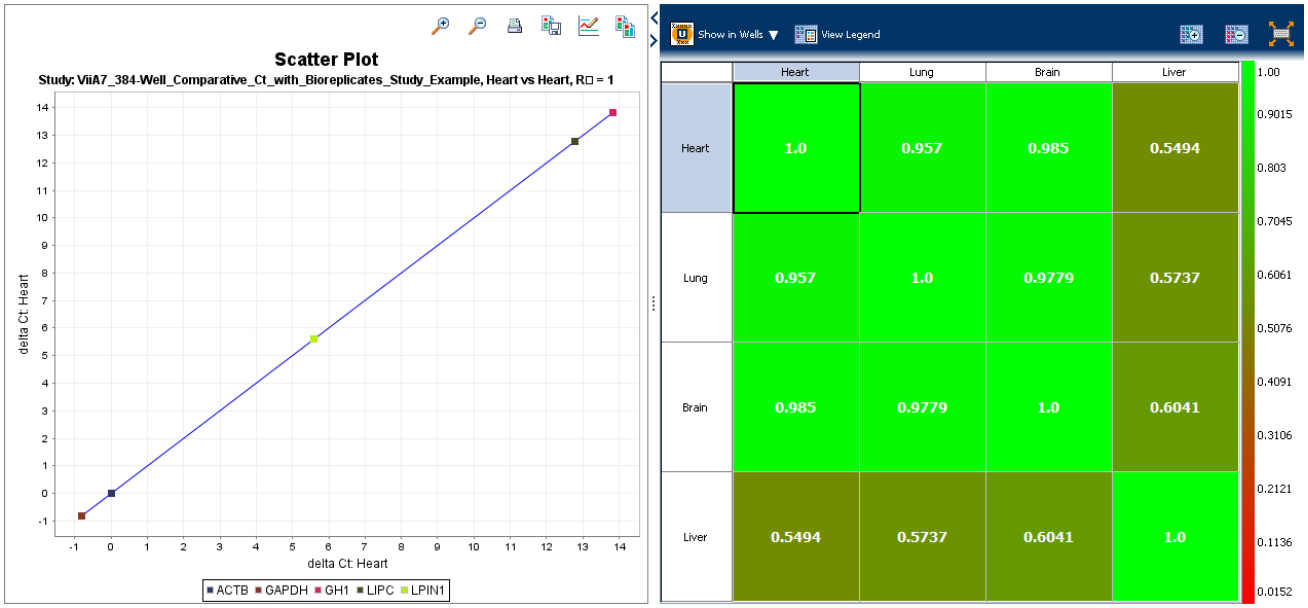
- LPIN1
- GAPDH
- LIPC
- GH1
- ACTB

Technical replicate samples of Brain cDNA belong to the biological group Brain, those of Lung cDNA belong to biological group Lung, technical replicate samples of Liver cDNA belong to the biological group Liver, and those of Heart cDNA belong to the biological group Heart.

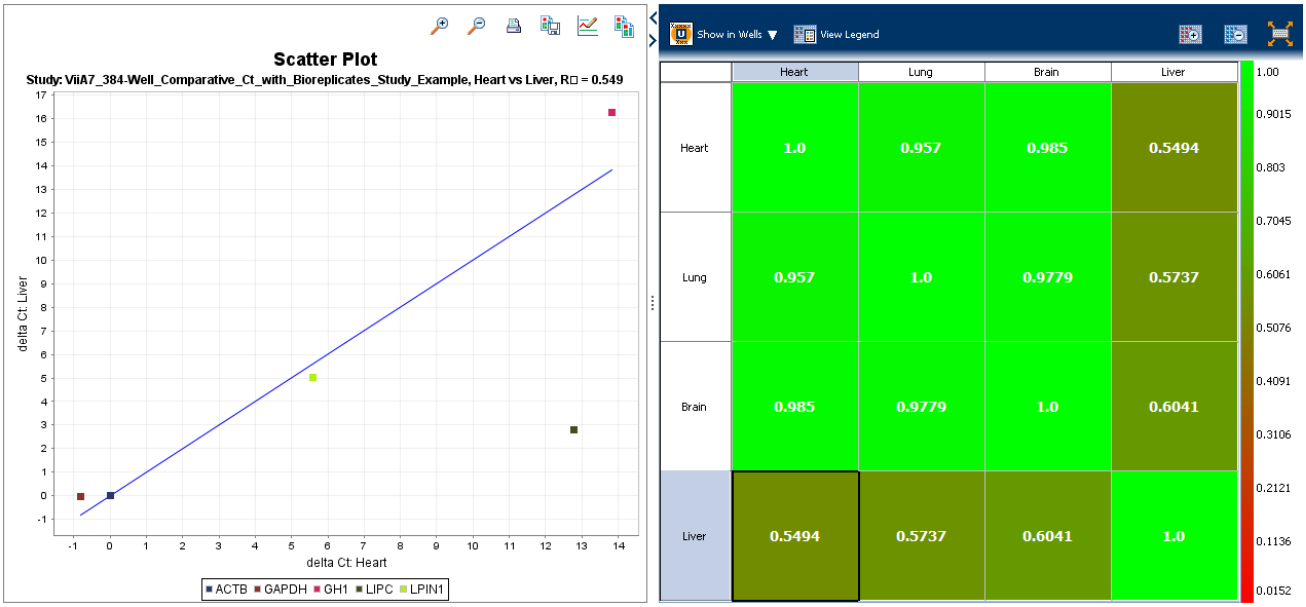
To view the Biological Group Correlation plot:

1. In the QC Plots pane, click **Biological Group Correlation** to access the heat map.

2. In the Heat Map, click the cell with a correlation value, R^2 =/≈ 1. The corresponding scatter plot pane displays the scatter plot of the targets in that biological replicate group along the line of reference.



3. Click the cell with a correlation value, R^2 =/≈ 0. The corresponding scatter plot pane displays the scatter plot of the targets in that biological replicate group away from the line of reference.



Tips for viewing your own scatter plots and heat maps

When you analyze your study, look for scatter plots and heat maps that display a correlation value that is =/≈ 1 for samples that come from the same source or tissue (technical or biological replicates). If the replicates do not correlate well, that could be a sign that there is a problem with a sample.

If your study does not meet the guidelines above, you can omit individual wells from the analysis. See "Omit wells from the analysis" on page 175.

View the QC Summary

The QC Summary screen displays a list of the ViiA™ 7 Software flags, and it includes the flag frequency and location for any experiment that is added to a study.

About the example study

In the Comparative C_T example study, you review the QC Summary screen for any flags generated by the study data. In the example study, several wells produced data that generated flags.

View the QC Summary

1. From the Study Menu pane, select **Analysis ▸ QC Summary**.

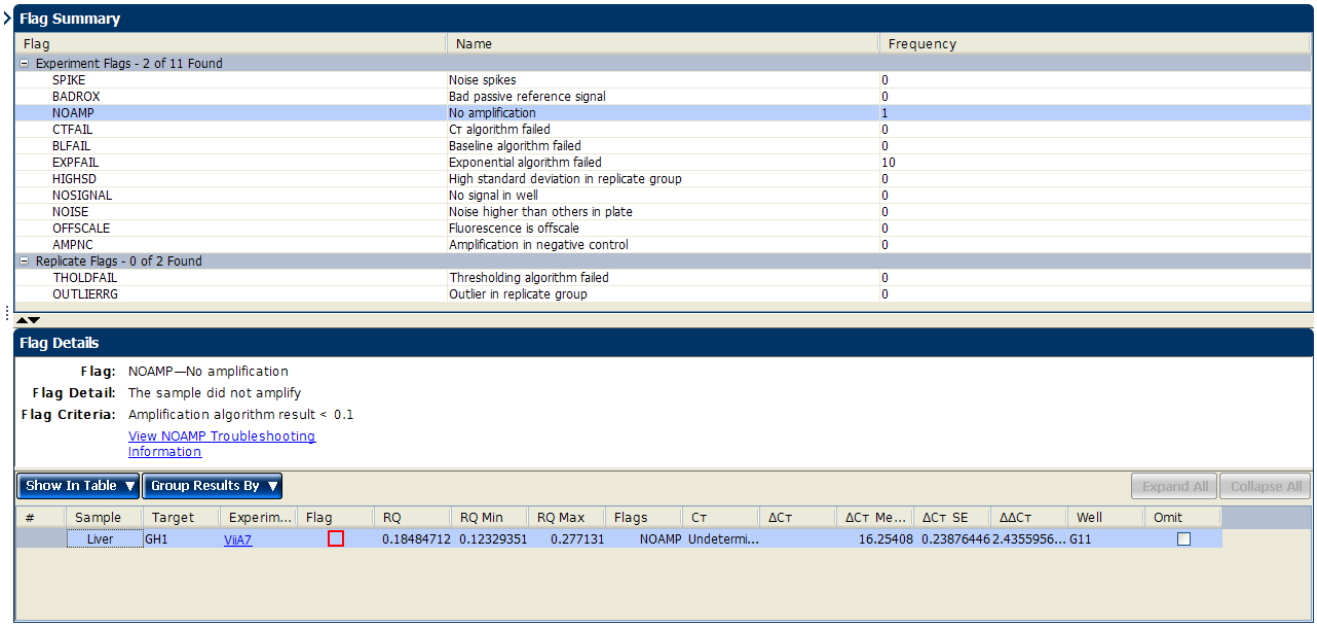
   Note: If no data are displayed, click **Analyze**.

2. In the Flags Summary table, look in the Frequency column to determine which flags appear in the study. In the example study. The EXPFAIL flag appears 10 times and the NOAMP flag appears once.

   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the study.

3. For each flag that appears in the study, click the flag row to display details about the flag in the Flag Details table. In the example study, the NOAMP flag indicates no amplification and the EXPFAIL flag indicates that the exponential algorithm failed.

4. Consider removing the NOAMP well from the analysis. See "Omit wells from the analysis" on page 175.



Possible flags

For Comparative C_T studies, the flags listed below may be generated by the study data.

If a flag does not appear in the study, its frequency is 0. If the frequency is >0, the flag appears somewhere in the study, and the associated well position is listed in the Wells column.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed

Flag	Description
Secondary analysis flags	
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the Frequency column for each flag).

Tips for using flags to evaluate your study

- In the Flag Summary table, click each flag that has a frequency >0 to display details about the flag in the Flag Details table. If needed, click the troubleshooting link in the Flag Details table to view information on correcting the flag.

  Note: In the Flag Details table, the numbers on each flag symbol indicate the number of flags generated for that well. For example, indicates that two flags have been generated for that well.

- You can change the flag settings. For more information, see "Flag Settings" on page 145:
  - Adjust the sensitivity so that more wells or fewer wells are flagged.
  - Change the flags that are applied by the ViiA™ 7 Software.
- You can omit individual wells from the analysis. See "Omit wells from the analysis" on page 175.

Compare analysis settings

Use the Compare Settings screen to perform a side-by-side comparison of analysis settings for a comparative $_{CT}$ study. You can change one or more of the analysis settings, then compare the new results with the previous results. For example, you can compare the effects of:

- Using multiple endogenous controls versus a single endogenous control.
- Changing the amplification efficiency of a specific target versus keeping it at 100%.

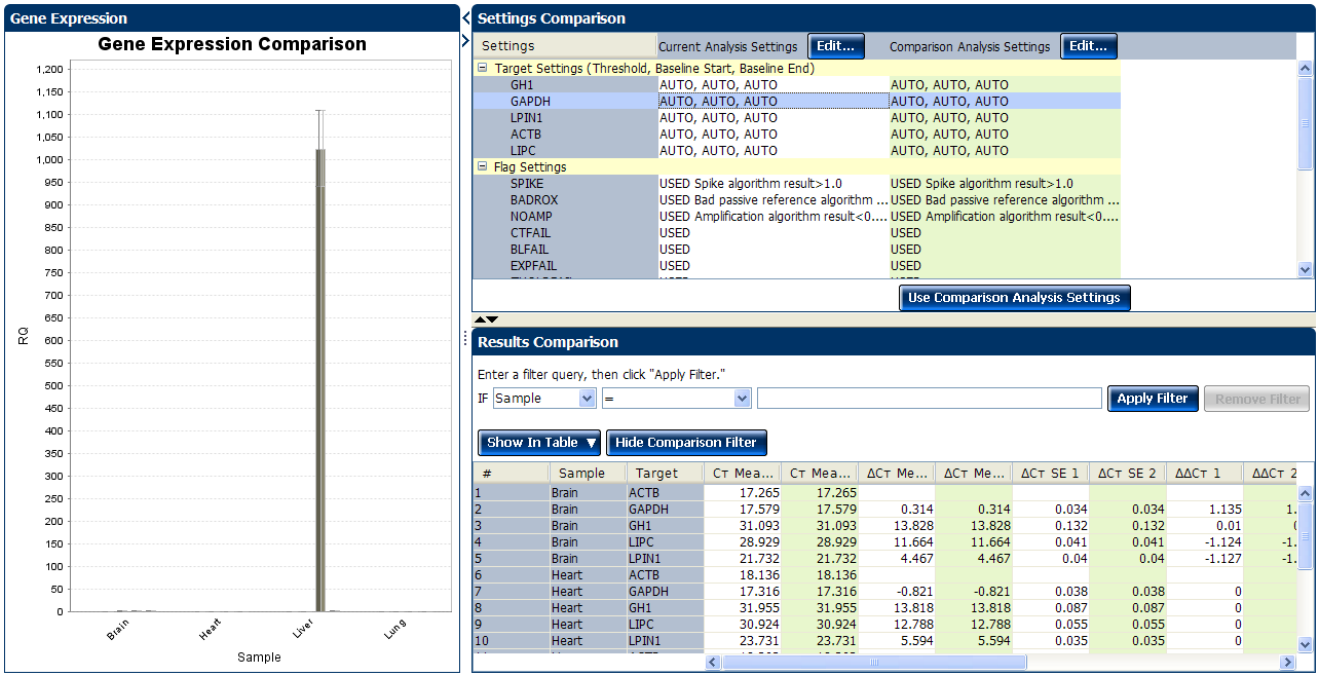
About the example study

In the comparative C_T example study, you change the endogenous control to LPIN1, then compare results.

Modify comparison criteria

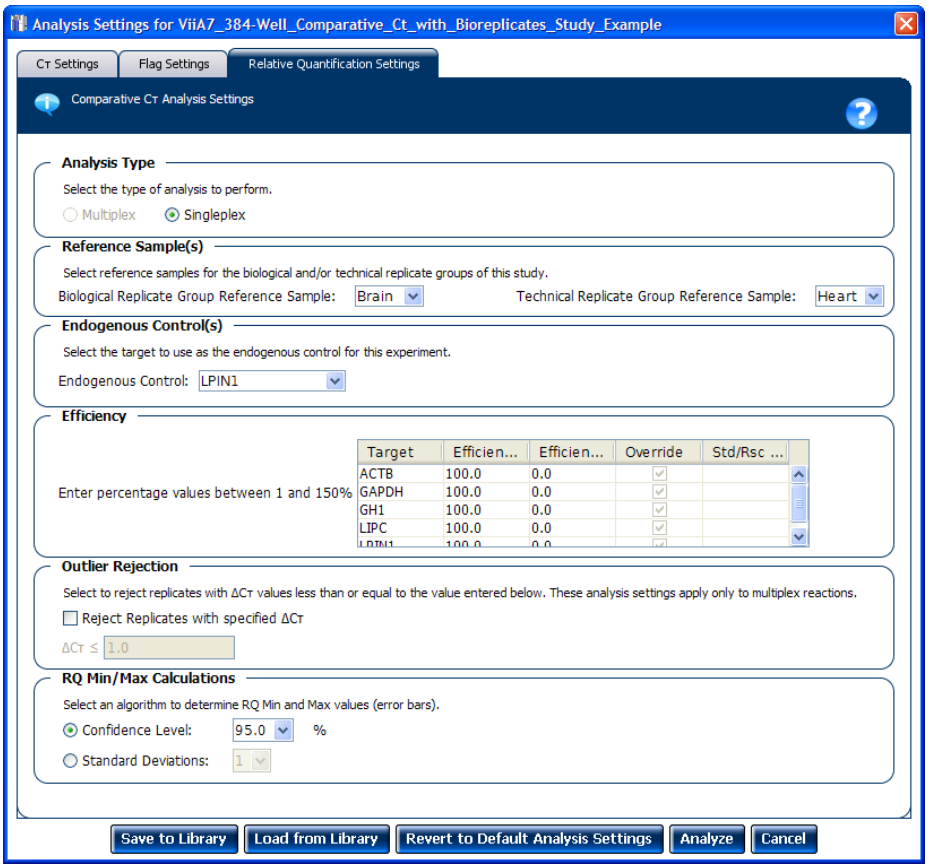
1. From the Study Menu pane, select **Analysis ▸ Compare Settings**. When the Compare Settings screen is initially displayed (before you make any changes):
   - In the Settings Comparison pane, the green column is titled "Current Analysis Settings," the white column is titled "Comparison Analysis Settings," and the Use Comparison Analysis Settings button is under the green column.
   - In the Results Comparison pane, values in the white columns and values in the green columns are the same.
   - The Gene Expression Comparison plot is the same plot that is displayed in the Gene Expression screen (**Analysis ▸ Gene Expression**).

2. In the Settings Comparison pane, click **Edit** in the green column to open the Comparison Analysis Settings dialog box.



3. In the Comparison Analysis Settings dialog box, change the endogenous control:
   a. Select the **Relative Quantification Settings** tab.
   b. In the Endogenous Control(s) pane, select **LIPN1** from the Endogenous Control drop-down menu.

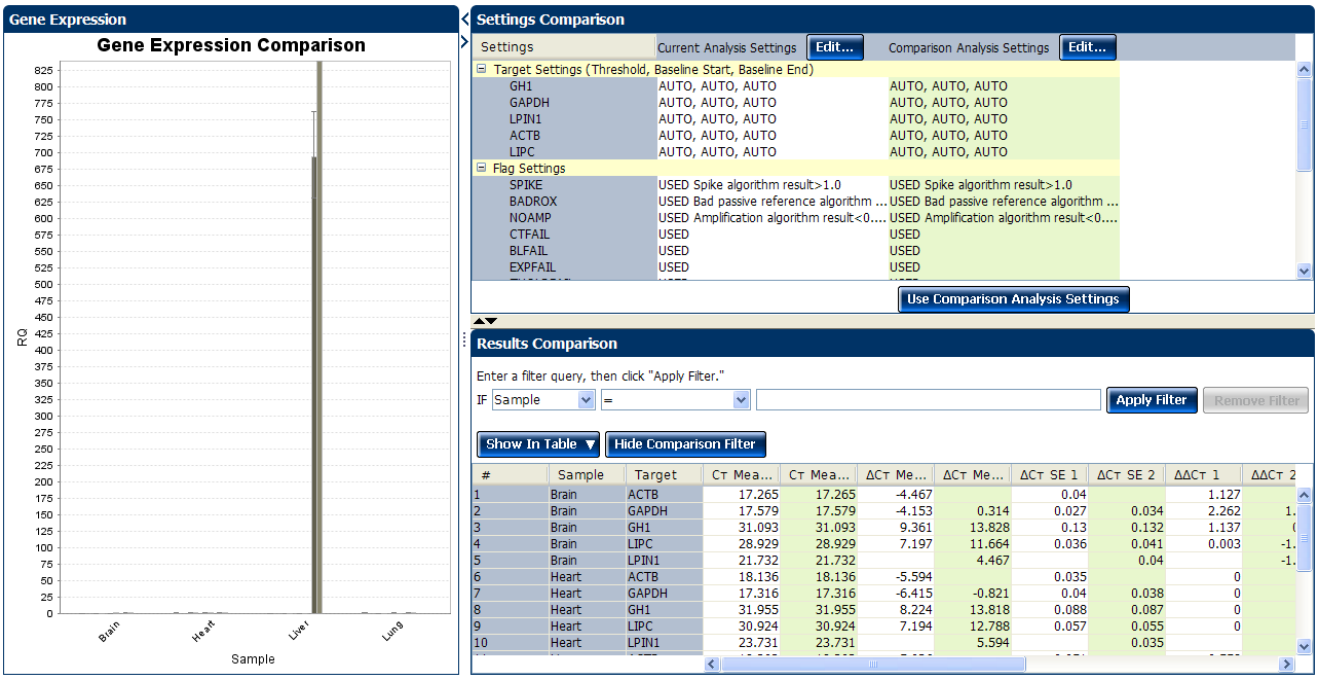
c. Click **Analyze** to analyze the data and close the dialog box.



4. In the Settings Comparison pane, click **Use Comparison Analysis Settings** under the green column, then compare the results:
   - In the Settings Comparison pane, the white column is titled "Comparison Analysis Settings," the green column is titled "Current Analysis Settings," and the Use Comparison Analysis Settings button is under the white column.
   - In the Results Comparison pane, values in the white columns are based on the default analysis settings, and values in the green columns are based on the modified analysis settings. In the example experiment, changing the endogenous control to 18S affects the RQ values. To view the RQ values, scroll to the left. If desired, you can click and drag the RQ column headings so that they appear first in the table.
   - The Gene Expression Comparison plot displays the default analysis settings (the values from the white columns).

     Note: The Gene Expression Comparison plot has limited functions. For example, you cannot change to log scale and you cannot view by target.

Note: The default analysis settings are the settings automatically made by the software when the study is initially analyzed.



5. From the Study Menu pane, select **Analysis ▸ Gene Expression** to view the gene expression plot using the modified analysis settings.

   Note: In the Gene Expression screen you can view the modified data in log scale, by target, and so on. See "Assess the gene expression profile using the Gene Expression Plot" on page 150.

6. *(Optional)* View the data in the other analysis screens. All other analysis screens for the study display the data using the modified analysis settings.

7. Close the study.
   - Save your changes before closing the study.

     Or
   - Close the study without saving your changes. If you do not save your changes, the software reverts to the default analysis settings the next time you open the study.

Tips for managing your own comparison

- Edit the comparison analysis settings as desired. For information on editing the settings, see "View the analysis settings" on page 143.
- After making your first round of changes to the analysis settings, you can continue making changes using one of the following methods:
  - (Recommended) Revert to the saved analysis settings, then make new changes. To do this: In the Settings Comparison pane, click **Use Comparative Analysis Settings** (now under the white column) to revert to the saved analysis settings, then repeat steps 2 through 6 above. This method ensures that you do not lose the saved analysis settings.

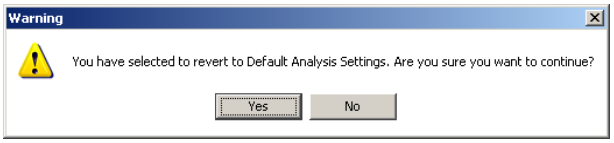
    Note: If you have made changes, but have not saved them, the software reverts to the default analysis settings when you click **Use Comparative Analysis Settings**. The default analysis settings are the settings automatically made by the software when the study is initially analyzed.

  - Continually compare new settings with previous settings. To do this: In the Settings Comparison pane, alternate clicking **Edit** in the white and green columns, then repeat steps 3 through 6 above. This method does not allow you to return to your saved settings; subsequent comparisons are made with the previous analysis settings, building upon any changes that you have already made.

Revert to the default analysis settings

IMPORTANT! The default analysis settings are defined by the software. If you make changes to the analysis settings and save the study, the saved changes are lost when you revert to the default analysis settings.

1. In the Settings Comparison pane, click **Edit** next to the settings you want to revert to the default: *Current Analysis Settings* or *Comparison Analysis Settings*.
2. In the Analysis Settings dialog box, revert to defaults and reanalyze the data:
   a. Click **Revert to Default Analysis Settings**.
   b. At the prompt, click **Yes**.



   c. Click **Analyze** to analyze the data and close the dialog box.
3. In the Settings Comparison pane, click **Use Comparison Analysis Settings**. In the Results Comparison pane, values for the settings you selected to edit in step 1 ("Current Analysis Settings" or "Comparison Analysis Settings") are generated according to the default analysis settings.

Omit wells from the analysis

You can use the Well Table to omit individual wells from the analysis. To omit a well:

1. From the Study Menu pane, select one of the following analysis screens:
   - **Analysis ▸ Amplification Plot**
   - **Analysis ▸ Multicomponent Plot**
   - **Analysis ▸ Multiple Plots View**
2. In the Experiment Data pane, select the experiment that contains the well to omit.
3. In the Well Results Data pane, click the **Well Table** tab, then select the check box in the Omit column for the well to omit.

   Note: You cannot omit all technical replicates that belong to a reference sample or a reference biological group, or that serve as the endogenous control.

Export the study

Note: If you are using RealTime StatMiner® Software to analyze the exported data, make sure you have assigned a sample to all the wells containing data in the individual experiments. If a sample is not assigned, the RealTime StatMiner® Software reports an error during import.

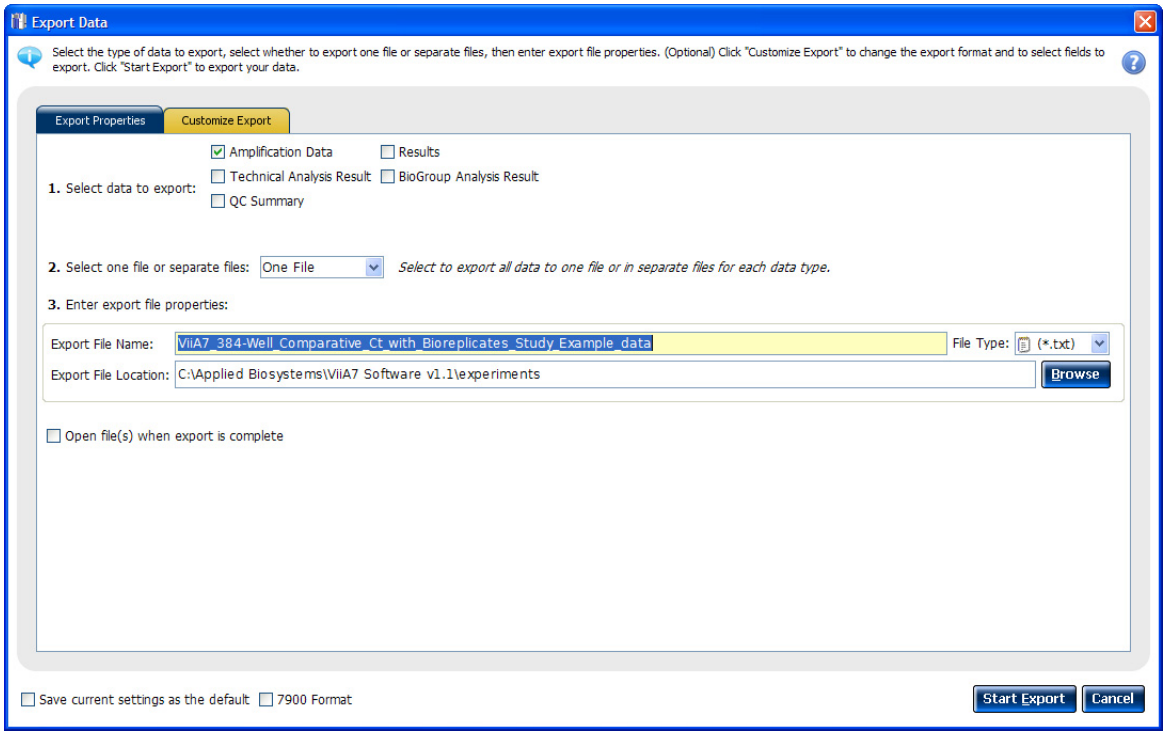
You can export the data within a study from the Analysis screen. To export a study:

1. On the Analysis screen, click **Export** to access the Export Properties tab.
2. Define export properties.
   a. Select the data to export. Options are:
      - Amplification Data
      - Results
      - Technical Analysis Result
      - BioGroup Analysis Result
      - QC Summary
   b. Select **One File** or **Separate Files** from the drop-down menu to export all data to one file or in separate files for each data type respectively.
   c. Enter the export file properties and file name.
   d. Select the file type from the **File Type** drop-down menu. You can choose from ***.txt**, ***.xls**, and ***.xlsx**.
   e. Enter the Export File Location. The default location is C:\Applied Biosystems\ViiA7 Software v1.1\experiments.
   f. Select the Open file(s) when export is complete check box to automatically open the file when export is complete.

For the example study, enter:

Field or Selection	Entry
Select Data to export	Amplification Data
Select one file or separate files	One File
Export File Name	ViiA7_384-Well_Comparative_Ct_with_Bioreplicates_Study_Example_data
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\ViiA7 Software v1.1\experiments
Open file(s) when export is complete	Unchecked
Save current settings as the default	Unchecked
7900 Format	Unchecked

The Export Data screen looks like this:



3. To change the export format, complete the tasks on the Customize Export tab.

   Note: To complete the tasks on the Customize Export tab you must have at least one type of data to export.

   a. Select the data from the Export Properties tab. The type of data that you selected in the Export Properties tab appears in the Customize field in the Customize Export tab.

   b. Select the data type content.

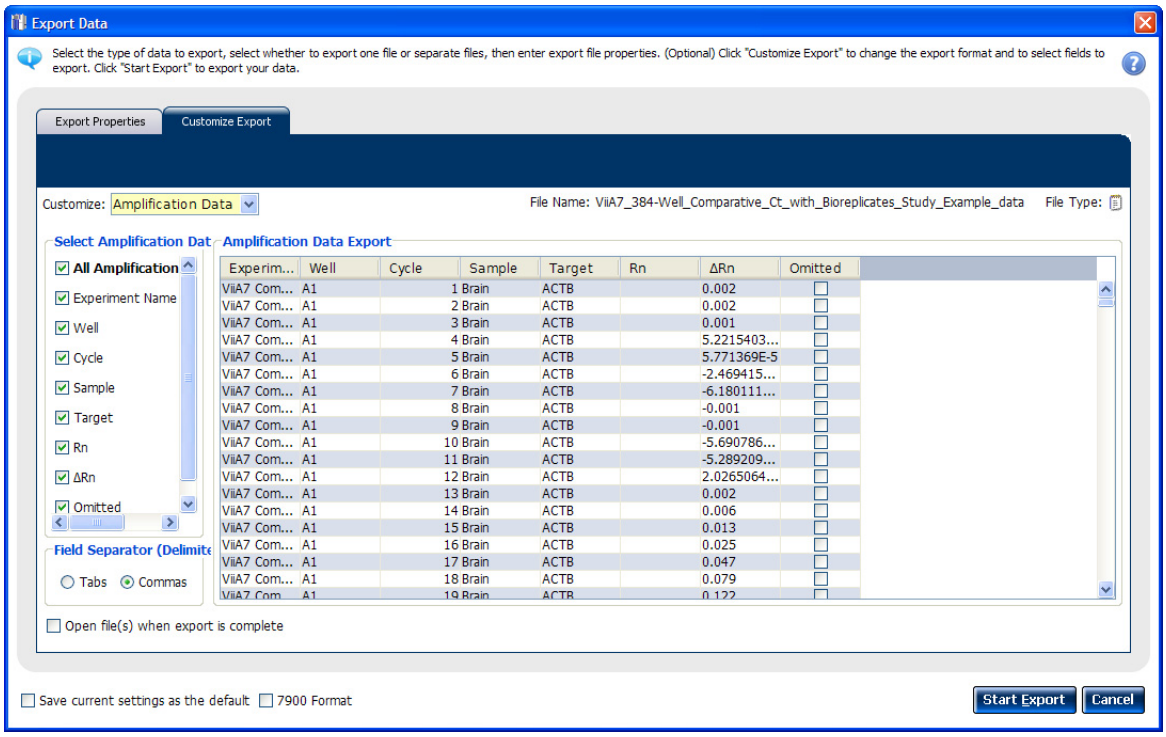
   c. Select the Tabs or Commas radio button to select the Field Separator (Delimiter).

   d. Select the Open file(s) when export is complete check box to automatically open the file when export is complete.

   For the example study, enter:

Field or Selection	Entry
Customize	Amplification Data
Field Separator (Delimited)	Commas
Open file(s) when export is complete	Unchecked
Save current settings as the default	Unchecked
7900 Format	Unchecked

The Export Data screen for customized export of Amplification Data looks like this:



4. Select the **Save current settings as the default** check box to save the export settings that you have modified. Alternatively, select the **7900 Format** check box to save the export settings in the 7900 format.

5. Click **Start Export**.

For more information

For more information on	Refer to	Part number
Calculating Relative Quantification Values	*User Bulletin #2: Relative Quantitation of Gene Expression.*	4303859
Consumables	Chapter 1 in Booklet 1, *Getting Started with ViiA™7 System Experiments.* Appendix A in Booklet 7, *ViiA™ 7 System Experiments - Appendixes*	4441434
Publishing data	*Chapter 1 in Booklet 1, Getting Started with ViiA™7 System Experiments.*	4441434

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BOOKLET 4

Running Genotyping Experiments

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

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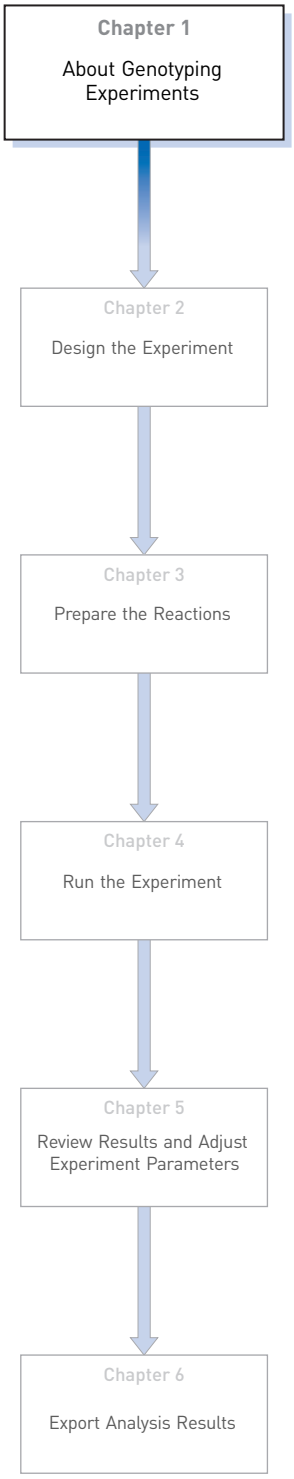
1 About Genotyping Experiments



This chapter covers:

- About data collection 8
- About TaqMan® SNP Genotyping assays 8
- About TaqMan® MGB probes 9
- 5¢ nuclease assay 9
- Minimizing non-specific fluorescence 10
- Reading and analyzing the plates 10
- About the example experiment 11

IMPORTANT! First-time users of the ViiA™ 7 System, please read Booklet 1, *Getting Started with ViiA™ 7 System Experiments* and Booklet 7, *ViiA™ 7 System Experiments - Appendixes* of this binder thoroughly. The booklets provide information and general instructions that are applicable to all the experiments described in this binder.

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems ViiA™ 7 Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ ViiA™ 7 Software Help**.

About data collection

Genotyping experiments are performed to detect single nucleotide polymorphism (SNP) variants of a target nucleic acid sequence in samples. The PCR reactions contain primers designed to amplify the sequence containing the SNP and reagents to detect two different alleles.

You can collect the results of a genotyping experiment in two different ways: At the end of the experiment, or continuously during the experiment. Data collection at the end of the experiment is called end-point data collection. Data collection during the experiment run is considered real-time PCR. The real-time data helps further data analysis.

In end-point data collection, the normalized intensity of the reporter dye, or Rn, is the data collected. Some end-point experiments also include pre-PCR (data collected before the amplification process) data collection. The system calculates the delta Rn (ΔRn) value per the following formula:

ΔRn = Rn (post-PCR read) – Rn (pre-PCR read), where Rn = normalized readings.

About TaqMan® SNP Genotyping assays

A Genotyping assay detects variants of a single nucleic acid sequence, without quantifying the target. The presence of two probes in each reaction allows Genotyping of the two possible variants at the single nucleotide polymorphism (SNP) site in a target sequence.

Each TaqMan® SNP Genotyping Assay consists of a single, ready-to-use tube containing:

- Two sequence-specific primers for amplifying the polymorphism of interest
- Two allele-specific TaqMan® MGB probes for detecting the alleles for the specific polymorphism of interest

About TaqMan® MGB probes

Each allele-specific TaqMan® MGB probe has:

- A reporter dye at its 5′ end:
  - VIC® dye is linked to the 5′ end of the Allele 1 probe.
  - FAM™ dye is linked to the 5′ end of the Allele 2 probe.

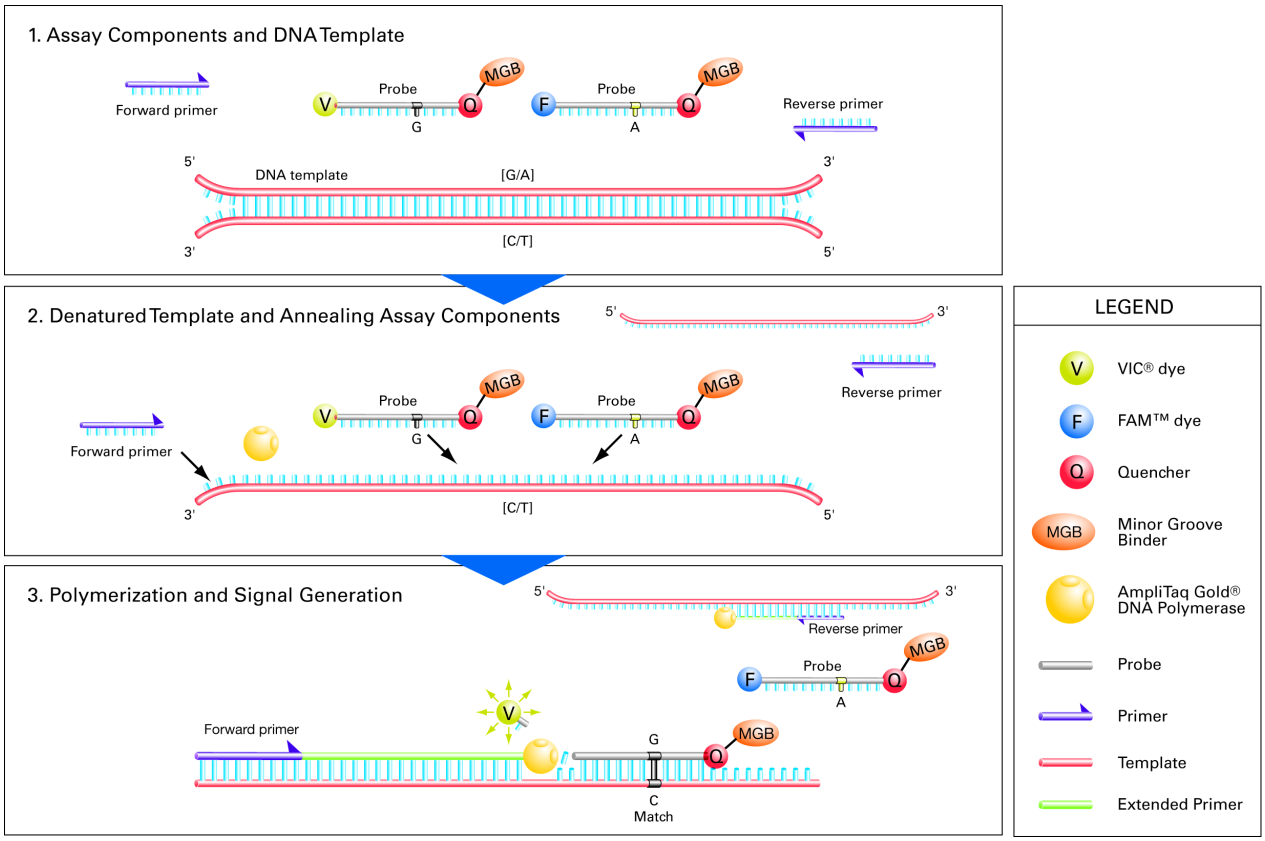
  The Allele 1 VIC® dye-labeled probe corresponds to the first nucleotide inside the square brackets of the context sequence in the assay information file (AIF) shipped with each order. The Allele 2 FAM™ dye-labeled probe corresponds to the second nucleotide inside the square brackets of the context sequence in the AIF. For the context sequence ATCGATT[G/T]ATCC, the VIC® dye-labeled probe binds to the G allele, and the FAM™ dye-labeled probe to the T allele.
- A minor groove binder (MGB), which increases the melting temperature (T_m) for a given probe length and allows the design of shorter probes. The use of shorter probes results in greater differences in T_m values between matched and mismatched probes, and more robust genotyping.
- A non-fluorescent quencher (NFQ) at its 3′ end, which allows for detection of the reporter dye fluorescence with greater sensitivity than with a fluorescent quencher.

5′ nuclease assay

The figure below is a schematic depiction of the 5′ nuclease assay. During PCR:

- Each TaqMan® MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites.
- When the oligonucleotide probe is intact, the proximity of the quencher dye to the reporter dye quenches the reporter signal.
- AmpliTaq Gold® DNA polymerase extends the primers bound to the genomic DNA template.

- AmpliTaq Gold® DNA polymerase (with its 5′ nuclease activity) cleaves probes that are hybridized to the target sequence.
- Cleavage of the probes hybridized to the target sequence separates the quencher dye from the reporter dye, resulting in increased fluorescence by the reporter. The fluorescence signal generated by PCR amplification indicates which alleles are present in the sample.



Minimizing non-specific fluorescence

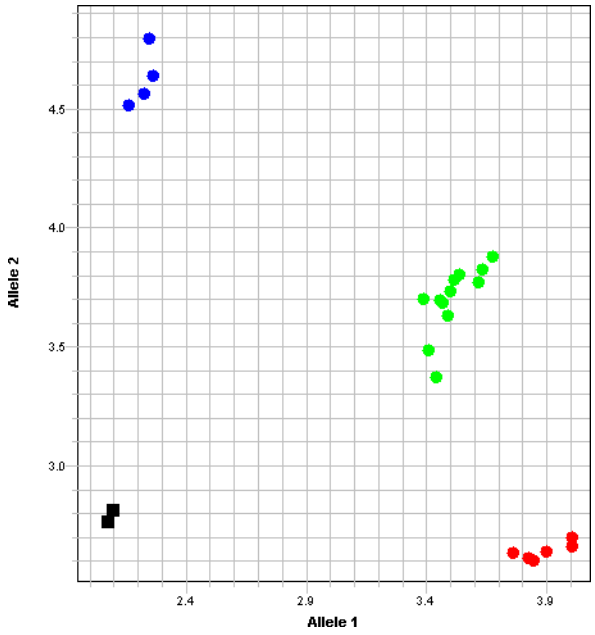
In TaqMan® assays, fluorescence from nonspecifically bound probes is reduced, because nucleotide mismatches between a probe and a sequence reduce the chances that the probe will be cleaved. The probe's short length means that a one-base-pair mismatch will a have a larger negative effect on the binding. The mismatched probe will not bind tightly to the allele; the AmpliTaq Gold® DNA polymerase will likely displace the probe without cleaving the dye.

Reading and analyzing the plates

The ViiA™ 7 Software genotypes the DNA samples from the reaction plate simultaneously. First, the software normalizes the fluorescence of the reporter dyes to the fluorescence of the passive reference dye in each well. Next, the software plots the normalized intensities (Rn) of the reporter dyes in each sample well on an Allelic

Discrimination Plot, which contrasts the reporter dye intensities of the allele-specific probes. Finally, the ViiA™ 7 Software algorithmically clusters the sample data, and assigns a genotype call to the samples of each cluster according to its position on the plot.

Note: The ViiA™ 7 Software clustering algorithm does not call genotypes when only one genotype is present in an experiment.



The clustering of datapoints can vary along the horizontal axis (Allele 1), vertical axis (Allele 2), or diagonal (Allele 1/Allele 2). This variation results from differences in the extent of reporter dye fluorescent intensity after PCR amplification. The table below shows the correlation between fluorescence signals and sequences in a sample.

A substantial increase in...	Indicates...
VIC® dye-labeled probe fluorescence only	Homozygosity for Allele 1
FAM™ dye-labeled probe fluorescence only	Homozygosity for Allele 2
Both VIC® and FAM™ dye-labeled probes fluorescence	Allele 1-Allele 2 heterozygosity

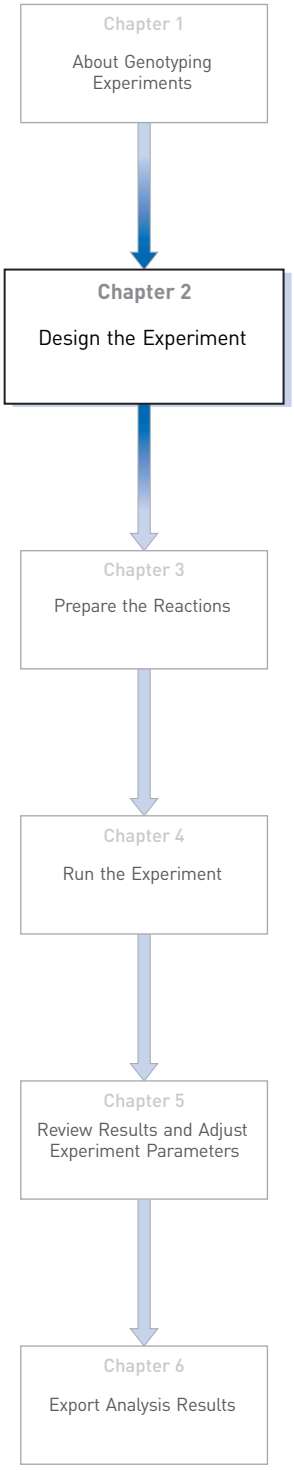
About the example experiment

To illustrate how to perform Genotyping experiments, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with a ViiA™ 7 System.

The objective of the example Genotyping experiment is to investigate SNP rs8039, where possible genotypes are AA, AC, and CC. In the example, two unknown genomic DNA (gDNA) samples were genotyped using TaqMan® Drug Metabolism Genotyping Assay ID C__11711420_30. The reactions were set up so that the PCR

primers and probes that target both alleles of SNP rs8039 were present in the same well. The PCR was performed using the TaqMan® Genotyping Master Mix and run according to the protocol that is described in the *Performing a TaqMan® Drug Metabolism Genotyping Assay.*

2 Design the Experiment



This chapter explains how to design the example experiment from the Experiment Setup menu.

This chapter covers:

- Define the experiment properties. 14
- Define SNPs and samples 15
- Assign markers and samples 17
- Set up the run method 19
- Order materials for the experiment 21
- For more information. 21

Note: To automatically export the analyzed data to a specified location, select the Auto Export task from the Export screen, before running the experiment. For more information on Auto Export, refer to Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*.

Define the experiment properties

Click **Experiment Setup** ▸ **Experiment Properties** to create a new experiment in the ViiA™ 7 Software. Enter:

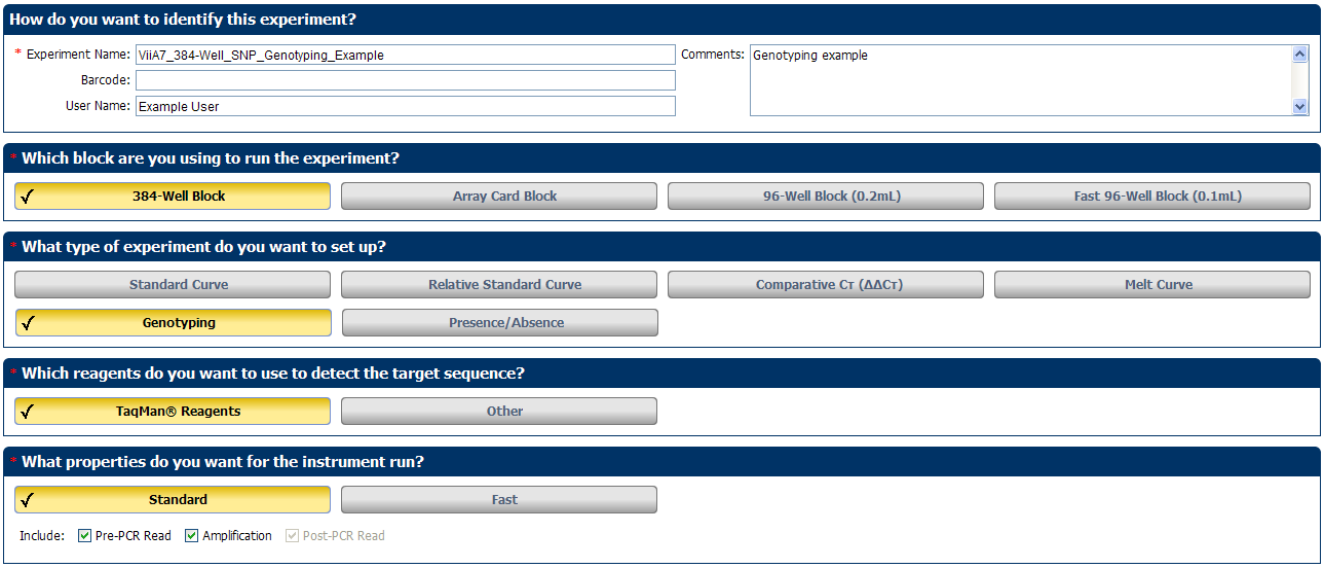
Field	Entry
Experiment Name	ViiA7_384-Well_SNP_Genotyping_Example
Barcode	Leave field empty
User Name	Example User
Comments	Genotyping example
Block	384-Well Block
Experiment Type	Genotyping
Reagents	TaqMan® Reagents
Ramp speed	Standard

Select all three data-collection check boxes: Pre-PCR, Amplification, and Post-PCR collection methods:

Pre-PCR Read	Checked
Amplification	Checked
Post-PCR Read	Checked

Save the experiment.

Your Experiment Properties screen should look like this:



Define SNPs and samples

Click **Define** to access the Define screen. Enter:

1. SNP Assays

SNP assay name	NCBI SNP reference	Context sequence	Allele 1	Reporter	Quencher	Allele 2	Reporter	Quencher	Color
SNP Assay 1			Allele1	VIC	NFQ-MGB	Allele2	FAM	NFQ-MGB	
SNP Assay 2			Allele1	VIC	NFQ-MGB	Allele2	FAM	NFQ-MGB	

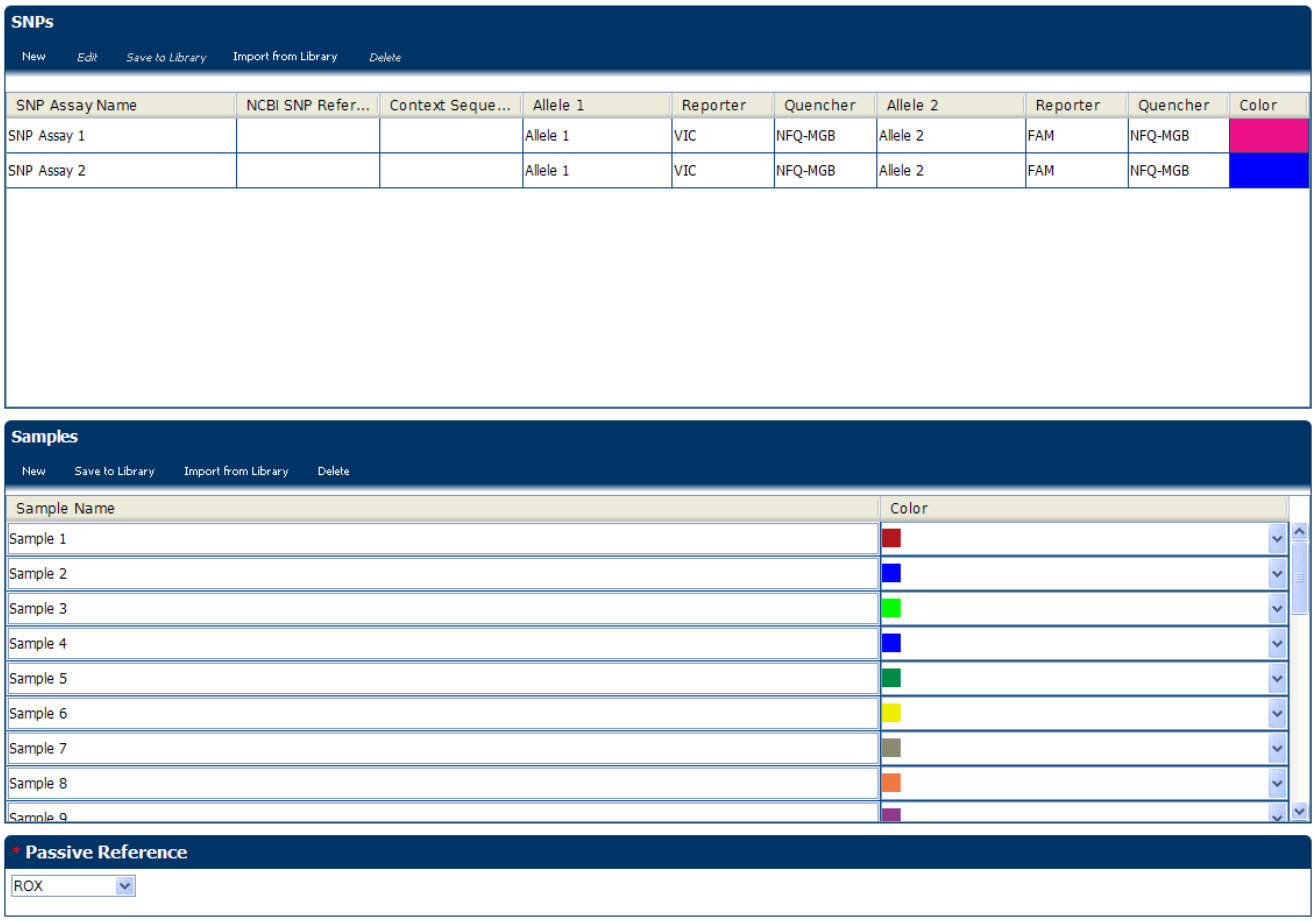
Note: The NCBI SNP reference and Context sequence fields are optional fields and are used for reference. They are not required to run an experiment.

2. Samples

Sample name	Color	Sample name	Color	Sample name	Color
Sample 1		Sample 11		Sample 21	
Sample 2		Sample 12		Sample 22	
Sample 3		Sample 13		Sample 23	
Sample 4		Sample 14		Sample 24	
Sample 5		Sample 15		Sample 25	
Sample 6		Sample 16		Sample 26	
Sample 7		Sample 17		Sample 27	
Sample 8		Sample 18		Sample 28	
Sample 9		Sample 19		Sample 29	
Sample 10		Sample 20		Sample 30	

3. Dye to be used as a Passive Reference
   ROX

Your Define screen should look like this:



Note: This example experiment does not define biological replicate groups. Leave Biological Replicate Groups blank.

Assign markers and samples

Click **Assign** to access the Assign screen. Enter the SNP assays and samples:

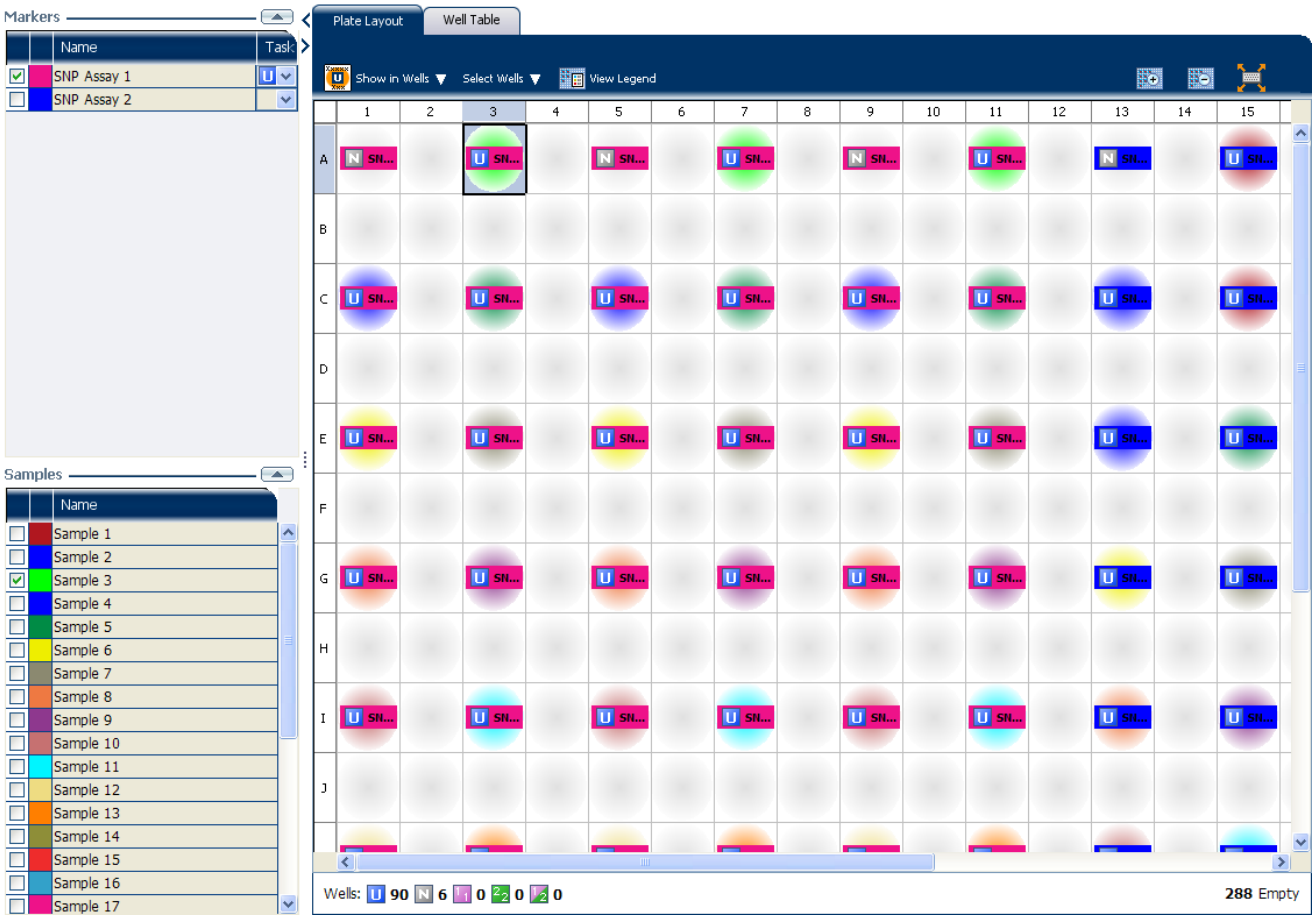
- SNP Assay 1

Target name	Well number	Task	Sample
SNP Assay 1	A3, A5, A7	Unknown	Sample 3
	C1, C5, C9		Sample 4
	C3, C7, C11		Sample 5
	E1, E5, E9		Sample 6
	E3, E7, E11		Sample 7
	G1, G5, G9		Sample 8
	G3, G7, G11		Sample 9
	I1, I5, I9		Sample 10
	I3, I7, I11		Sample 11
	K1, K5, K9		Sample 12
	K3, K7, K11		Sample 13
	M1, M5, M9		Sample 14
	M3, M7, M11		Sample 15
	O1, O5, O9		Sample 16
	O3, O7, O11		Sample 17
SNP Assay 1	A1, A5, A9	Negative	NTC

- SNP Assay 2

Target name	Well number	Task	Sample
SNP Assay 2	A15, A19, A23	Unknown	Sample 1
	C13, C17, C21		Sample 2
	C15, C19, C23		Sample 18
	E13, E17, E21		Sample 19
	E15, E19, E23		Sample 20
	G13, G17, G21		Sample 21
	G15, G19, G23		Sample 22
	I13, I17, I21		Sample 23
	I15, I19, I23		Sample 24
	K13, K17, K21		Sample 25
	K15, K19, K23		Sample 26
	M13, M17, M21		Sample 27
	M15, M19, M23		Sample 28
	O13, O17, O21		Sample 29
	O15, O19, O23		Sample 30
SNP Assay 2	A13, A17, A21	Negative	NTC

Your Assign screen should look like this:



Set up the run method

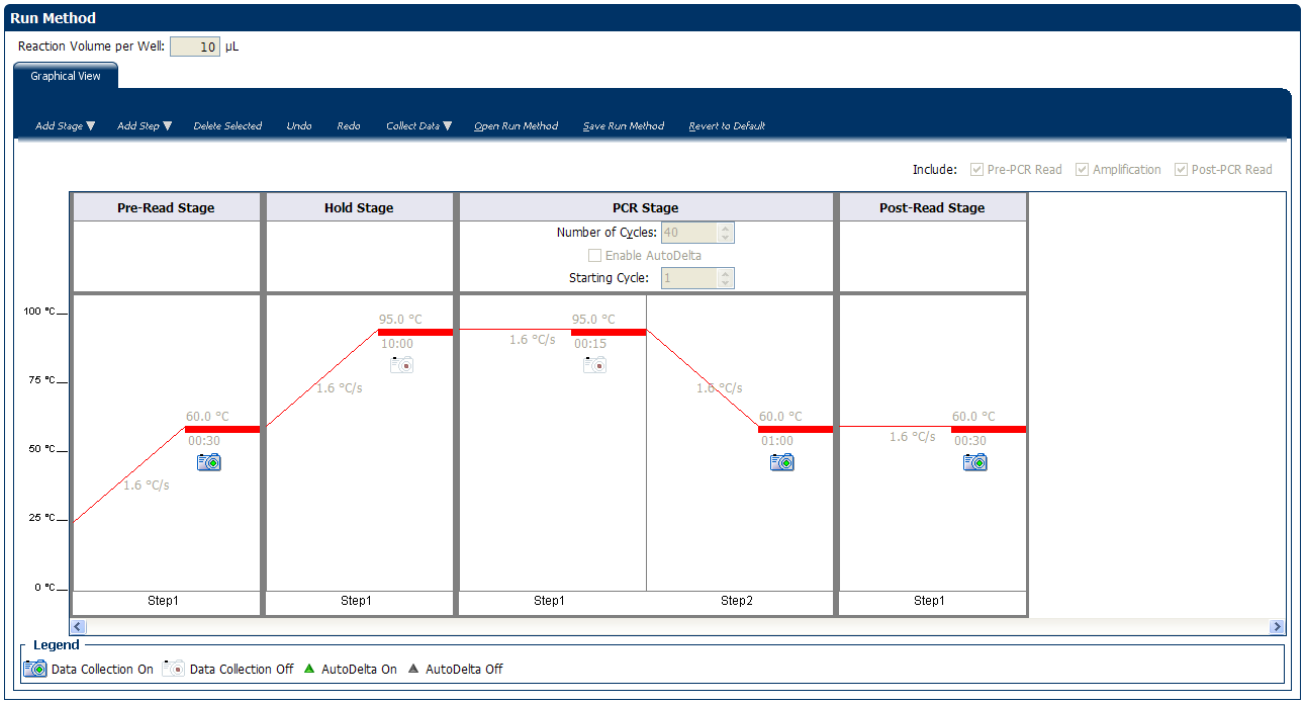
Click **Run Method** to access the Run Method screen. Set the thermal profile under the Graphical View tab. Enter:

- Reaction Volume Per Well: 10µl
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Pre-Read Stage	Step 1	1.6 °C/s	60 °C	30 seconds
Hold Stage	Step 1	1.6 °C/s	95°C	10 minutes

Stage	Step	Ramp rate	Temperature	Time
PCR Stage • Number of Cycles: 40 (default) • Enable AutoDelta: Unchecked (default) • Starting Cycle: Disabled when Enable AutoDelta is unchecked	Step 1	1.6 °C/s	95°C	15 seconds
	Step 2	1.6 °C/s	60°C	1 minute
Post-Read Stage	Step 1	1.6 °C/s	60°C	30 seconds

Your Run Method screen should look like this:

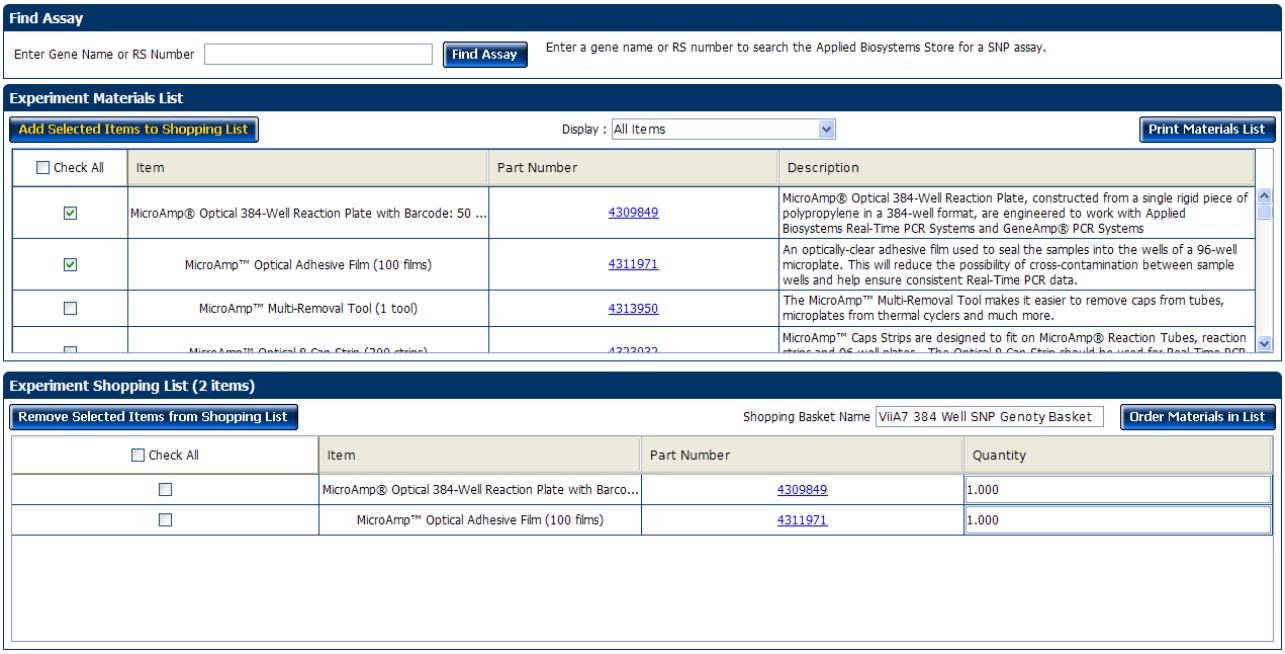


Order materials for the experiment

The recommended materials are:

- MicroAmp® Optical 384-Well Reaction Plate
- MicroAmp® Optical Adhesive Film
- TaqMan® Genotyping Master Mix (PN 4371355)

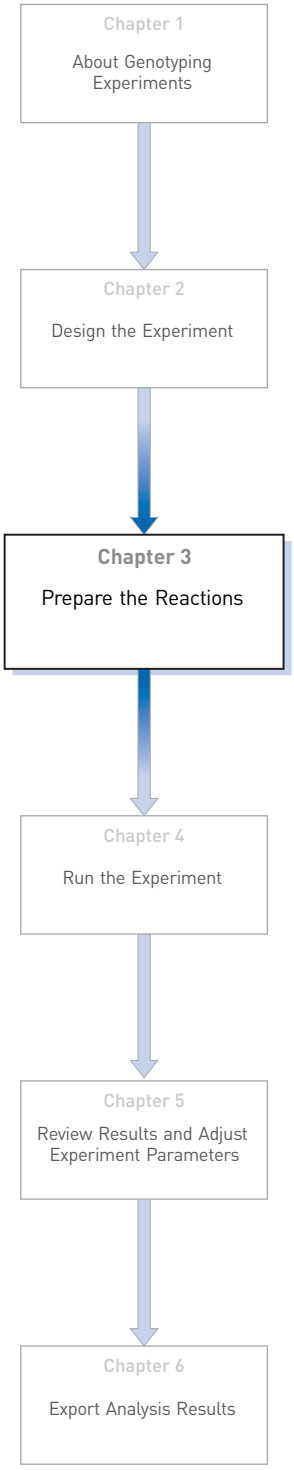
Your Materials List screen should look like this:



For more information

For more information on...	Refer to...	Part number
Consumables	Chapter 1 in Booklet 1, Getting Started with ViiA™ 7 System Experiments Appendix A in Booklet 7, *ViiA™ 7 System Experiments - Appendixes*	4441434
Data collection	Chapter 1 in Booklet 1, Getting Started with ViiA™ 7 System Experiments	4441434
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*	127AP05-03
Using Alternative Setup	Chapter 2 in Booklet 1, *Getting Started with* ViiA™ 7 *System Experiments*	4441434

3 Prepare the Reactions



This chapter explains how to prepare the PCR reactions for the Genotyping example experiment.

This chapter covers:

- Assemble required materials 24
- Prepare the sample dilutions 24
- Prepare the reaction mix (“cocktail mix”). 24
- Prepare the reaction plate 25
- Tips for preparing reactions for your own experiments. 27
- For more information. 27

Assemble required materials

- Items listed in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*
- Samples - Sample 1 - Sample 30
- Example experiment reaction mix components:
  - **TaqMan® Genotyping Master Mix (2X)**
  - SNP 1 Assay Mix (20X)
  - SNP 2 Assay Mix (20X)

Prepare the sample dilutions

For the example experiment, two targets are assigned to 45 wells each. Each well contains 20 ng of Coriell DNA diluted from 100 ng/µL of stock.

To prepare the sample dilutions:

1. Label a separate microcentrifuge tube for each sample to be diluted.
2. Add 5µL of sample stock to each empty tube.
3. Add 45µL of sterile water (diluent) to each tube, such that each working stock tube has a final concentration of 10 ng/µL
4. Vortex each diluted sample for 3 to 5 seconds, then centrifuge the tubes briefly.
5. Place the diluted samples on ice until you prepare the reaction plate.

Prepare the reaction mix ("cocktail mix")

1. Label an appropriately sized tube for each reaction mix:
   - SNP 1 Reaction Mix
   - SNP 2 Reaction Mix
2. For SNP Assay 1, prepare a cocktail by adding the required volumes of each component to the SNP 1 reaction tube, as detailed below.

Reaction component	Reaction volume			
	Per well (µL)		45 Reactions + 10% excess (µL)	
	Dry	Wet	Dry	Wet
TaqMan® Genotyping Master Mix (2X)	10.0	10.0	500.0	500.0
SNP Assay Mix (20X)	1.0	1.0	50.0	50.0
H_2O, DNase-free	9.0	7.0	450.0	350.0
Total Reaction Mix Volume	20.00	18	1000.0	900.0

3. Gently pipette the reaction mix up and down, then cap the tube.
4. Centrifuge the tube briefly.
5. Place the reaction mixes on ice until you prepare the reaction plate.
6. Repeat step 2 through 5 for the SNP 2 assay.

   Note: Do not add the sample at this time.

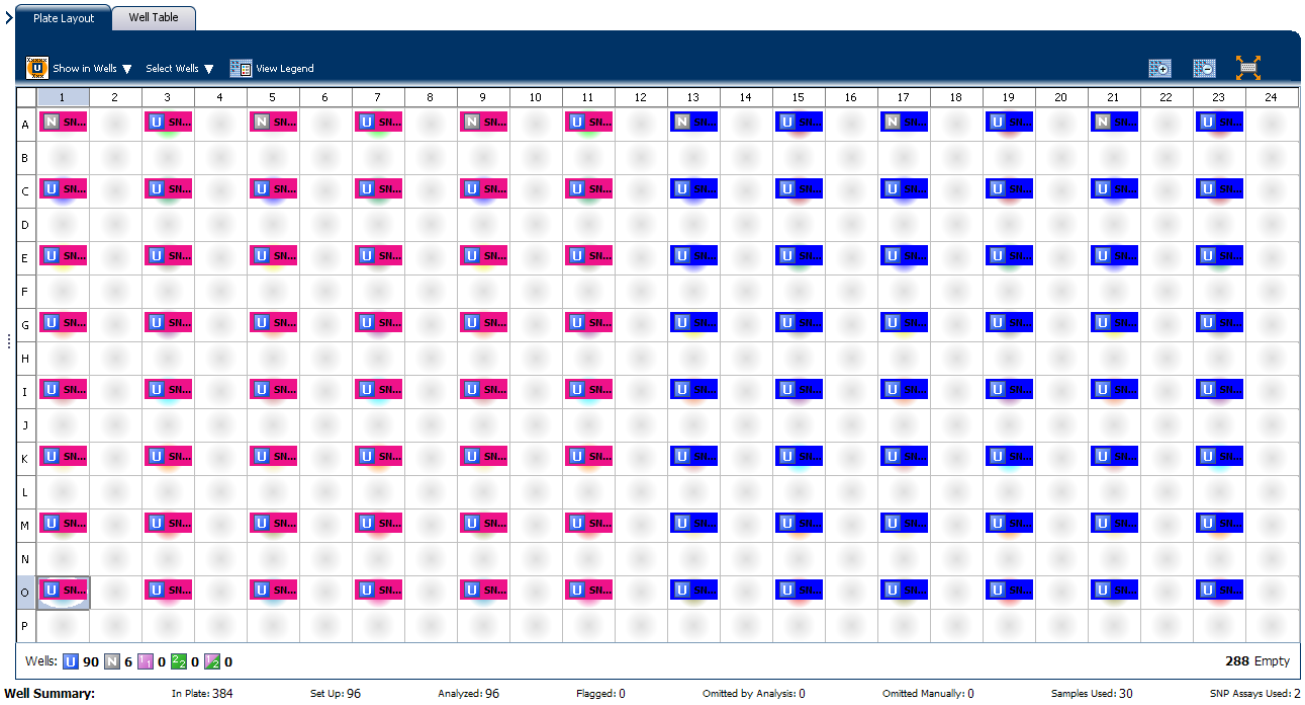
Prepare the reaction plate

Example experiment reaction plate components

The reaction plate for the Genotyping example experiment contains:

- A MicroAmp® Optical 384-Well Reaction Plate
- Reaction volume: 10 µL/well
- 90 Unknown wells

The reaction plate for the example experiment looks like this:



To prepare the reaction plate: dried gDNA

1. Pipette 2.0 µL of the appropriate sample (20 ng of purified genomic DNA) into each well of the reaction plate.

   All wells belonging to the same Genotyping assay must contain approximately the same quantity of sample or control.

   Note: While preparing the reaction plate for your own Genotyping experiment, add between 1 and 20 ng of purified DNA per reaction.

2. Dry down the samples by evaporation at room temperature in a dark, amplicon-free location. (Cover the reaction plate with a lint-free tissue while drying.)

3. Transfer 20 µL of reaction mix to each well.

   IMPORTANT! Make sure that no cross-contamination occurs from well to well.

4. Seal the reaction plate with adhesive film.

5. Vortex the reaction plate for 3 to 5 sec.

6. Briefly centrifuge the reaction plate.

7. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the plate again at a higher speed and for a longer period of time.

To prepare the reaction plate: wet gDNA

1. Add 2 µL of DNA to the appropriate wells.

2. Add 2 µL of water to wells containing the NTCs.

3. Transfer 18 µL of reaction mix to the appropriate wells.

4. Seal the reaction plate with optical adhesive film.

5. Vortex the reaction plate for 3 to 5 seconds, then briefly centrifuge it.

6. Centrifuge the reaction plate briefly.

7. Until you are ready to perform the run, place the reaction plate at 4 °C, in the dark.

Tips for preparing reactions for your own experiments

Tips for preparing samples

When you prepare the samples for your own experiment:

- Use DNAse-free water to dilute the samples.
- Use the same quantity of DNA per well for each experiment.

Tips for preparing the reaction mix

When you prepare the reaction mix for your own experiment, make sure you prepare the reactions for each SNP separately.

Prior to use:

- Mix the master mix thoroughly by swirling the bottle.
- Resuspend the assay mix by vortexing, then centrifuge the tube briefly.
- Thaw frozen samples by placing them on ice. When thawed, resuspend the samples by vortexing, then centrifuge the tubes briefly.

Tips for preparing the reaction plate

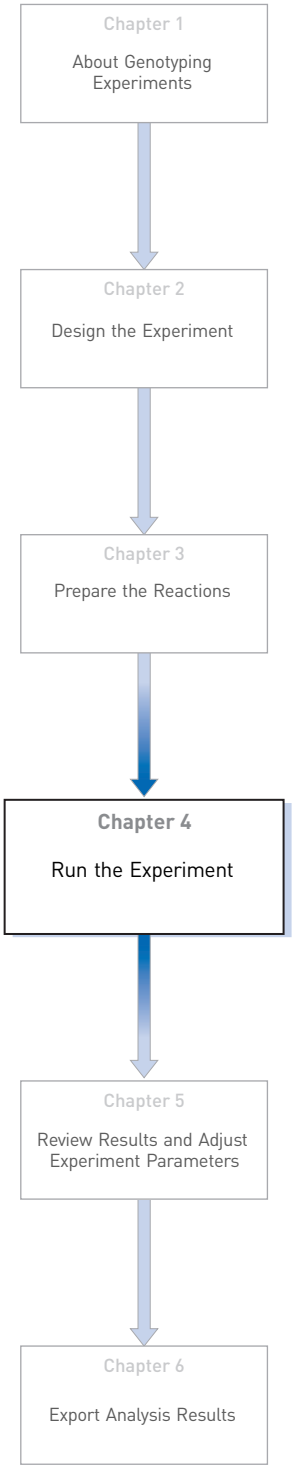
When you prepare the reaction plate for your own experiment:

- Make sure the reaction locations match the plate layout in the ViiA™ 7 Software.
- Load 1 to 20 ng of purified genomic DNA per reaction
- All wells belonging to the same Genotyping assay must contain approximately the same quantity of sample or control.
- Multiple assays may be run on one reaction plate, but must be analyzed separately.

For more information

For more information on...	Refer to...	Part number
Assigning the reaction plate components	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

4 Run the Experiment



This chapter explains how to run the example experiment on the ViiA™ 7 Instrument.

This chapter covers:

- Start the run 30
- Monitor the run. 30

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the ViiA™ 7 Instrument is in operation.

Start the run

1. Open the Genotyping example file that you created using instructions in Chapter 2.
2. Load the reaction plate into the instrument.
3. Start the run.

   Note: To collect real-time data during a run, click the button on the Run Method screen in the Experiment Setup menu.

Monitor the run

Monitor the example experiment run:

- From the ViiA™ 7 Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the ViiA™ 7 Software (to monitor an experiment started from another computer or from the ViiA™ 7 Instrument touchscreen).
- From the ViiA™ 7 Instrument touchscreen.

From the Instrument Console of the ViiA™ 7 Software

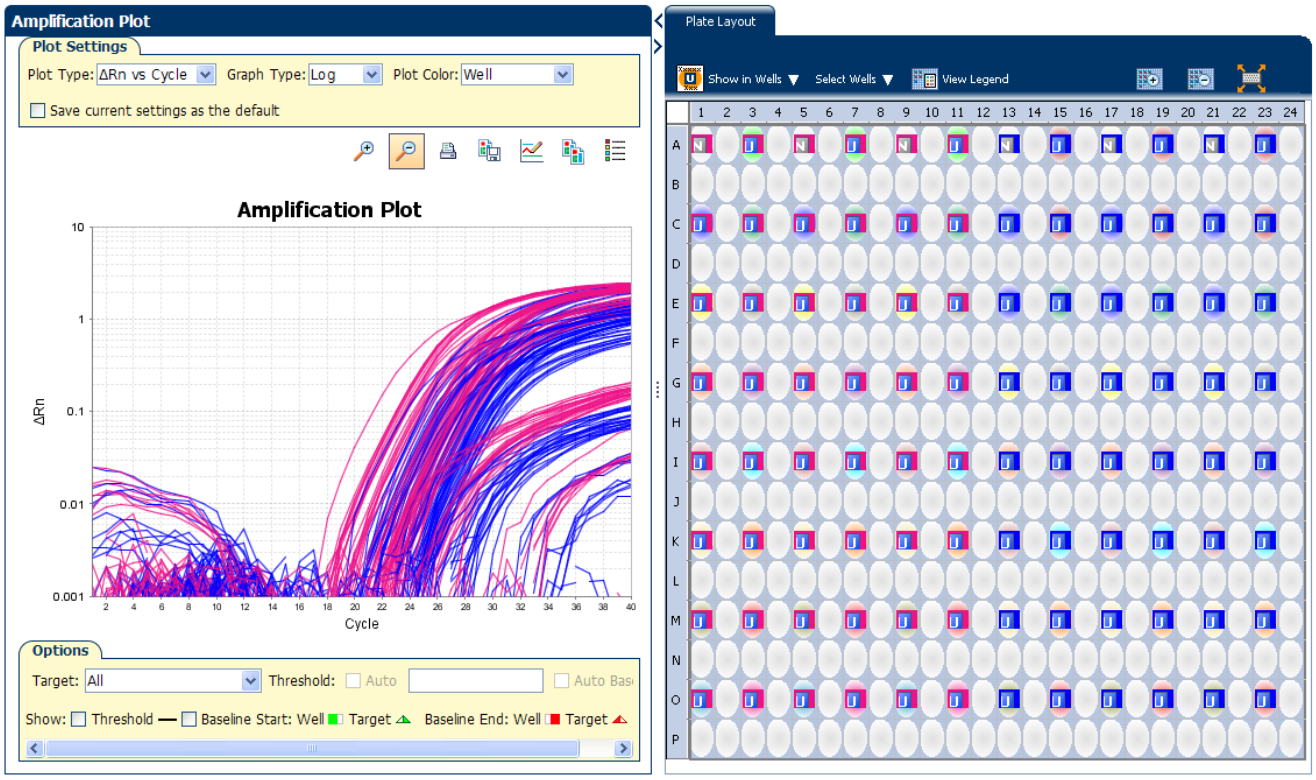
1. In the Instrument Console screen, select the instrument icon.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. On the Manage Instrument screen, click **Monitor Running Instrument** to access the Run screen.

View the Amplification Plot

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the ViiA™ 7 Software for potential problems.

Click **Amplification Plot** from the Run Experiment Menu, select the Plate Layout tab, then select the wells to view.

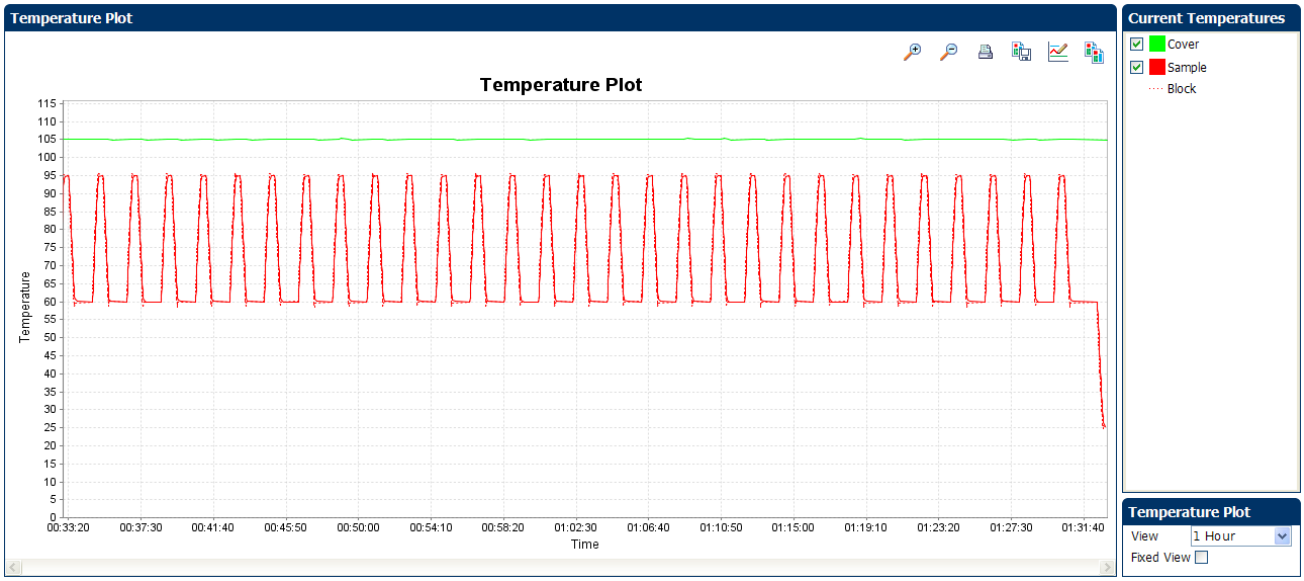
The figure below shows the Amplification Plot screen as it appears at the end of the example experiment.



View the Temperature Plot

Click **Temperature Plot** from the Run Experiment Menu.

The figure below shows the Temperature Plot screen as it appears during the example experiment.

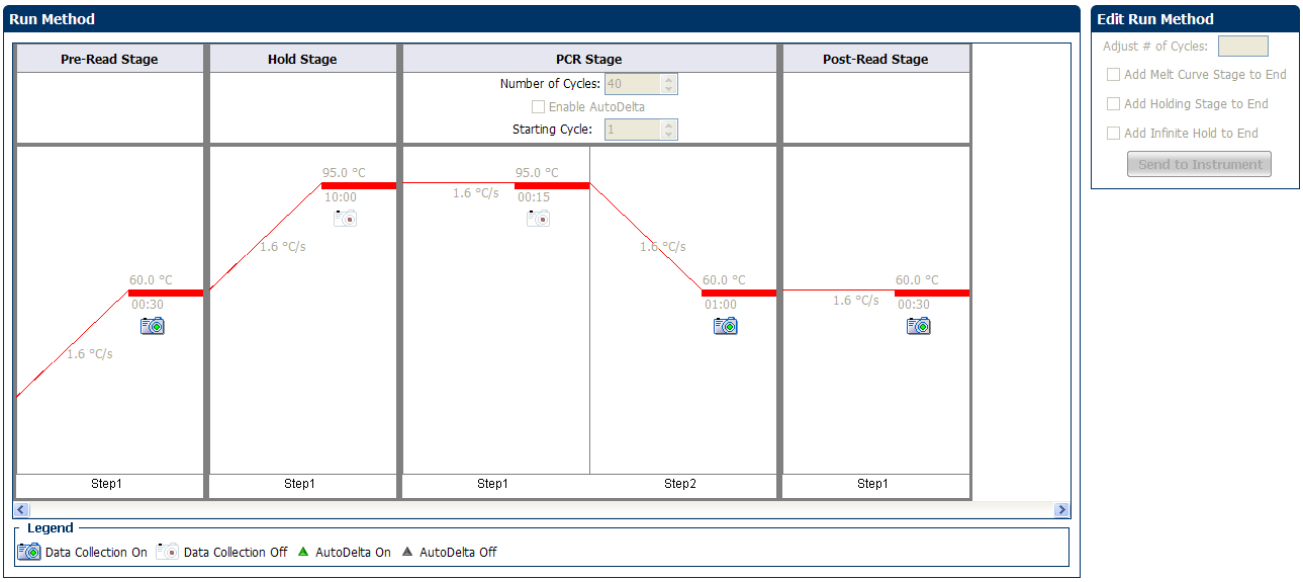


Note: The sample temperature displayed in the Current Temperatures group is a calculated value.

View the Run Method

Click **Run Method** from the Run Experiment Menu.

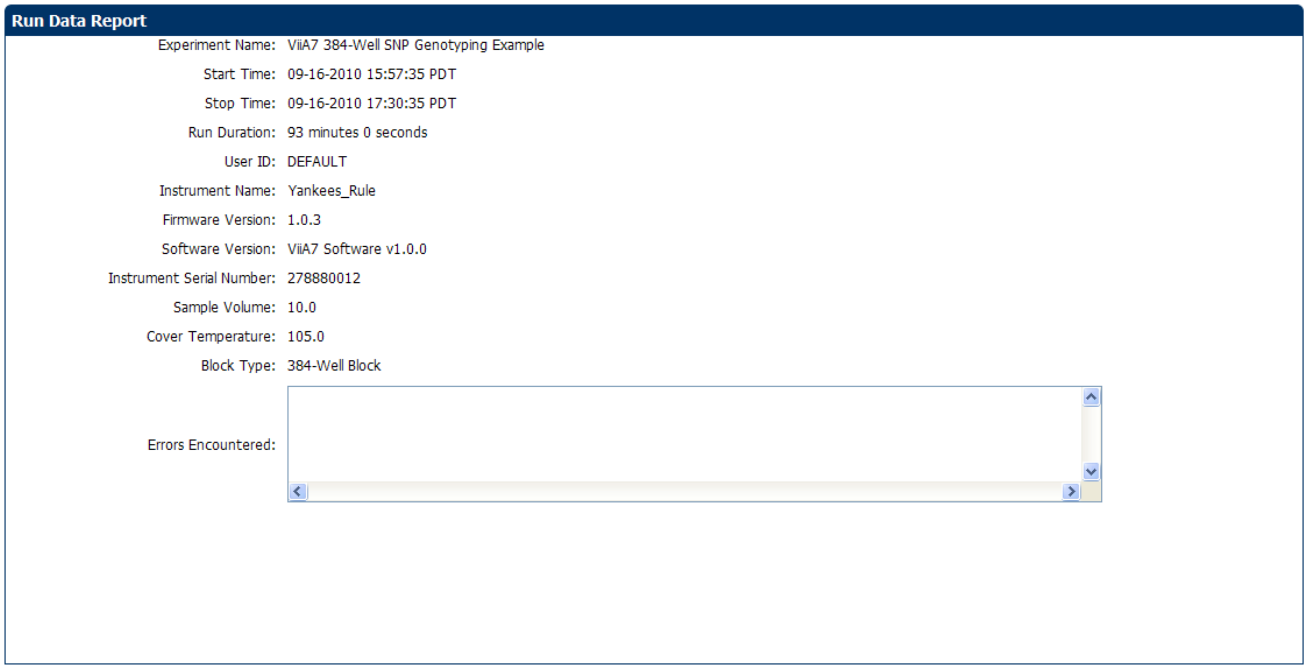
The figure below shows the Run Method screen as it appears in the example experiment.



View run data

Click **View Run Data** from the Run Experiment Menu.

The figure below shows the View Run Data screen as it appears in the example experiment.

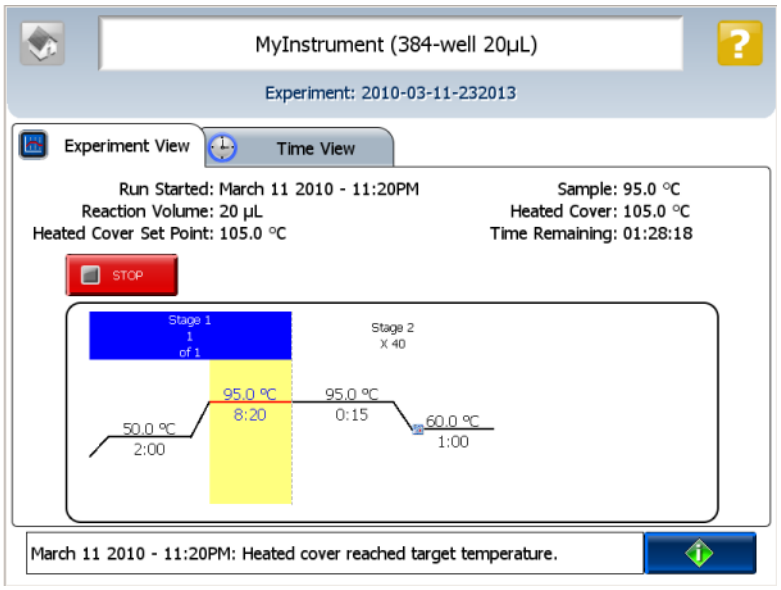


From the ViiA™ 7 Instrument touchscreen

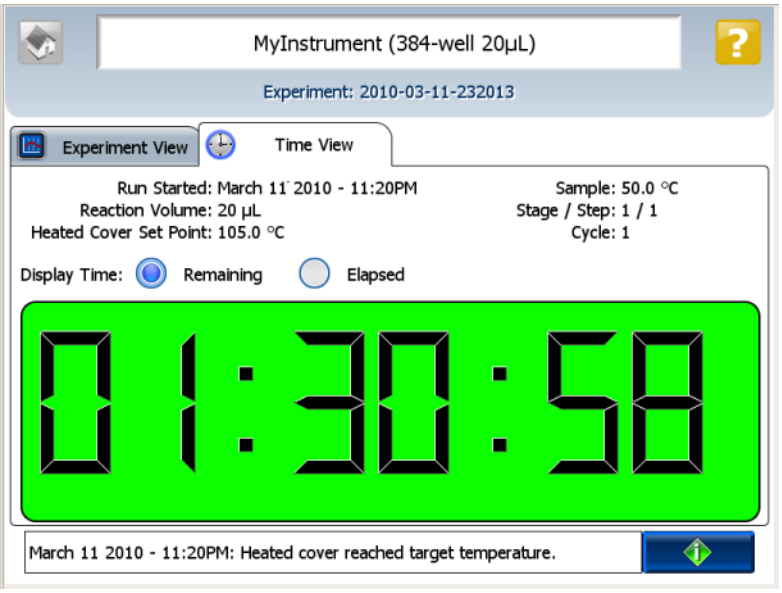
You can also view the progress of the run from the touchscreen of the ViiA™ 7 Instrument.

The Run Method screen on the **ViiA™ 7 Instrument** touchscreen looks like this:

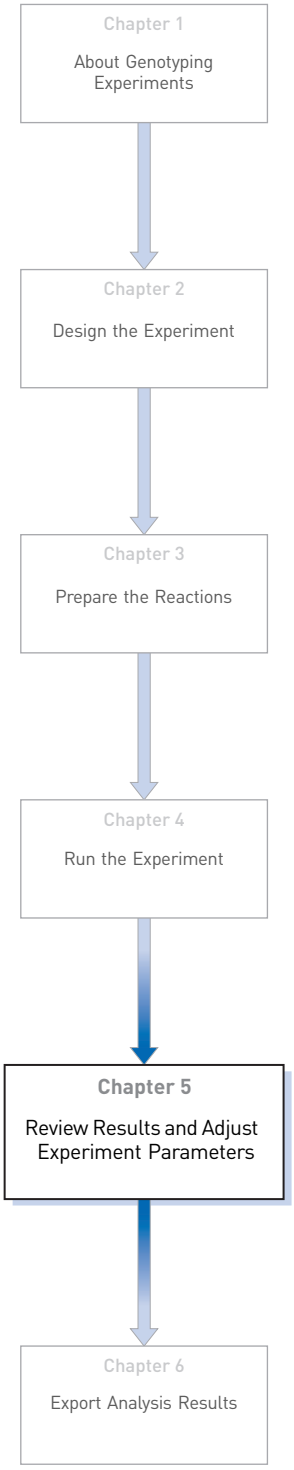
Experiment View



Time View


MyInstrument (384-well 20µL)
Experiment: 2010-03-11-232013
Experiment View
Time View
Run Started: March 11 2010 - 11:20PM
Reaction Volume: 20 µL
Heated Cover Set Point: 105.0 °C
Sample: 50.0 °C
Stage / Step: 1 / 1
Cycle: 1
Display Time:
Remaining
Elapsed
01:30:58
March 11 2010 - 11:20PM: Heated cover reached target temperature.

5 Review Results and Adjust Experiment Parameters



In Section 5.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 5.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

Section 5.1 Review Results 37

- Analyze the example experiment. 37
- View clusters in the Allelic Discrimination Plot 37
- Confirm setup accuracy using Plate Layout. 40
- Assess amplification results using the Amplification Plot. 43
- Identify well problems using the Well Table 46
- Confirm accurate dye signal using the Multicomponent Plot. 49
- Determine signal accuracy using the Raw Data Plot 51
- Review the flags in the QC Summary 52
- For more information. 54

Section 5.2 Adjust parameters for re-analysis of your own experiments. 55

- Adjust analysis settings 55
- For more information. 60

5.1

Section 5.1 Review Results

Analyze the example experiment

1. Open the example experiment file that you ran in Chapter 4.
2. Click **Analyze**. The software analyzes the data using the default analysis settings.

   Note: You can also access the experiment to analyze from the Home screen.

View clusters in the Allelic Discrimination Plot

The Allelic Discrimination Plot contrasts the normalized reporter dye fluorescence (Rn) for the allele-specific probes of the SNP assay.

View the allelic discrimination plot to identify:

- Clusters for the three possible genotypes (Allele 1 homozygous, Allele 2 homozygous, and Allele 1/2 heterozygous).
- A cluster for the negative controls.

To view and assess the allelic discrimination plot

1. From the Experiment menu pane, select **Analysis ▸ Allelic Discrimination Plot.**
2. Click the **Plate Layout** tab, then click any empty well to select it.

   Note: In the Allelic Discrimination Plot, the software highlights all wells that are selected in the Plate Layout tab. If the plot displays a single color for all wells, then all wells in the plate layout are selected.

3. In the allelic discrimination plot, select **SNP Assay 1** from the SNP Assay menu, then enable Autocaller.

   The Allelic Discrimination Plot displays allele symbols for each sample evaluated for the selected SNP. The samples are grouped on the plot as follows:

Genotype	Symbol	Location
Homozygous for Allele 1 of the selected SNP assay.	(red)	X-axis of the plot
Homozygous for Allele 2 of the selected SNP assay.	(blue)	Y-axis of the plot
Heterozygous for both alleles of the selected SNP assay (Allele 1 and Allele 2).	(green)	Midway between the homozygote clusters

Genotype	Symbol	Location
A negative control.	■ (black)	Bottom-left corner of the plot
Undetermined.	× (black)	Anywhere on plot

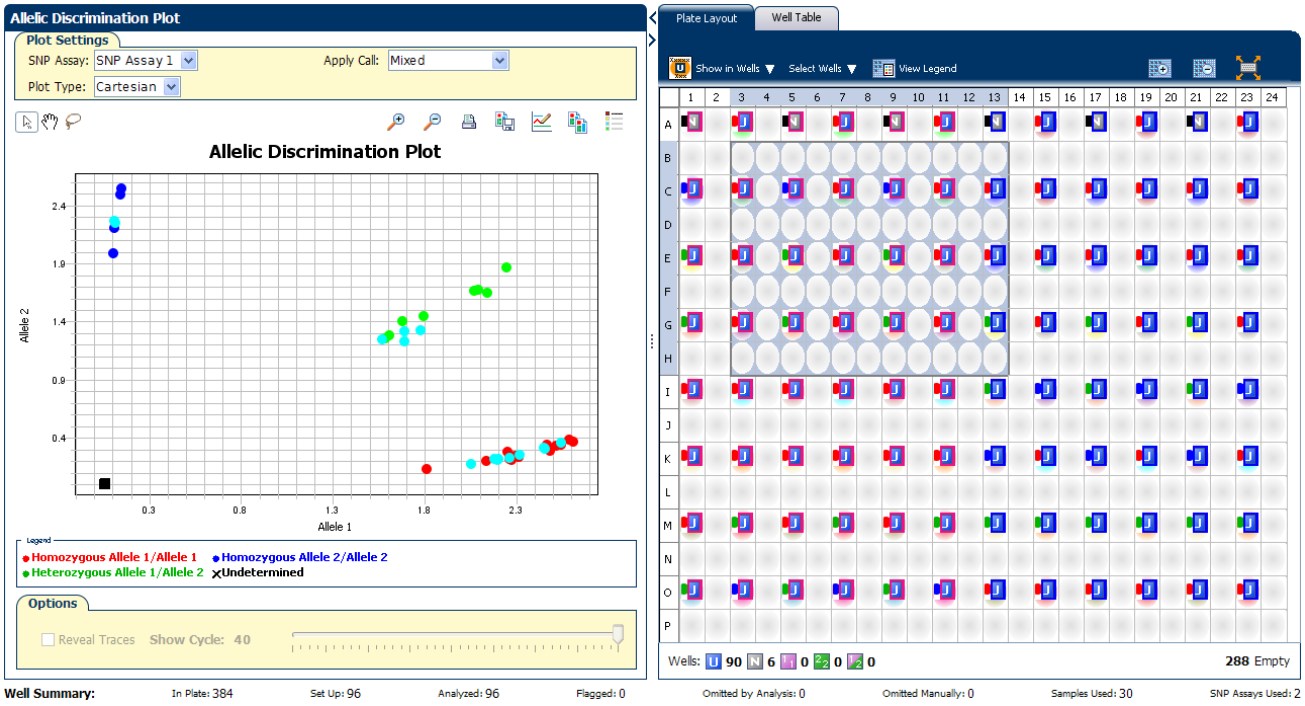
Note: If the Autocaller is not enabled, the Allelic Discrimination Plot displays a crossmark (× – Undetermined) for each sample.

4. Review each cluster in the plot:
   a. Click and drag a box around the cluster to select the associated wells in the plate layout and well table.
   b. Confirm that the expected wells are selected in the well table.

      For example, if you select the cluster at the bottom-left corner of the plot, only the negative controls should be selected. The presence of an unknown among the negative controls may indicate that the sample failed to amplify.
   c. Repeat steps 4a and 4b for all other clusters in the plot.
   d. The table below describes the elements of the Allelic Discrimination Plot.

Element	Description
SNP Assay drop-down menu	Determines the SNP assay data that the ViiA™ 7 Software displays in the plot.
Plot Type drop-down menu	Determines the type of plot (Cartesian or Polar) that the ViiA™ 7 Software uses to display the data.
Apply Call drop-down menu	When a datapoint is selected, this menu allows you to assign an allele call to the datapoint within the scatterplot.
Toolbar	Contains tools for manipulating the scatterplot: • – Selection tool. • – Selection tool. • – Repositioning tool. • – Zooms in. • – Zooms out.
Legend	Explains the symbols in the scatterplot.
Options	The Reveal Traces option allows you to trace the clusters throughout the PCR process. This option is not activated for the example experiment. To activate the feature, see "Adjust analysis settings" on page 56.

The Allelic Discrimination plot for the example experiment looks like this:



Troubleshoot clustering on the Allelic Discrimination Plot

Do all controls have the correct genotype?

In the example experiment and in your own experiments, confirm that data points cluster as expected.

Clustering in positive controls

1. From the well table, select the wells containing a positive control to highlight the corresponding data points (symbols) in the Allelic Discrimination Plot.
2. Check that the data points for the positive controls cluster along the expected axis of the plot. For example, if you select the Positive Control Allele 1/Allele 1, then the controls should cluster along the X-axis.
3. Repeat steps 1 and 2 for the wells containing the other positive controls.

Failed amplification in the unknown samples

1. Select the data points of the cluster in the lower left corner of the Allelic Discrimination Plot to select the corresponding wells in the well table.
2. Check that the selected wells in the well table are negative controls, and not unknown samples.

Samples clustered with negative controls

Samples that clustered with the negative controls may:

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

Confirm the results of these samples by retesting them.

Are outliers present?

If the Allelic Discrimination Plot contains clusters other than the three representative genotype clusters (heterozygous, homozygous allele 1, and homozygous allele 2), then those can be classified as outliers.

Confirm the results of the associated samples by retesting them.

Note: The results displays are synchronized. For example, selecting a well in the plate layout selects the corresponding data in the well table and Allelic Discrimination Plot.

Confirm setup accuracy using Plate Layout

Review the experiment results in the Plate Layout. The plate layout displays the assay-specific setup and analysis properties for the experiment in a well format corresponding to the type of reaction plate used for the run.

Example experiment plate layout values

For the example experiment, confirm that the ViiA™ 7 Software called:

- 54 samples as Allele 1 homozygous ( )
- 12 samples as Allele 2 homozygous ( )
- 24 samples as heterozygous ( )
- 0 samples as undetermined (✕)

Confirm that no wells of the reaction plate triggered QC flags (▲). The example experiment does not display any flags.

View the layout

1. Click the ‹ icon beside the Allelic Discrimination Plot to maximize the plate layout.
2. Click **Show in Wells**, then select or deselect a parameter that you want the wells to display. Repeat this step until the plate layout contains all of the desired parameters.

Parameter	Description
Sample Name	The name of the sample applied to the well.

Parameter	Description
Task	The task assigned to the well: – Unknown – Negative Control – Positive Control - Allele 1 – Positive Control - Allele 2 – Positive Control - Allele 1/2
SNP Assay Name	The name of the SNP evaluated by the well.
Assay ID	The Assay ID number of the SNP evaluated by the well.
Allele 1 / Allele 2	The name of the associated allele for the SNP evaluated by the well
Allele 1 Dyes / Allele 2 Dyes	The name of the reporter and quencher dyes of the associated allele for the SNP evaluated by the well
SNP Assay Color	The color of the SNP evaluated by the well.
Sample Color / Task Color	The color of the sample or task applied to the well.
Genotype Call	The allele call assigned to the sample: • Homozygous 1/1 • Homozygous 2/2 • Heterozygous 1/2 • Negative Control • Undetermined
Flag	The number of QC flags the well triggered as listed in the ▲ symbol.

The following figure shows the plate layout of the example Genotyping experiment.

Tips for troubleshooting plate setup in your own experiment

You can adjust your view of the plate layout:

- Note the location of any samples that trigger QC flags (▲). Understanding the position of errors can aid in diagnosing any failures that may occur.
- You can select the entire reaction plate, areas of the reaction plate, or specific wells:
  - Click the upper left corner of the reaction plate to select all 384 wells.
  - Left-click the mouse and drag across the area to select it.
  - Select **Sample**, **SNP Assay**, or **Task** from the Select Wells menu in the Plate Layout tab to select wells of a specific type using the well-selection criteria.
- Use the (Zoom In), (Zoom Out), and (Fit Plate) buttons to magnify or compress the view of the wells shown.
- Use the arrow tabs to expand the plate layout to cover the entire screen.

Assess amplification results using the Amplification Plot

IMPORTANT! Amplification plots are not used to make SNP calls. Examine the plots to help with troubleshooting and quality control.

If you collected real-time data for your experiment, review the amplification data to further understand the flags triggered by the experiment data.

About amplification plots

The Amplification Plot screen displays amplification of all samples in the selected wells. Use the amplification plots to confirm the results of the experiment:

- **ΔRn vs. Cycle** – ΔRn is the difference in normalized fluorescence signal generated by the reporter between the pre-PCR read and the post-PCR read. This plot displays Δ_{Rn} as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.

  Note: Viewing the ΔRn vs. Cycle plot is discussed in this booklet as an example of how to view the plot.

- **Rn vs. Cycle** – Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays Rn as a function of cycle number. You can use this plot to identify and examine irregular amplification.
- **C_T vs. Well** – C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. You can use this plot to locate outlying amplification (outliers).

Each plot can be viewed as a linear or log10 graph type.

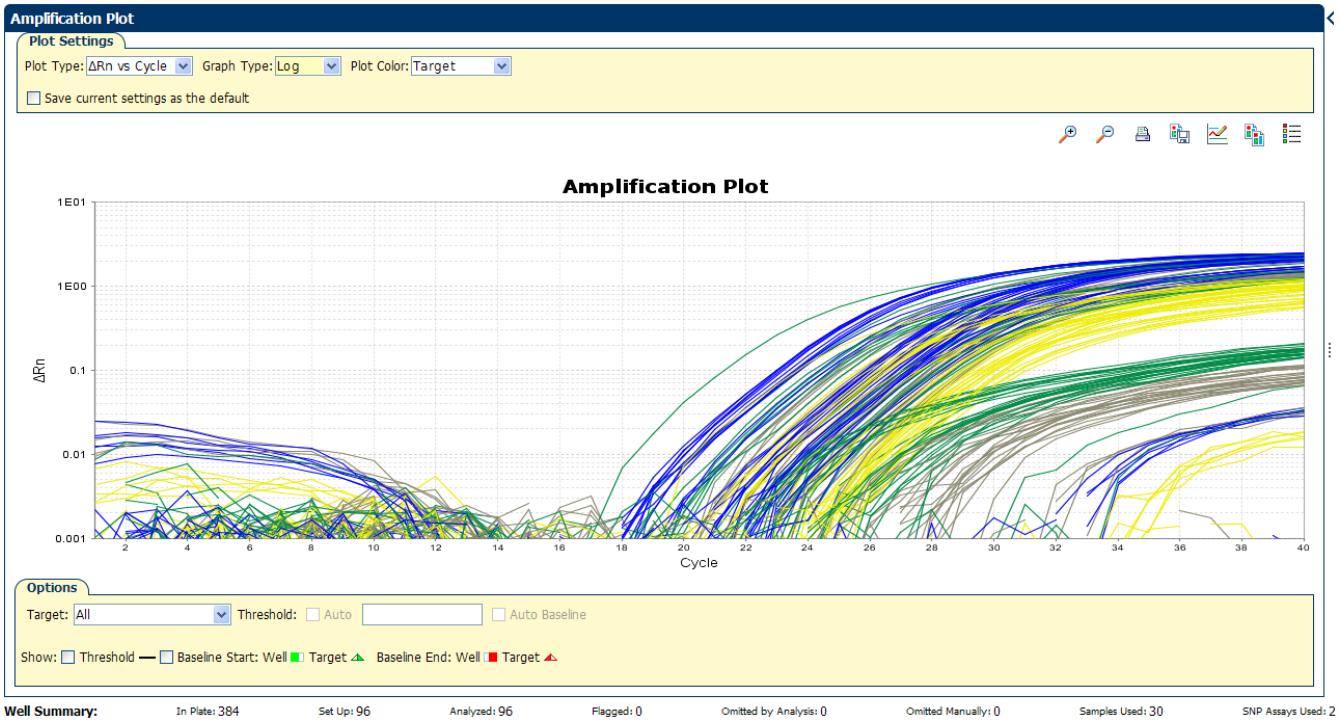
View the ΔRn vs. Cycle plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.

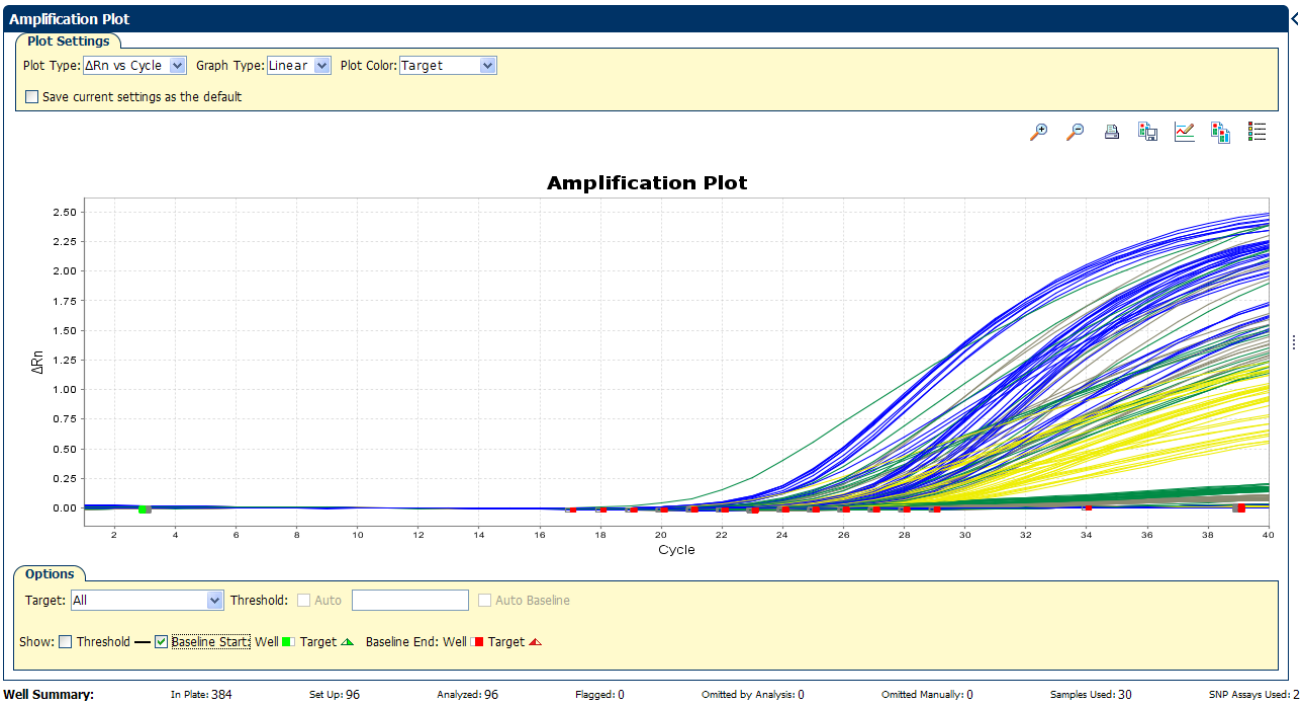
2. Select the plot type and format:

Menu	Selection
Plot Type	ΔRn vs. Cycle
Plot Color	Target
(This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.)	Check (default)

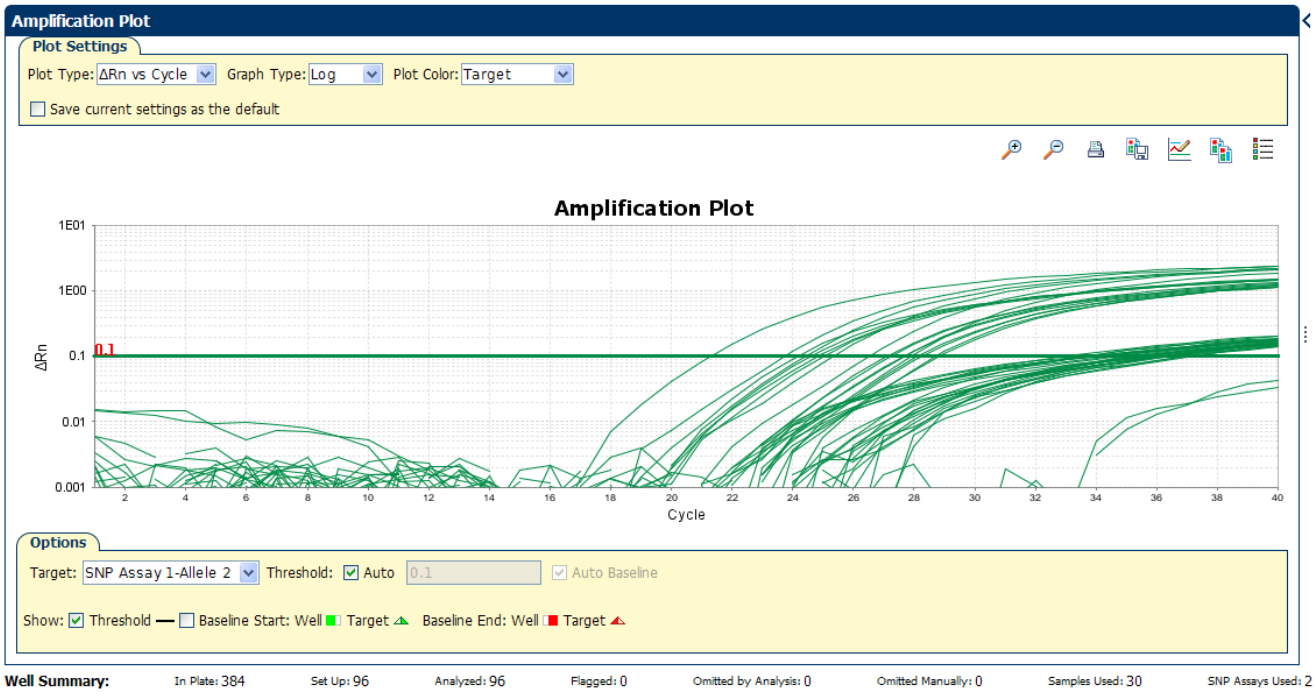


3. View the baseline values:
   a. From the Graph Type drop-down menu, select **Linear**.

b. Select Baseline to show the start cycle and end cycle.



4. View the threshold values:
   a. From the Graph Type drop-down menu, select **Log**.
   b. From the Target drop-down menu, select **SNP Assay 1-Allele 2**.
   c. Select the Threshold check box to show the threshold.



Identify well problems using the Well Table

Review the details of the experiment results in the Well Table and identify any flagged wells. The Well Table displays the assay-specific setup and analysis properties for the experiment in a tabular format.

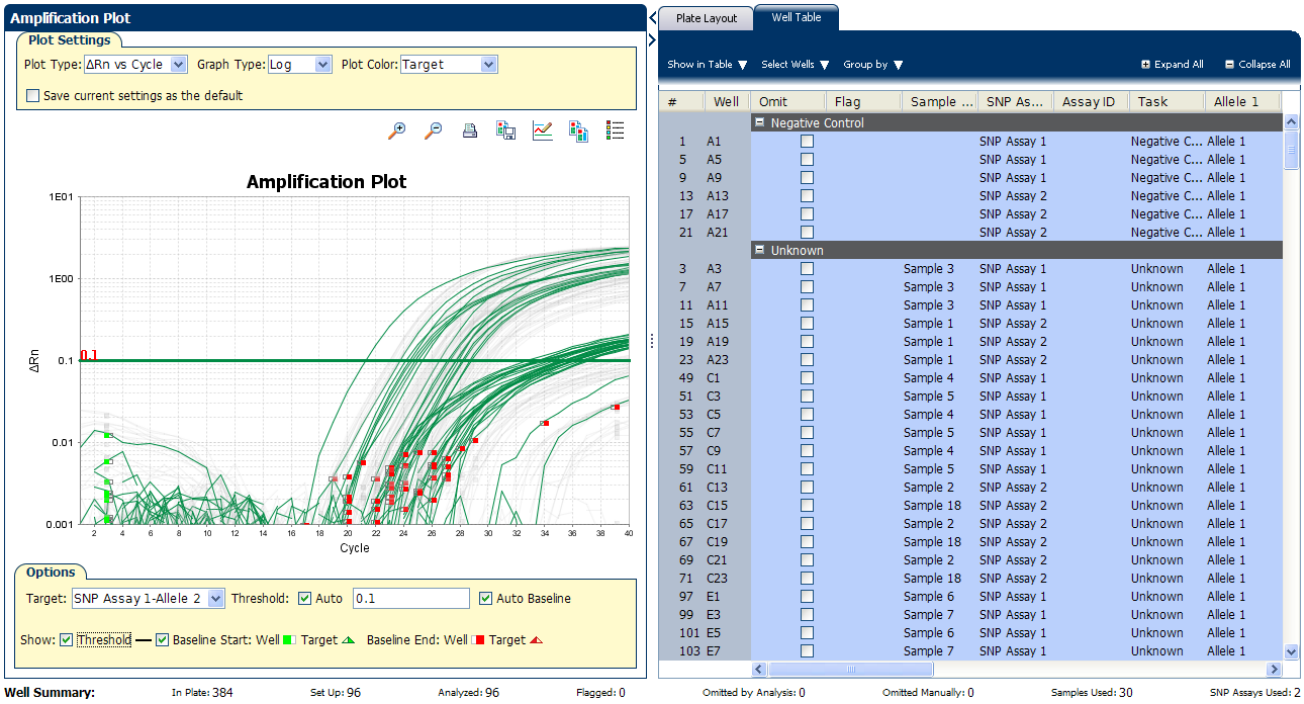
Example experiment values and flags

For the example experiment, look for wells that triggered QC flags (▲). The example experiment has no flags.

View the well table

1. Select the **Well Table** tab.
2. Click the **Flag** column header to sort the data so that the wells that triggered flags appear at the top of the table.
3. Confirm the integrity of the controls:
   a. From the Group By menu, select **Task** to organize the table rows by their function on the reaction plate.
   b. Confirm that each of the controls do not display flags (▲1).
   c. Click the ▪ icon to collapse the negative and positive controls.

The figure below shows the well table of the example Genotyping experiment.



The following table gives the names and description of the columns in the well table:

Column	Description
Well	The position of the well on the reaction plate.
Omit	A check mark indicates that the well has been removed from the analysis.
Flag	A ⚠1 indicates that the well triggered the number of flags listed inside the symbol.
Sample Name	The name of the sample.
SNP Assay Name	The name of the SNP assay evaluated by the well.
Assay ID	The Assay ID number of the SNP evaluated by the well.
Task	The task assigned to the well (Unknown, Negative Control, or Positive Control).
Allele 1 / 2	The name of the associated allele for the SNP evaluated by the well.
Allele 1 / 2 Dyes	The name of the reporter and quencher dyes of the associated allele for the SNP evaluated by the well.
Allele 1 / 2 $_{Rn}$	Normalized signal ($_{Rn}$) of the reporter dye of the associated allele for the SNP evaluated by the well.
Pass Ref	The signal of the passive reference dye for the well.
Call	The allele call assigned to the sample, where possible calls are: • ● Homozygous 1/1 - Homozygous for allele 1 • ● Homozygous 2/2 - Homozygous for allele 2 • ● Heterozygous 1/2 - Heterozygous • ■ Negative Control • ✕ Undetermined
Quality(%)	The quality value calculated for the genotype call.
Method	The method used to assign the call to the sample (Auto if assigned by the ViiA™ 7 Software, or Manual if applied by a user).
Comments	Comments entered for the associated sample well.
Allele 1 / 2 C_T	Threshold cycle (C_T) of the sample for the associated allele for the SNP evaluated by the well.

Identify quality control (QC) problems

The Well Table displays columns for QC flags that are triggered by the experimental data. If the experiment data does not trigger a QC flag, then the ViiA™ 7 Software does not display a corresponding column for the flag.

A (⚠) in one of the following columns indicates that the associated well triggered the flag.

Flag	Description
BADROX	The well produced a passive reference signal greater than the limit defined in the analysis settings.
OFFSCALE	The well produced a level of fluorescence greater than the ViiA™ 7 System can measure.
NOSIGNAL	The well did not produce a detectable level of fluorescence.
CLUSTER#	For the SNP evaluated by the well, the number of clusters generated from the experiment data is greater than the limit defined in the analysis settings.
PCFAIL	The positive control did not produce an $_{Rn}$ for the associated allele greater than the limit defined in the analysis settings indicating that the control may have failed to amplify.
SMCLUSTER	The number of data points in the associated cluster is less than the limit defined in the analysis settings.
AMPNC	The negative control has produced a $_{Rn}$ greater than the limit defined in the analysis settings indicating possible amplification.
NOAMP	The well did not produce an $_{Rn}$ for either allele that is greater than the limit defined in the analysis settings indicating that the well may have failed to amplify.
NOISE	The background fluorescence (noise) produced by the well is greater than the other wells on the reaction plate by a factor greater than the limit **defined in the analysis settings**.
SPIKE	The amplification plot for the well contains one or more data points inconsistent with the other points in the plot.
EXPFAIL	The software cannot identify the exponential region of the amplification plot for the well.
BLFAIL	The software cannot calculate the best fit baseline for the data for the well.
THOLDFAIL	The software cannot calculate a threshold for the associated well.
CTFAIL	The software cannot calculate a threshold cycle (C_T) for the associated well.

Tips for analyzing your own experiments

Confirm the integrity of positive controls

When you analyze the example experiment or your own experiment, if you are using positive controls, confirm the integrity of the positive controls:

1. From the Group By menu, select **Task** to organize the table rows by their function on the reaction plate
2. Confirm that the positive controls do not display flags (▲) and that their normalized reporter dye fluorescence (R_n) is appropriate for the genotype (for example, if evaluating the Positive Control Allele 1/Allele 1, you would expect to see significant increase in R_n for the Allele 1 probe and very little for the Allele 2 probe).

Adjust the Well Table

- Review the data for the Unknown samples. For each row that displays (▲) in the Flag column, note the data and the flag(s) triggered by the associated well.
- Select areas of the table or wells of a specified type by:
  - Left-clicking the mouse and dragging across the area you want to select an area of the table.
  - Selecting **Sample**, **SNP Assay**, or **Task** from the Select Wells menu in the Well Table tab to select wells of a specific type using the well-selection tool.
- Group the rows of the plate layout by selecting an option from the Group By menu. You can then collapse or expand the lists either by clicking the **+/-** icon next to individual lists, or by clicking **Collapse All** or **Expand All**.
- Omit a well from the analysis by selecting the **Omit** check box for that well. To include the well in the analysis, deselect the **Omit** check box.

  Note: You must reanalyze the experiment each time you omit or include a well.

Confirm accurate dye signal using the Multicomponent Plot

The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the example experiment, you review the Multicomponent Plot screen for:

- ROX™ dye (passive reference)
- FAM™ dye (reporter)
- VIC® dye (reporter)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

View the Multicomponent Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Multicomponent Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the unknown wells in the plate layout to display the corresponding data in the Multicomponent Plot screen:

   a. Click the **Plate Layout** tab.

   b. Select one well in the plate layout; the well is shown in the Multicomponent Plot screen.

   Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

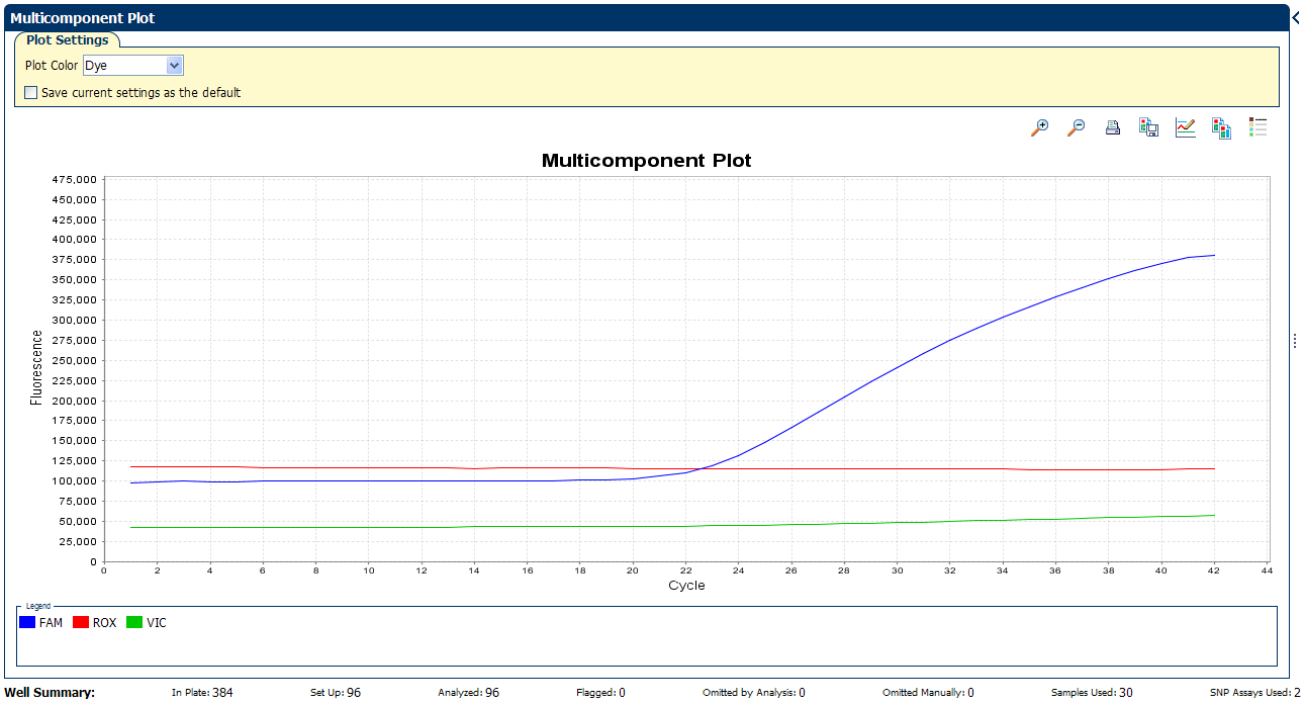
3. From the Plot Color drop-down menu, select **Dye**.

4. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

5. Check the ROX dye signal. In the example experiment, the ROX dye signal remains constant throughout the PCR process; a constant ROX dye signal indicates typical data.

6. Check the FAM dye signal. In the example experiment, the FAM dye signal increases throughout the PCR process; increase in FAM dye signal indicates normal amplification.



7. Select the negative control wells one at time and check for amplification. Wells with the negative control should not show amplification. The example experiment does not have negative controls.

Tips for confirming dye accuracy in your own experiment

When you analyze your own Genotyping experiment, look for:

- **Passive Reference** – The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter Dye** – The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- **Irregularities in the signal** – There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative Control wells** – There should not be any amplification in the negative control wells.

Determine signal accuracy using the Raw Data Plot

The Raw Data Plot screen displays the raw fluorescence signal (not normalized) for each optical filter for the selected wells during each cycle of the real-time PCR.

Purpose

In the Genotyping example experiment, you review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the Raw Data Plot

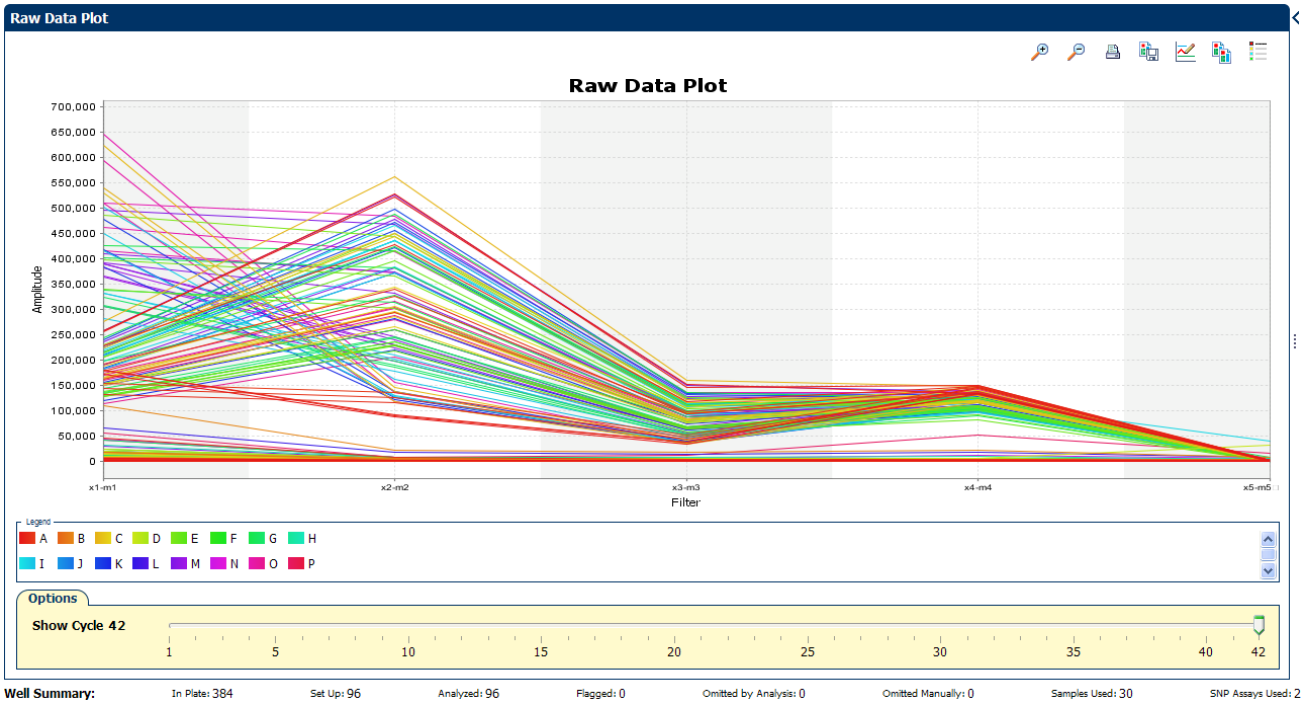
1. From the Experiment Menu pane, select **Analysis** ▸ **Raw Data Plot**.

   Note: If no data are displayed, click **Analyze**.

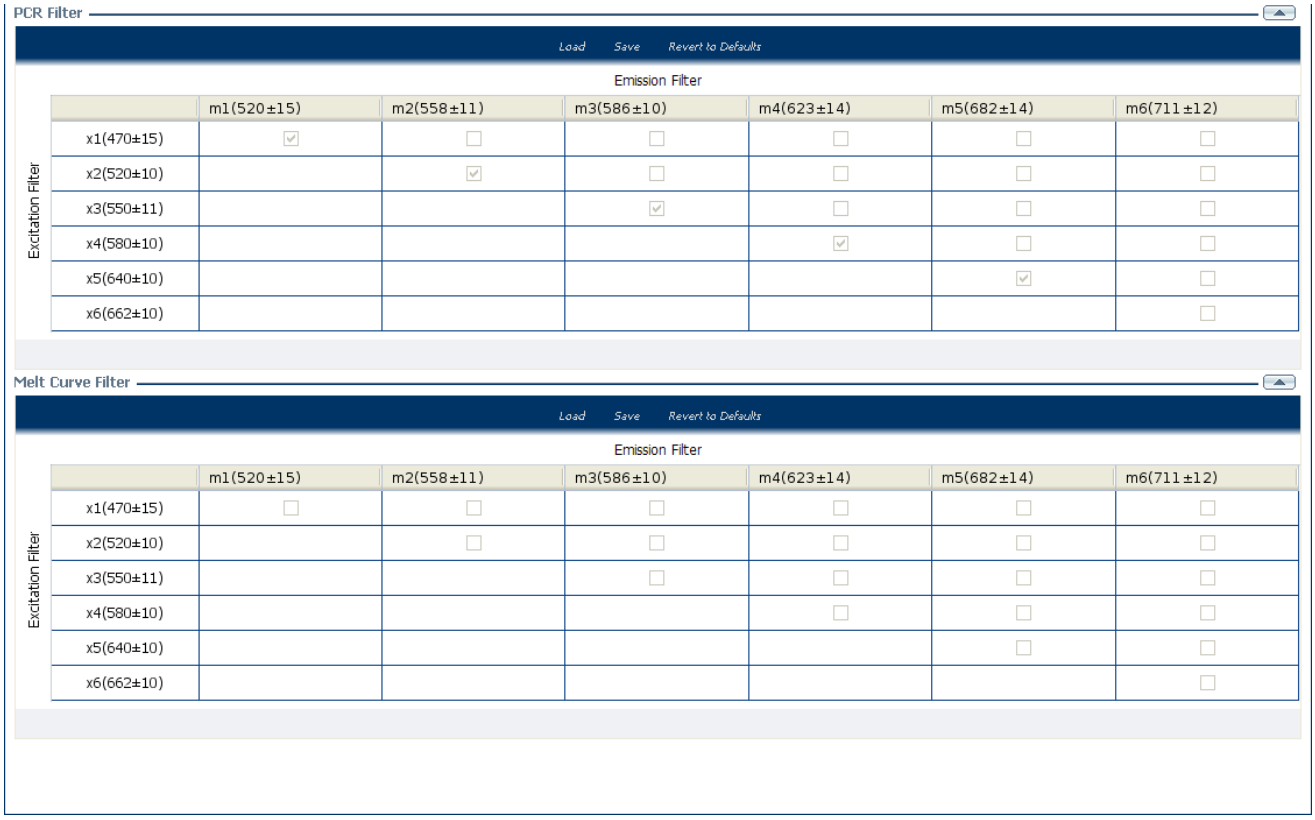
2. Display all 384 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the Plate Layout tab.

3. Click **Show a legend for the plot** (default). The legend displays the color code for each row of the reaction plate (see the legend in the Raw Data Plot shown below).

4. Click and drag the Show Cycle pointer from cycle 1 to cycle 42. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.



The filters are:



Tips for determining signal accuracy in your own experiment

When you analyze your own Genotyping experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

Review the flags in the QC Summary

The QC Summary screen displays a list of the ViiA™ 7 Software flags, including the flag frequency and location for the open experiment.

For Genotyping experiments, flag appearance is triggered by experiment data or the assay. If a flag has been triggered by the assay, the Plate Layout does not display the ⚠ icon. The flag details appear in the QC Summary.

In the example experiment, there are no flags.

View the QC Summary

1. From the Experiment Menu pane, select **Analysis ▸ QC Summary**.

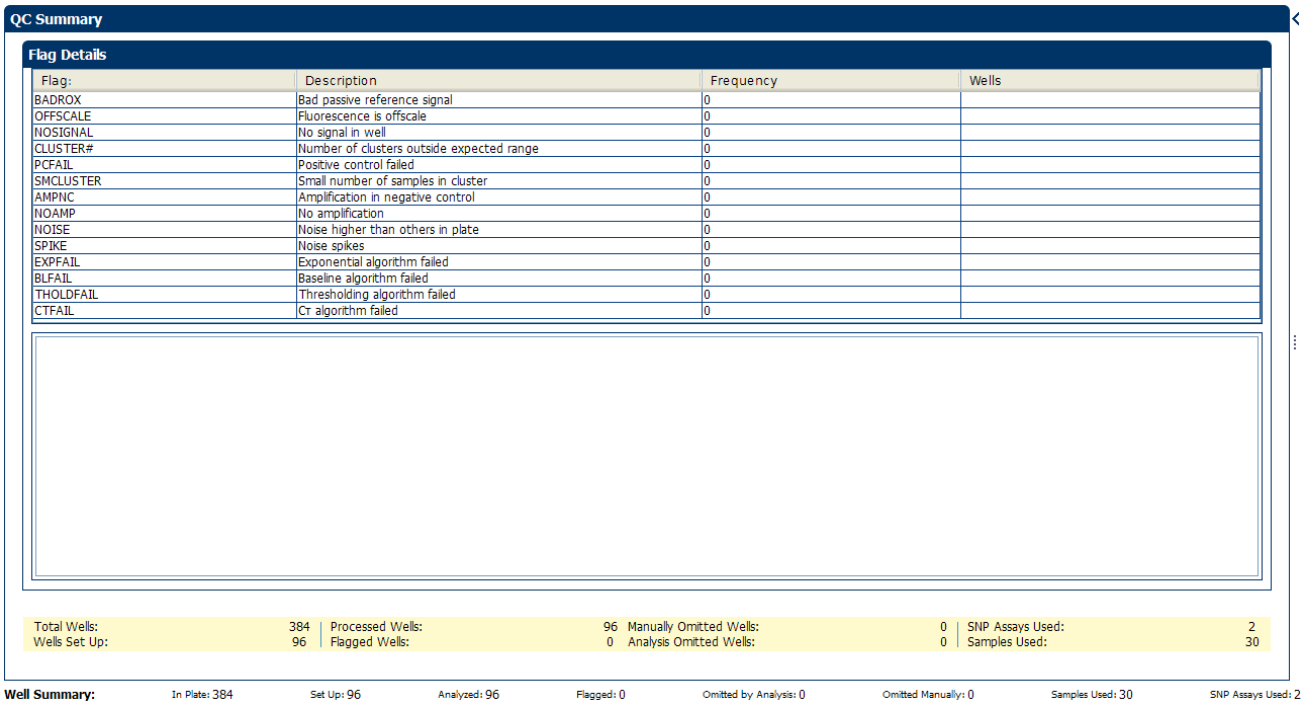
   Note: If no data are displayed, click **Analyze**.

2. Review the Flags Summary.

   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment. If the frequency is >0, the flag appears somewhere in the experiment; the well position is listed in the Wells column.

3. In the Flag Details table, click each flag with a frequency >0 to display detailed information about the flag.

4. *(Optional)* For those flags with frequency >0, click each flag with a frequency >0 to display detailed information about the flag.



Possible flags

The flags listed below may be triggered by the experiment data or the assay.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
Secondary analysis flags	
AMPNC	Amplification in negative control
CLUSTER#	Number of clusters outside expected range
PCFAIL	Positive Control failed
SMCLUSTER#	Small number of samples in clusters

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the Frequency column for each flag).

For more information

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with ViiA™7 System Experiments*.	4441434

5.2 Section 5.2 Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

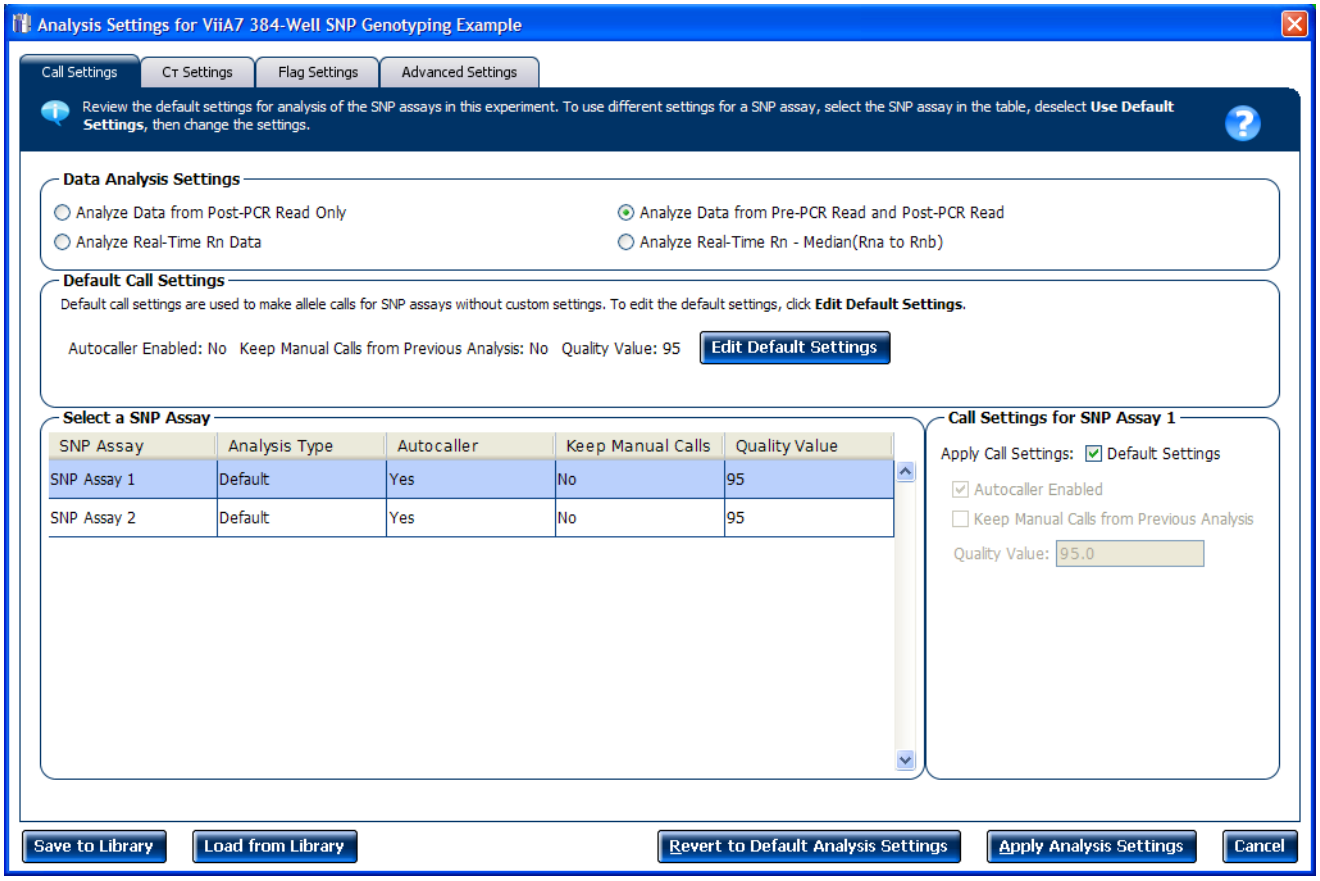
The Analysis Settings dialog box displays the analysis settings for the call, threshold cycle (C_T), flags, and advanced options.

You can change the settings in the Analysis Settings dialog box, then reanalyze your experiment.

View the analysis settings

1. From the Experiment Menu pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the Analysis Settings dialog box.
   In the example experiment, the default analysis settings are used for each tab:
   - Call Settings
   - C_T Settings
   - Flag Settings
   - Advanced Settings

The Analysis Settings dialog box for a Genotyping experiment looks like this:



3. View and, if necessary, change the analysis (see "Adjust analysis settings" below).

   Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see About the Analysis Settings Library in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*.

4. Click **Apply Analysis Settings** to apply the current analysis settings.

   Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

Call Settings

Use the Call Settings tab to:

- Change the default data analysis settings. You can select from:
  - Analyze data from Post-PCR Read only - Select if you do not want to use data from the pre-PCR read to determine genotype calls.
  - Analyze data from Pre-PCR Read and Post-PCR Read - If you included the pre-PCR read in the run, select if you want to use data from the pre-PCR read to determine genotype calls.

  - Analyze Real-Time Rn Data - If you included amplification in the run, select if you want to use the normalized reporter (Rn) data from the cycling stage to determine genotype calls.
  - Analyze data from Rn - Avg (Rna to Rnb) - If you included amplification in the run, select if you want to use the subtracted median of the normalized reporter (Rn) data from the cycling stage to determine genotype calls, where Rna to Rnb refers to all the cycles from the Start Cycle Number to the End Cycle Number. The average subtraction provides improved data accuracy.

    Note: To activate the Reveal Traces feature on the Allelic Discrimination Plot scree, select either **Analyze Real-Time Rn Data** or **Analyze data from Rn - Avg (Rna - Rnb)**.

- Edit the default call settings. Click **Edit Default Settings**, then specify the default settings:
  - **Autocaller Enabled** - Select for the software to make genotype calls using the autocaller algorithm.
  - **Keep Manual Calls from Previous Analysis** - If the autocaller is enabled, select to maintain manual calls after reanalysis
  - **Quality Value** - Enter a value to use to make genotype calls. If the confidence value is less than the call setting, the call is undetermined.
- Use custom call settings for a SNP assay.
  - Select one or more SNP assays in the table, then deselect the **Default Settings** checkbox.
  - **Define the custom call settings**.

C_T Settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method.
- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

  The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for gene quantification.

  The Relative Threshold algorithm lets you compare the data on a per-well or per-target basis. This setting is ideal for analyzing a single gene across samples or a single sample across genes with no dependence on targets, thereby reducing variability. Any settings for baseline or threshold do not affect the analysis when you use the Relative Threshold algorithm.

- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the alleles that do not have custom settings. To edit the default settings, click **Edit Default Settings**.

- **C_T Settings for Target**

  When you manually set the threshold and baseline, Applied Biosystems recommends:

Setting	Recommendation
Threshold	Enter a value for the threshold so that the threshold is: • Above the background. • Below the plateau and linear regions of the amplification curve. • Within the exponential phase of the amplification curve.
Baseline	Select the Start Cycle and End Cycle values so that the baseline ends before significant fluorescent signal is detected.

Note: This setting is applicable only to the Baseline Threshold algorithm.

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag Settings

Use the Flag Settings tab to:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the ViiA™ 7 Software.

  To adjust the flag settings:

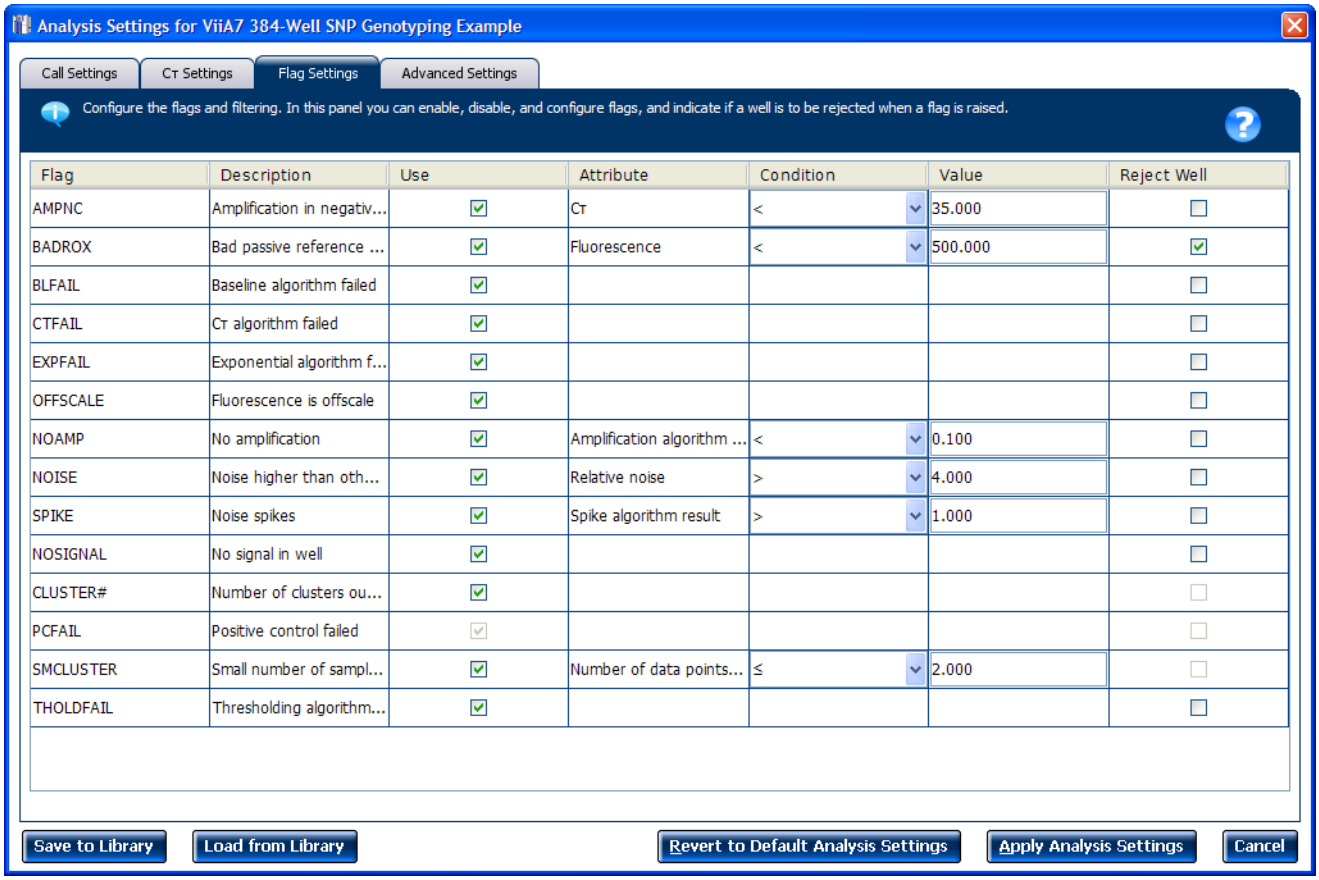
To adjust the flag settings:

1. In the Use column, select the check boxes for flags to apply during analysis.
2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.
3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations may change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.
4. Click **Apply Analysis Settings** in the Analysis Settings dialog box. If the run status is complete, the data are reanalyzed.

The Flag Settings tab looks like this:



Advanced Settings

Use the Advanced Settings tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold setting.

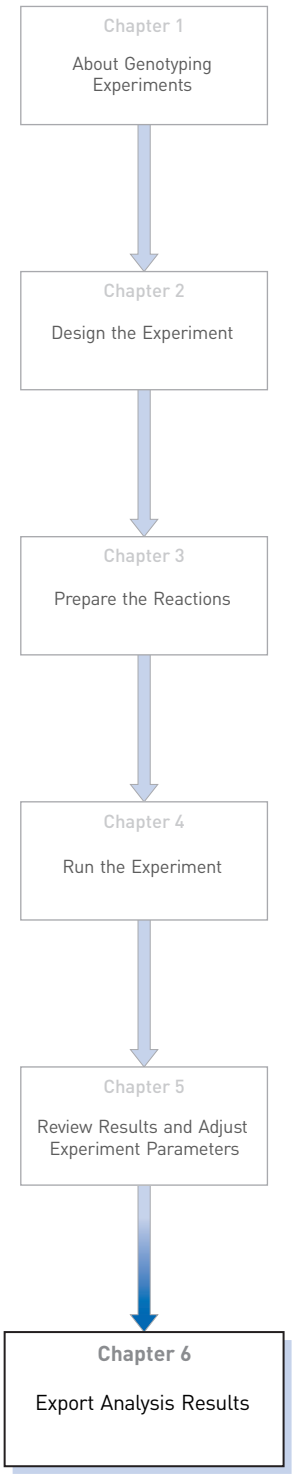
To use custom baseline settings for a well-target combination:

1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** check box.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, then enter the baseline start cycle and baseline end cycle.

For more information

For more information on...	Refer to...	Part number
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*.	127AP05-03

6 Export Analysis Results

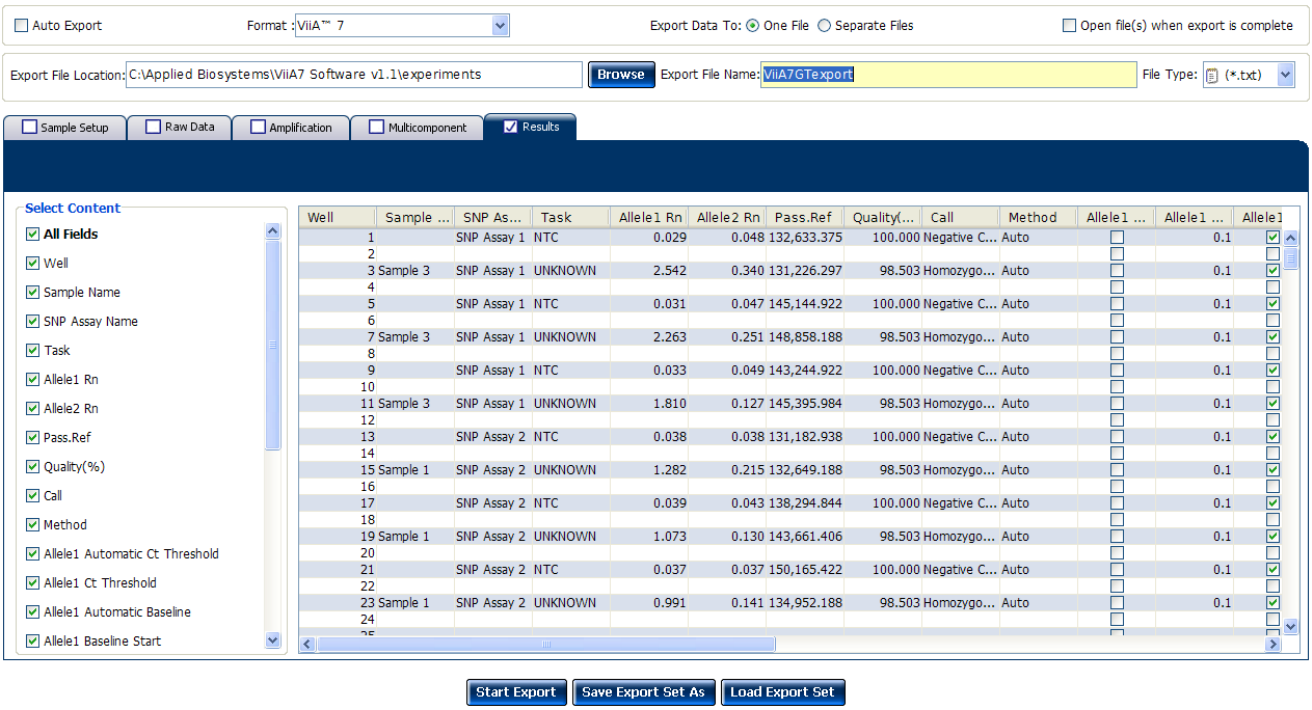


1. Open the Genotyping example experiment file that you analyzed in Chapter 5.
2. In the Experiment Menu, click **Export.**

   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.
3. Select **ViiA™7 Format**.
4. Complete the Export dialog box as shown below:

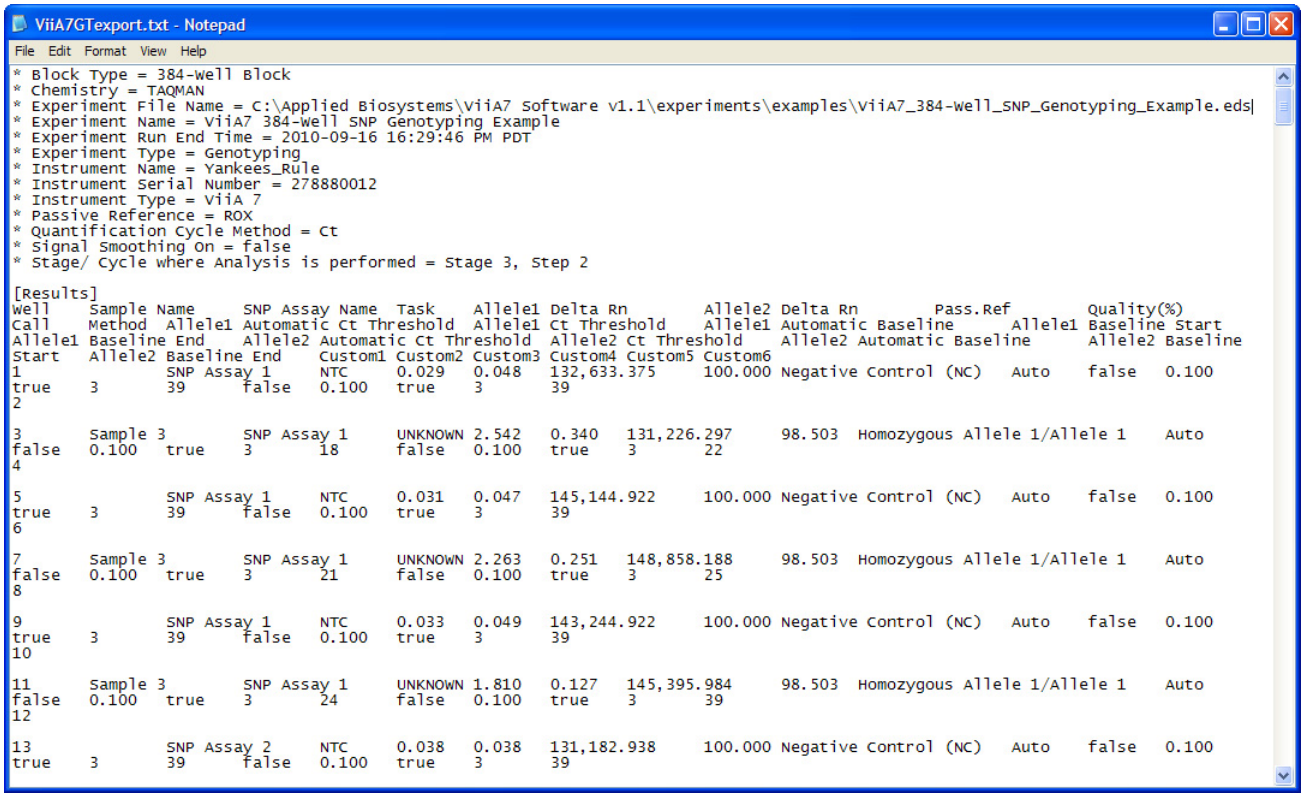
Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	ViiA7GTexport
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\ViiA7 Software v1.1\experiments

Your Export screen should look like this:



Well	Sample ...	SNP As...	Task	Allele1 Rn	Allele2 Rn	Pass.Ref	Quality(...	Call	Method	Allele1 ...	Allele1 ...	Allele1
1		SNP Assay 1	NTC	0.029	0.048	132,633.375	100.000	Negative C...	Auto		0.1	
2												
3	Sample 3	SNP Assay 1	UNKNOWN	2.542	0.340	131,226.297	98.503	Homozygo...	Auto		0.1	
4												
5		SNP Assay 1	NTC	0.031	0.047	145,144.922	100.000	Negative C...	Auto		0.1	
6												
7	Sample 3	SNP Assay 1	UNKNOWN	2.263	0.251	148,858.188	98.503	Homozygo...	Auto		0.1	
8												
9		SNP Assay 1	NTC	0.033	0.049	143,244.922	100.000	Negative C...	Auto		0.1	
10												
11	Sample 3	SNP Assay 1	UNKNOWN	1.810	0.127	145,395.984	98.503	Homozygo...	Auto		0.1	
12												
13		SNP Assay 2	NTC	0.038	0.038	131,182.938	100.000	Negative C...	Auto		0.1	
14												
15	Sample 1	SNP Assay 2	UNKNOWN	1.282	0.215	132,649.188	98.503	Homozygo...	Auto		0.1	
16												
17		SNP Assay 2	NTC	0.039	0.043	138,294.844	100.000	Negative C...	Auto		0.1	
18												
19	Sample 1	SNP Assay 2	UNKNOWN	1.073	0.130	143,661.406	98.503	Homozygo...	Auto		0.1	
20												
21		SNP Assay 2	NTC	0.037	0.037	150,165.422	100.000	Negative C...	Auto		0.1	
22												
23	Sample 1	SNP Assay 2	UNKNOWN	0.991	0.141	134,952.188	98.503	Homozygo...	Auto		0.1	
24												

Your exported file when opened in Notepad should look like this:



ViiA7GTexport.txt - Notepad

File Edit Format View Help

```
* Block Type = 384-Well Block
* Chemistry = TAQMAN
* Experiment File Name = C:\Applied Biosystems\ViiA7 Software v1.1\experiments\examples\ViiA7_384-Well_SNP_Genotyping_Example.eds
* Experiment Name = ViiA7 384-Well SNP Genotyping Example
* Experiment Run End Time = 2010-09-16 16:29:46 PM PDT
* Experiment Type = Genotyping
* Instrument Name = Yankees_Rule
* Instrument Serial Number = 278880012
* Instrument Type = ViiA 7
* Passive Reference = ROX
* Quantification Cycle Method = Ct
* Signal Smoothing On = false
* Stage/ Cycle where Analysis is performed = Stage 3, Step 2

[Results]
Well    Sample Name     SNP Assay Name  Task    Allele1 Delta Rn        Allele2 Delta Rn        Pass.Ref        Quality(%)
Call    Method  Allele1 Automatic Ct Threshold  Allele1 Ct Threshold    Allele1 Automatic Baseline      Allele1 Baseline Start
Allele1 Baseline End    Allele2 Automatic Ct Threshold  Allele2 Ct Threshold    Allele2 Automatic Baseline      Allele2 Baseline
Start   Allele2 Baseline End    Custom1 Custom2 Custom3 Custom4 Custom5 Custom6
1               SNP Assay 1     NTC     0.029   0.048   132,633.375     100.000 Negative Control (NC)   Auto    false   0.100
true    3       39      false   0.100   true    3       39
2

3       Sample 3        SNP Assay 1     UNKNOWN 2.542   0.340   131,226.297     98.503  Homozygous Allele 1/Allele 1    Auto
false   0.100   true    3       18      false   0.100   true    3       22
4

5               SNP Assay 1     NTC     0.031   0.047   145,144.922     100.000 Negative Control (NC)   Auto    false   0.100
true    3       39      false   0.100   true    3       39
6

7       Sample 3        SNP Assay 1     UNKNOWN 2.263   0.251   148,858.188     98.503  Homozygous Allele 1/Allele 1    Auto
false   0.100   true    3       21      false   0.100   true    3       25
8

9               SNP Assay 1     NTC     0.033   0.049   143,244.922     100.000 Negative Control (NC)   Auto    false   0.100
true    3       39      false   0.100   true    3       39
10

11      Sample 3        SNP Assay 1     UNKNOWN 1.810   0.127   145,395.984     98.503  Homozygous Allele 1/Allele 1    Auto
false   0.100   true    3       24      false   0.100   true    3       39
12

13              SNP Assay 2     NTC     0.038   0.038   131,182.938     100.000 Negative Control (NC)   Auto    false   0.100
true    3       39      false   0.100   true    3       39
```

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BOOKLET 5

Running Presence/Absence Experiments

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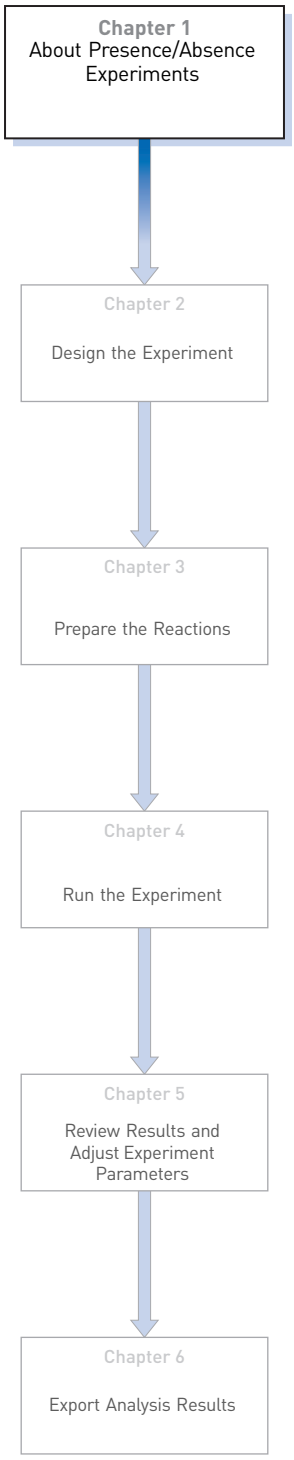
1 About Presence/Absence Experiments



This chapter covers:

- About data collection 6
- Setting up PCR reactions 6
- About the instrument run 7
- About the analysis 8
- About the example experiment 8

IMPORTANT! First-time users of the ViiA™ 7 System, please read Booklet 1, *Getting Started with ViiA™ 7 System Experiments* and Booklet 7, *ViiA™ 7 System Experiments - Appendixes* of this binder thoroughly. The booklet provides information and general instructions that are applicable to all the experiments described in this binder.

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems ViiA™ 7 Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ ViiA™ 7 Software Help**.

About data collection

Presence/Absence experiments are end-point experiments that are performed to detect a target nucleic acid sequence in a sample.

You can collect the experiment data at the end of the run or continuously in real time.

End-point PCR Data

The ViiA™ 7 System collects data at an end-point, that is after the process has completed.

The data collected is the normalized intensity of the reporter dye, or Rn.

Note: Some end-point experiments also include pre-PCR (data collected before the amplification process) datapoints. If so, the system calculates the delta Rn (ΔRn) value per the following formula:

ΔRn = Rn (post-PCR read) – Rn (pre-PCR read), where Rn = normalized readings.

Real-Time PCR Data

The ViiA™ 7 System provides the option of collecting real-time data, during the PCR process.

Note: Real-time data collection is used only for troubleshooting, and not for Presence/Absence analysis.

Setting up PCR reactions

With Presence/Absence experiments, you prepare PCR reactions that contain primers and probes to amplify the target and a reagent to detect amplification of the target. You can set up the PCR reactions for the Presence/Absence experiments two different ways.

Note: The example experiment uses IPC setup for setting up the PCR reactions.

IPC setup

Use an internal positive control (IPC) to monitor the PCR progress and ensure that a negative result is not caused by failed PCR in the sample. PCR reactions contain two primer/probe sets: One to detect the unknown target (unknown target primer set and TaqMan® probe to detect the unknown target) and one to detect the IPC (IPC primer set and a VIC dye-labeled TaqMan® probe to detect the IPC template). With this setup, there are three well types:

- **Unknown-IPC wells** contain sample template and IPC template; the presence of the target is not known.
- **Negative control-IPC wells** contain IPC template and water or buffer instead of sample template in the PCR reaction. Only the IPC template should amplify in negative control-IPC wells because the reaction contains no sample template. Also called **IPC+**.

- **Negative control-blocked IPC wells** do not contain sample template in the PCR reaction. Amplification is prevented by a blocking agent. As a result, no amplification should occur in negative control-blocked IPC wells because the reaction contains no sample template and amplification of the IPC is blocked. Negative control-blocked IPC is called *no amplification control (NAC)*.

  If the run method includes amplification, real-time data are plotted in an amplification plot.

No IPC, singleplex setup

Omit the IPC from your Presence/Absence experiment. PCR reactions contain one primer/probe set. PCR reactions do not contain the IPC. With this setup, there are two well types:

- **Unknown wells** – Wells contain sample template; the presence of the target is not known.
- **Negative controls** – Wells contain water or buffer instead of sample template.

About the instrument run

With Presence/Absence experiments, the instrument runs can include:

- **Pre-PCR read** – Perform the pre-PCR read on the ViiA™ 7 Software before PCR amplification to collect baseline fluorescence data.
- **Amplification** – Perform amplification on the ViiA™ 7 Software to collect fluorescence data during PCR amplification. If you do not include amplification in the run method, perform amplification on another instrument.
- **Post-PCR read** – To determine the results for Presence/Absence experiments, perform the post-PCR read on the instrument after PCR amplification to collect endpoint fluorescence data.

Fluorescence data collected during the instrument run are stored in an experiment data file (.eds).

About the analysis

Data from the instrument run are used to determine Presence/Absence calls. Results are plotted in a Presence/Absence plot. If the experiment includes amplification, results are plotted in an amplification plot.

- **Pre-PCR read** – If included, the data collected from the pre-PCR read can be used to normalize data collected from the post-PCR read.
- **Amplification** – If included, the data collected from the amplification can be used to troubleshoot.
- **Post-PCR read** – The data collected from the post-PCR read are used to make Presence/Absence calls:
  - **Presence** – The target amplified above the target's threshold. The target is present in the sample.
  - **Absence** – The target did not amplify above the target's threshold. The target is absent in the sample.
  - **Unconfirmed** – The data collected is below the target threshold, and the intensity of IPC is below the IPC threshold.

With the IPC setup, the data collected form the post-PCR read are used to make the following calls:

- **IPC Failed** – The IPC target did not amplify in the IPC wells and/or the IPC target amplified in the blocked IPC wells.
- **IPC Succeeded** – The IPC target amplified in the IPC wells and the IPC target did not amplify in the blocked IPC wells.

About the example experiment

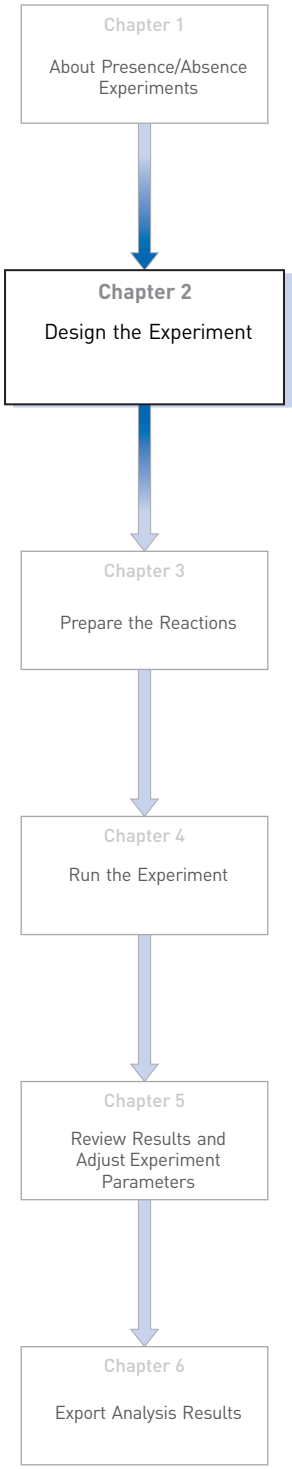
To illustrate how to perform Presence/Absence experiments, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with a ViiA™ 7 System.

The objective of the Presence/Absence example experiment is to determine if a pathogen is present or absent in each batch of ground beef.

In the Presence/Absence example experiment:

- DNA is extracted from samples using the PrepMan® Ultra Sample Preparation Reagent (PN 4318930). The DNA is extracted from each of the four samples of ground beef or from the bacteria found in the ground beef.
- The target is a pathogen.
- The experiment is designed for duplex PCR, where each reaction contains two primer/probe sets. One set detects the pathogen sequence, TGFB (primer set and FAM™ dye-labeled probe to detect the TGFB sequence). The other primer/probe set detects the IPC primer set and VIC® dye-labeled TaqMan® probe detects the IPC template.

2 Design the Experiment



This chapter explains how to design the example experiment from the Experiment Setup menu.

This chapter covers:

- Define the experiment properties. 10
- Define targets and samples 11
- Assign targets and samples. 12
- Set up the run method 13
- Order materials for the experiment 14
- For more information. 14

Note: To automatically export the analyzed data to a specified location, select the Auto Export task from the Export screen, before running the experiment. For more information on Auto Export, refer to Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*.

Define the experiment properties

Click **Experiment Setup** ▸ **Experiment Properties** to create a new experiment in the ViiA™ 7 Software. Enter:

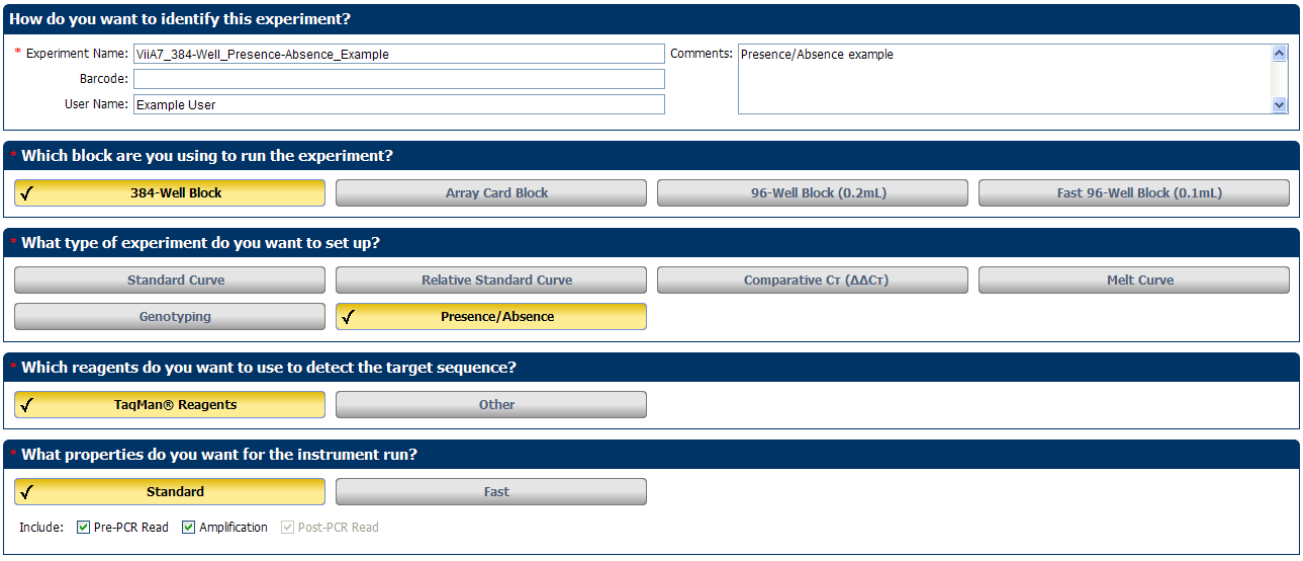
Field or Selection	Entry
Experiment Name	ViiA7_384-Well_Presence_Absence_ Example
Barcode	Leave field empty
User Name	Example User
Comments	Presence/Absence example
Block	384-Well Block
Experiment Type	Presence/ Absence
Reagents	TaqMan® Reagents
Ramp speed	Standard

Select all three data-collection check boxes: Pre-PCR, Amplification, and Post-PCR collection methods:

Pre-PCR Read	Checked
Amplification	Checked
Post-PCR Read	Checked

Save the experiment.

Your Experiment Properties screen should look like this:



Define targets and samples

Click **Define** to access the Define screen. Enter:

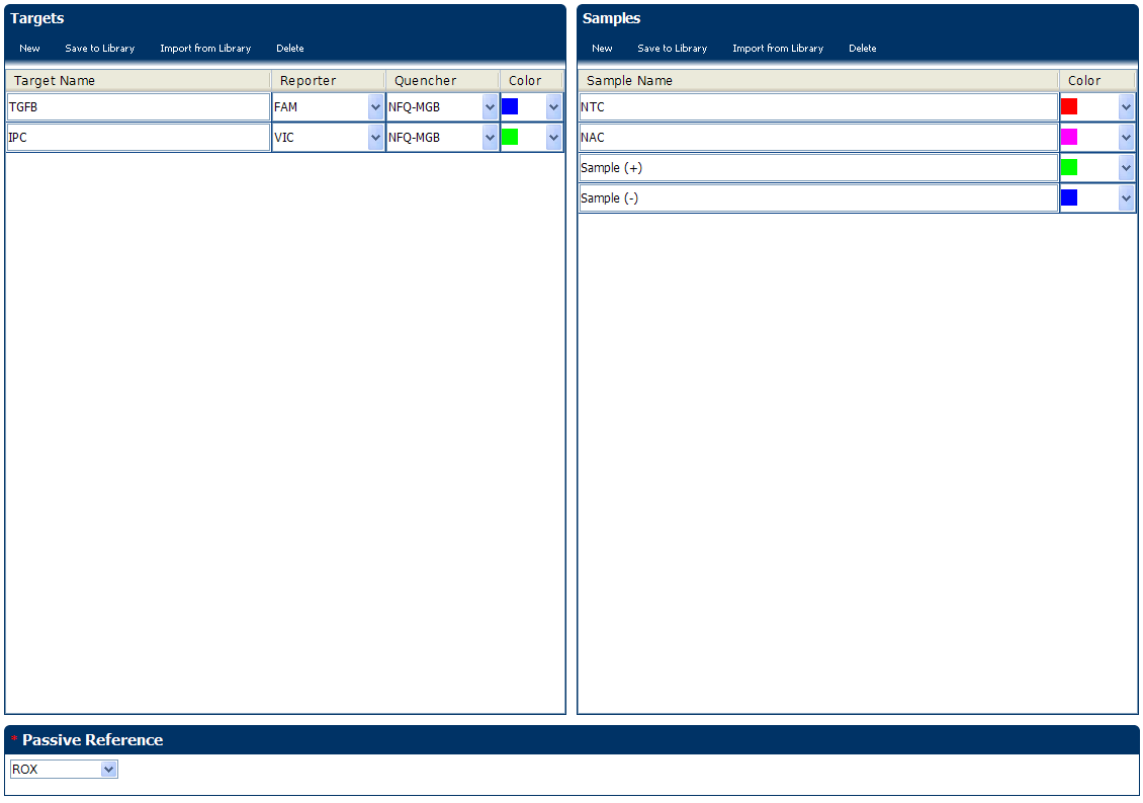
1. Targets

Target Name	Reporter	Quencher	Color
TGFB	FAM	NFQ-MGB	
IPC	VIC	NFQ-MGB	

2. Samples

Sample Name	Color
NTC	
NAC	
Sample (+)	
Sample (-)	

3. Dye to be used as a Passive Reference
   ROX
   Your Define screen should look like this:

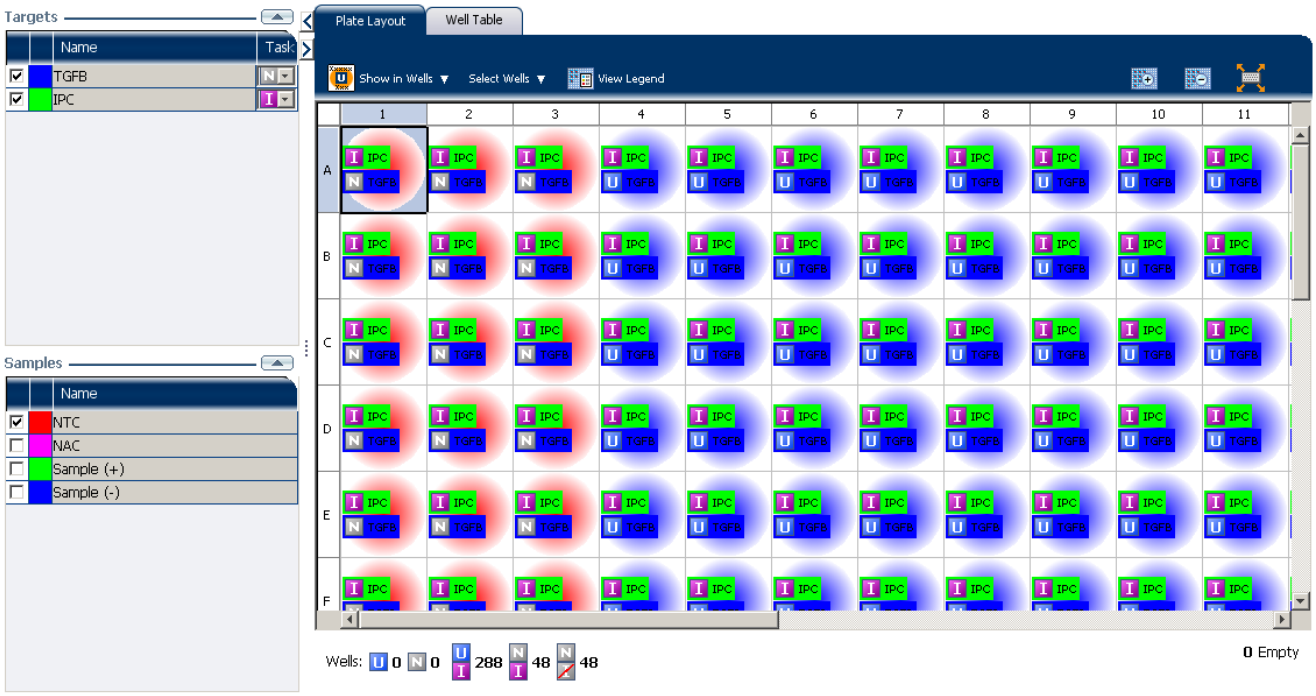


Assign targets and samples

Click **Assign** to access the Assign screen. Enter the targets and samples:

Target Name	Well Number	Task	Sample
TGFB IPC	A1 - P3 (Columns 1 - 3)	Negative IPC	NTC
TGFB IPC	A4 - P12 (Columns 4 - 12)	Unknown IPC	Sample (-)
TGFB IPC	A13 - P15 (Columns 13 - 15)	NTC Blocked IPC	NAC
TGFB IPC	A16 - P24 (Columns 16 - 24)	Unknown IPC	Sample (+)

Your Assign screen should look like this:



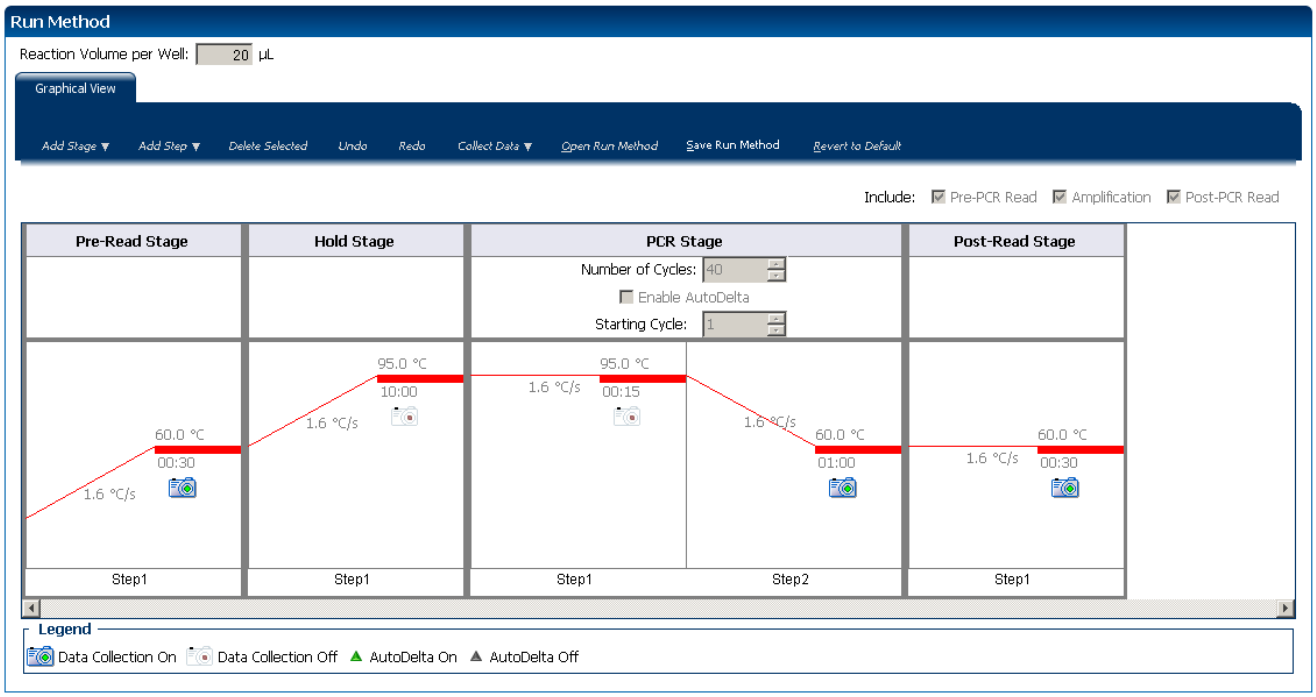
Set up the run method

Click **Run Method** to access the Run Method screen. Set the thermal profile under the Graphical View tab. Enter:

- Reaction Volume Per Well: 20µl
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Pre-Read Stage	Step 1	1.6 °C/s	60 °C	30 seconds
Hold Stage	Step 1	1.6 °C/s	95°C	10 minutes
PCR Stage • Number of Cycles: 40 (default) • Enable AutoDelta: Unchecked (default) • Starting Cycle: Disabled when Enable AutoDelta is unchecked	Step 1	1.6 °C/s	95°C	15 seconds
	Step2	1.6 °C/s	60°C	1 minute
Post-Read Stage	Step 1	1.6 °C/s	60°C	30 seconds

Your Run Method screen should look like this:

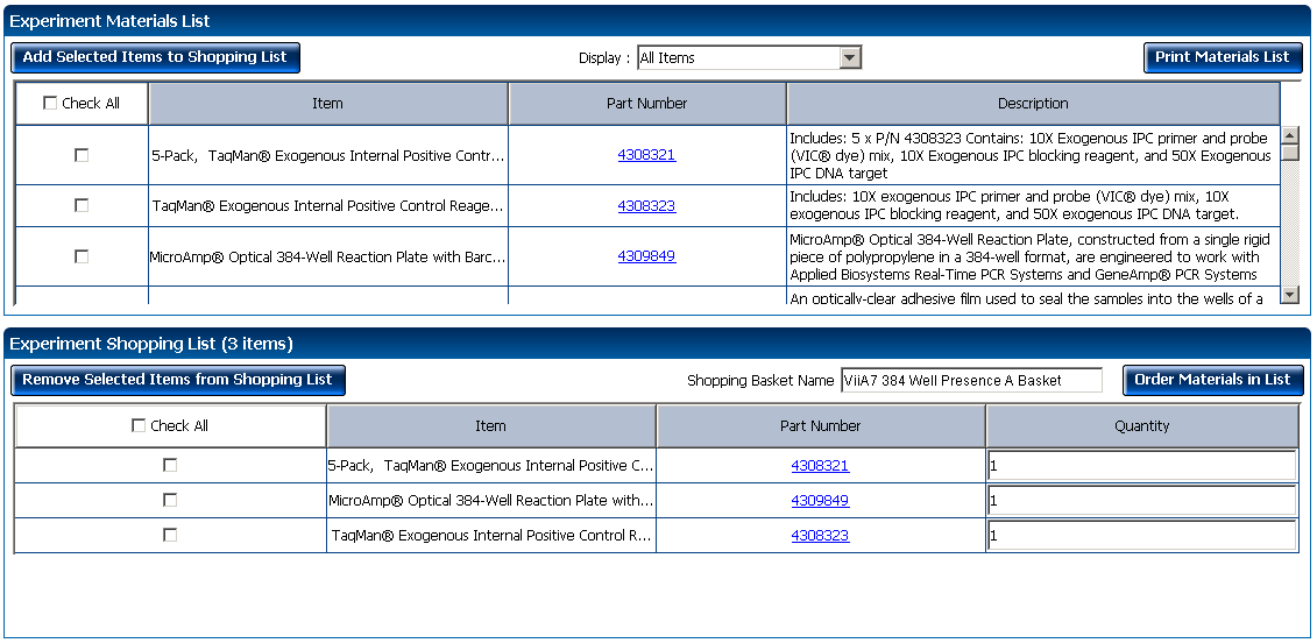


Order materials for the experiment

The recommended materials are:

- MicroAmp® Optical 384-Well Reaction Plate
- MicroAmp® Optical Adhesive Film
- TaqMan® Universal PCR Master Mix (2X)

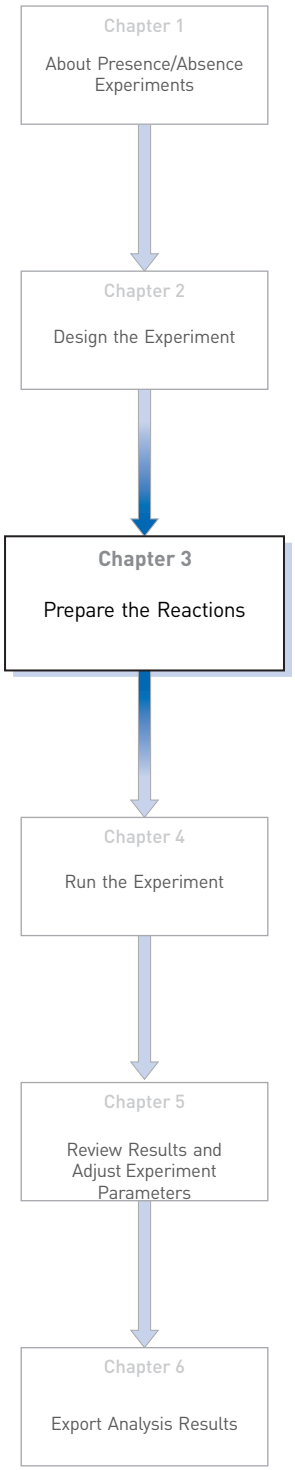
Your Materials list screen should look like this:



For more information

For more information on...	Refer to	Part number
Consumables	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments* Appendix A in Booklet 7, *ViiA™ 7 System Experiments - Appendixes*	4441434
Data collection	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*	127AP05-03
Using Alternative Setup	Chapter 2 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

3 Prepare the Reactions



This chapter explains how to prepare the PCR reactions for the Presence/Absence example experiment.

This chapter covers:

- Assemble required materials 16
- Prepare the reaction mix (“cocktail mix”). 16
- Prepare the reaction plate 17
- For more information. 19

Assemble required materials

- Items listed in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*
- Samples - DNA extracted from ground beef (100 ng/µL)
- Example experiment reaction mix components:
  - TaqMan® Universal PCR Master Mix
  - 10X IPC Mix
  - 50X IPC DNA
  - 20X Primer/ Probe Mix

Prepare the reaction mix ("cocktail mix")

For the Presence/ Absence example experiment, four cocktail mixes are used; one each for:

- Sample (+)
- Sample (-)
- NTC/ IPC+
- NAC/IPC-

The following tables list the universal assay conditions (volume and final concentration) for using the TaqMan® Universal PCR Master Mix for the four cocktail mixes.

Cocktail Mix	Reaction component	Volume for 1 reaction (µL)	Volume for 160 reactions (144 wells + 10% excess) (µL)
Cocktail Mix 1 for Sample (+)	TaqMan® Universal PCR Master Mix (2.0X)	12.50	2000.0
	10X IPC Mix	2.50	400.0
	50X IPC DNA	0.50	80.0
	20X Primer/ Probe Mix	1.25	200.0
	Water/ Buffer	5.75	920.0
	Diluted unknown 1	2.5	400
	Total reaction mix volume	25.0	4000.0
Cocktail Mix 2 for Sample (-)	TaqMan® Universal PCR Master Mix (2.0X)	12.50	2000.0
	10X IPC Mix	2.50	400.0
	50X IPC DNA	0.50	80.0
	20X Primer/ Probe Mix	1.25	200.0
	Water/ Buffer	5.75	920.0
	Diluted unknown 2	2.5	400

Cocktail Mix	Reaction component	Volume for 1 reaction (µL)	Volume for 160 reactions (144 wells + 10% excess) (µL)
Cocktail Mix 3 for NTC/ IPC+	TaqMan® Universal PCR Master Mix (2.0X)	12.50	662.5
	10X IPC Mix	2.50	132.5
	50X IPC DNA	0.50	26.5
	20X Primer/ Probe Mix	1.25	66.25
	Water/ Buffer	8.25	304.75
	Total reaction mix volume	25.0	1325.0
	TaqMan® Universal PCR Master Mix (2.0X)	12.50	662.5
Cocktail Mix 4 for NAC/ IPC-	TaqMan® Universal PCR Master Mix (2.0X)	12.50	662.5
	10X IPC Mix	2.50	132.5
	50X IPC DNA	0.50	26.5
	20X Primer/ Probe Mix	1.25	66.25
	IPC Block	2.5	132.5
	Water/ Buffer	5.75	304.75
	Total reaction mix volume	25.0	1325.0

To prepare the reaction mix for each of the four types:

1. Label four appropriately sized tubes for the reaction mixes:
   Sample (+), Sample (-), NTC, NAC.
2. Add the required volumes of each cocktail mix component to the tube.
3. Mix the cocktail thoroughly by gently pipetting up and down several times, then cap the tube.
4. Centrifuge the tube briefly to remove air bubbles.
5. Place the cocktail mix on ice until you prepare the reaction plate.

   Note: You can separately add the sample to the reaction plate, as opposed to preparing individual reaction mixes for each sample.

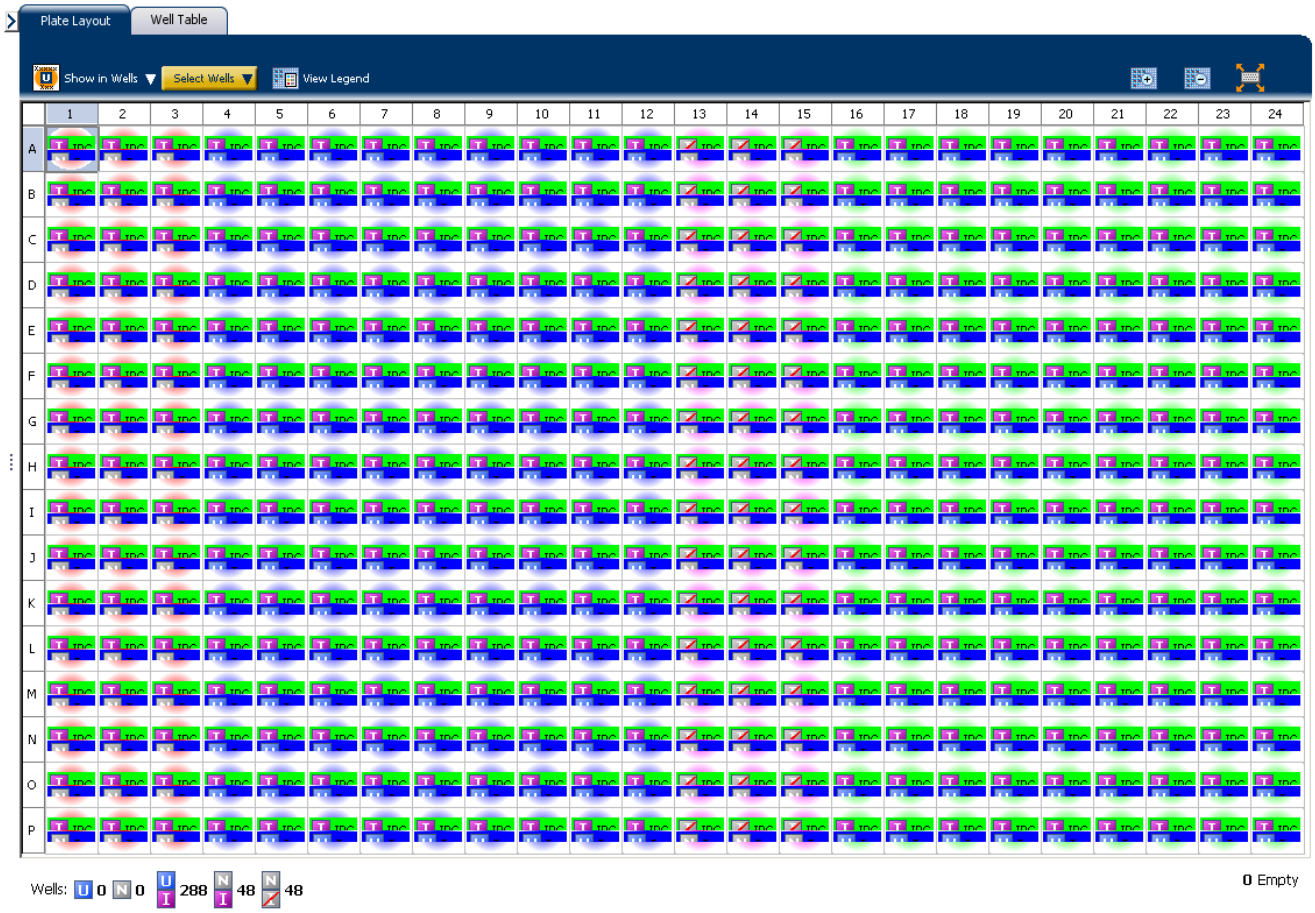
Prepare the reaction plate

The reaction plate for the Presence/Absence example experiment contains:

- A MicroAmp® Optical 384-Well Reaction Plate
- Reaction volume of 20 µL/well
- 144 Sample (+) wells
- 144 Sample (-) wells

- 48 NTC/ IPC+
- 48 NAC/IPC-

The plate layout looks like this:



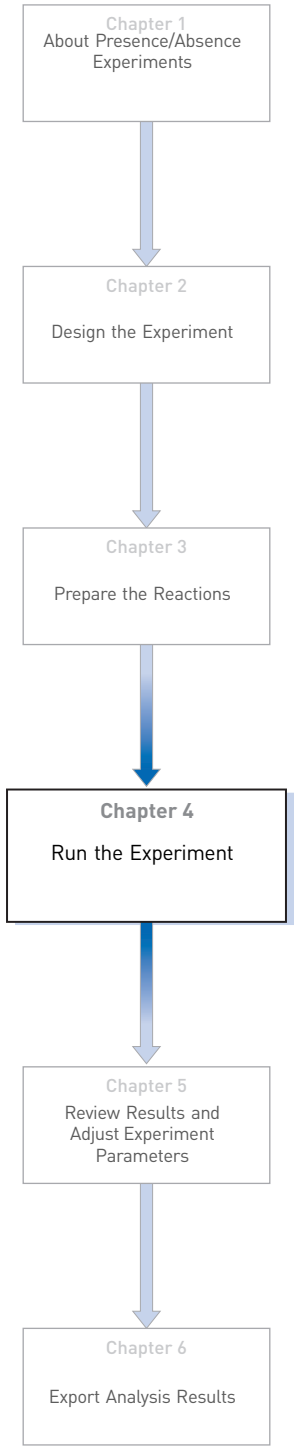
To prepare the reaction plate:

1. Add 25µL of Cocktail mix 1 to wells A16 - P24.
2. Add 25µL of Cocktail mix 2 to wells A4 - P12.
3. Add 25µL of Cocktail mix 3 to wells A13 - P15.
4. Add 25µL of Cocktail mix 4 to wells A1 - P3.
5. Seal the reaction plate with optical adhesive film.
6. Centrifuge the reaction plate briefly to remove air bubbles.
7. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.
8. Until you are ready to perform the run, place the reaction plate at 4 °C, in the dark.

For more information

For more information on...	Refer to...	Part number
Assigning the reaction plate components	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

4 Run the Experiment



This chapter explains how to run the example experiment on the ViiA™ 7 Instrument.

This chapter covers:

- Start the run 22
- Monitor the run. 22

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the ViiA™ 7 Instrument is in operation.

Start the run

1. Open the Presence/Absence example file that you created using instructions in Chapter 2.
2. Load the reaction plate into the instrument.
3. Start the run.

Monitor the run

Monitor the example experiment run:

- From the ViiA™ 7 Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the ViiA™ 7 Software (to monitor an experiment started from another computer or from the ViiA™ 7 Instrument touchscreen).
- From the ViiA™ 7 Instrument touchscreen.

From the Instrument Console of the ViiA™ 7 Software

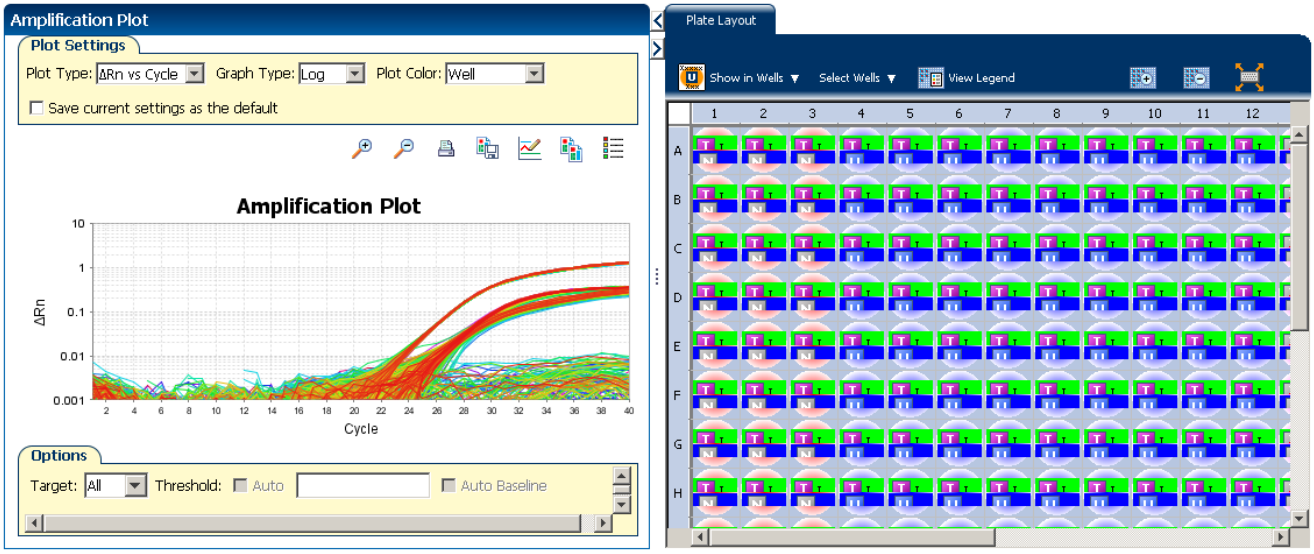
1. In the Instrument Console screen, select the instrument icon.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. On the Manage Instrument screen, click **Monitor Running Instrument** to access the Run screen.

View the Amplification Plot

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the ViiA™ 7 Software for potential problems.

Click **Amplification Plot** from the Run Experiment Menu, select the Plate Layout tab, then select the wells to view.

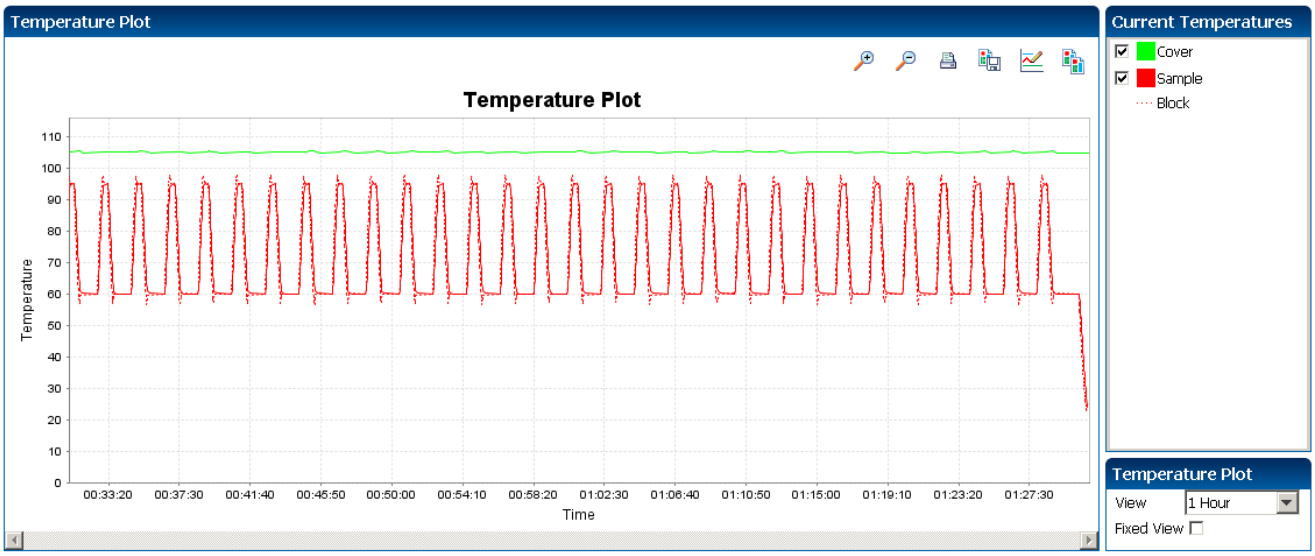
The figure below shows the Amplification Plot screen as it appears at the end of the example experiment.



View the Temperature Plot

Click **Temperature Plot** from the Run Experiment Menu.

The figure below shows the Temperature Plot screen as it appears during the example experiment.

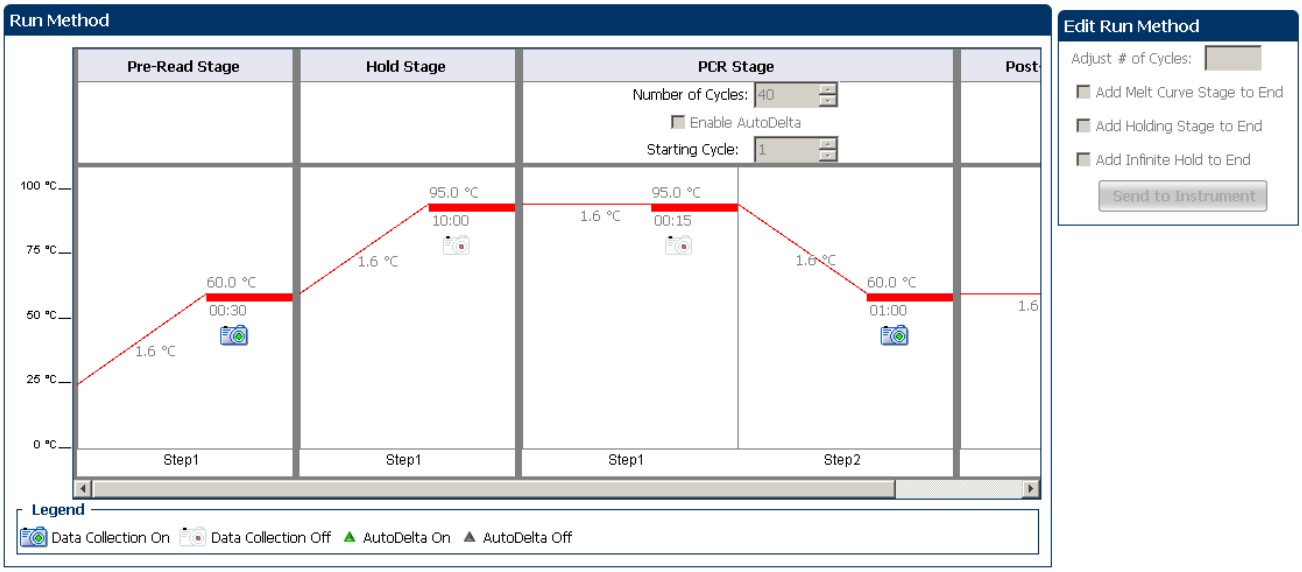


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

View the Run Method

Click **Run Method** from the Run Experiment Menu.

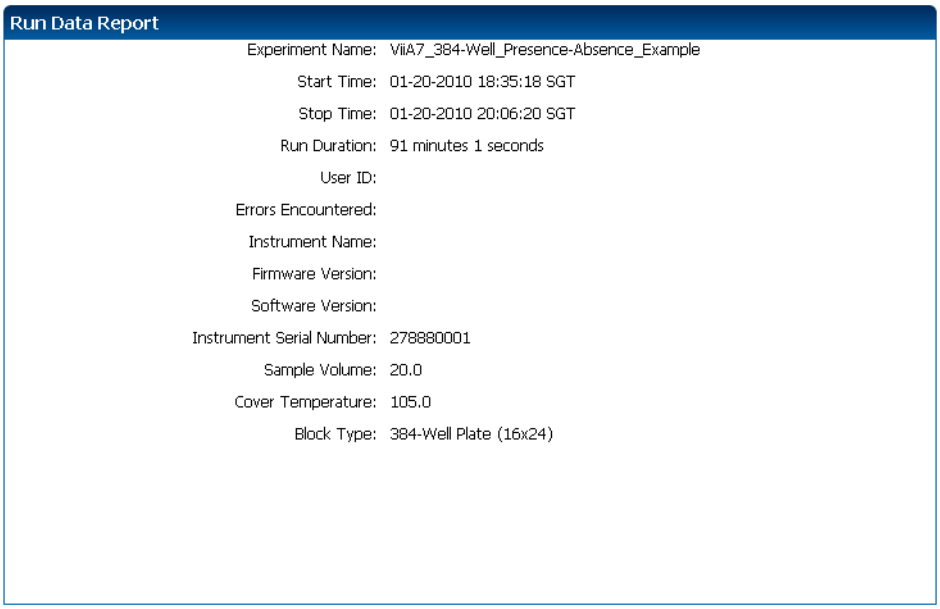
The figure below shows the Run Method screen as it appears in the example experiment.



View run data

Click **View Run Data** from the Run Experiment Menu.

The figure below shows the View Run Data screen as it appears in the example experiment.

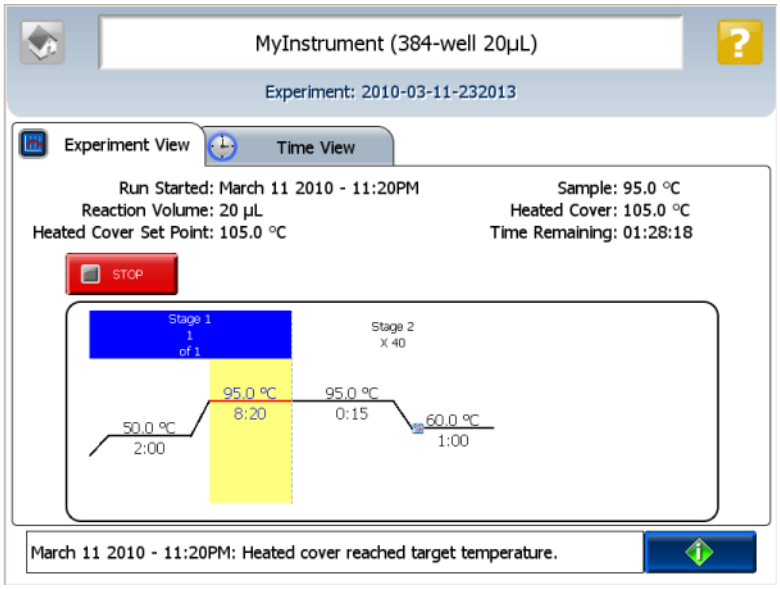


From the ViiA™ 7 Instrument touchscreen

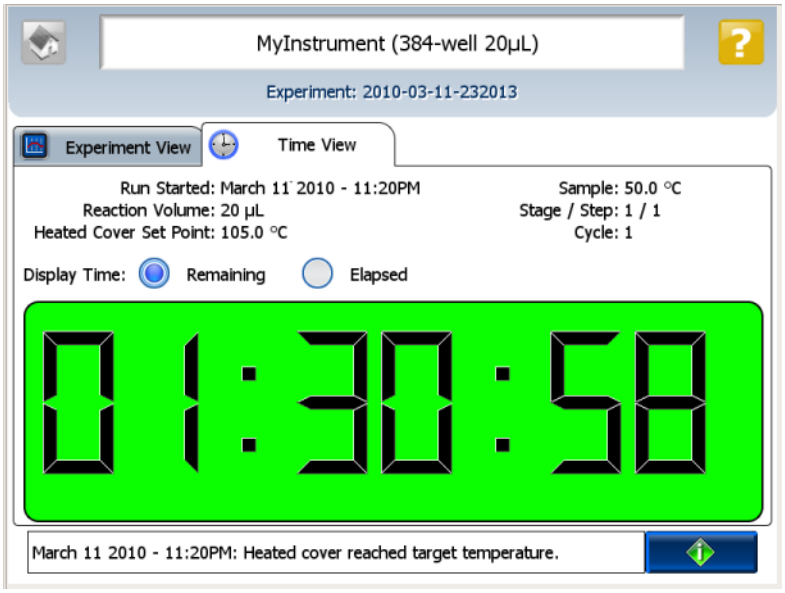
You can also view the progress of the run from the touchscreen of the ViiA™ 7 Instrument.

The Run Method screen on the **ViiA™ 7 Instrument** touchscreen looks like this:

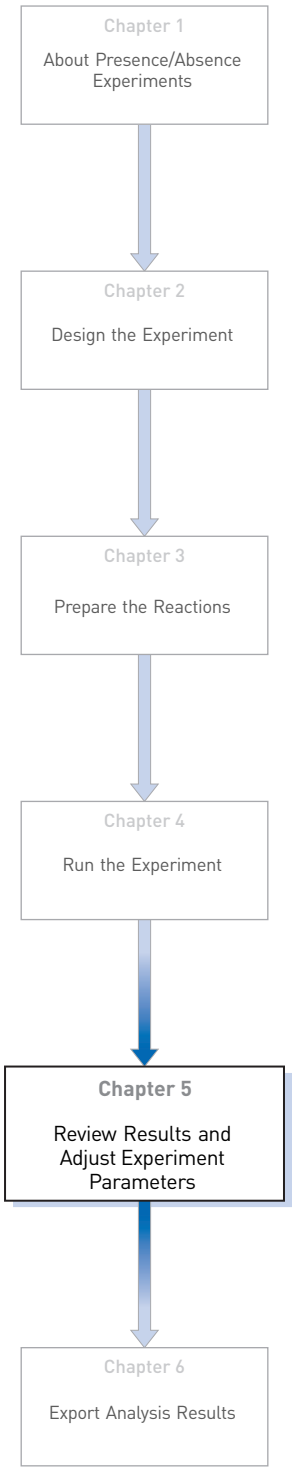
Experiment View



Time View



5 Review Results and Adjust Experiment Parameters



In Section 5.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 5.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

Section 5.1 Review Results 29

- Analyze the example experiment. 29
- View the Presence/Absence Plot 29
- Assess amplification results using the Amplification Plot. 31
- View the Well Table 35
- Confirm accurate dye signal using the Multicomponent Plot. 38
- Determine signal accuracy using the Raw Data Plot 41
- Review the flags in the QC Summary 43
- For more information. 45

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- Adjust analysis settings 47
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5.1

Section 5.1 Review Results

Analyze the example experiment

1. Open the Presence/Absence example experiment file that you ran in Chapter 4.
2. Click **Analyze**. The software analyzes the data using the default analysis settings.

   Note: You can also access the experiment to analyze from the Home screen.

View the Presence/Absence Plot

The Presence/Absence Plot displays the intensity of the fluorescence for each well position. There are four Presence/Absence plot views available:

- All Calls
- Presence calls only
- Absence calls only
- Unconfirmed calls

For each view you can choose to:

- Show IPC
- Show Controls

Purpose

The purpose of viewing the Presence/Absence Plot for the example experiment is to confirm that:

- The target is absent in samples NTC and Sample (-).
- The target is present in Sample (+).
- There are no unconfirmed wells.
- The IPC succeeded in all wells.
- There is no amplification in NAC wells.

To view and assess the Presence/Absence Plot

From the Experiment menu pane, select **Analysis ▸ Presence/Absence Plot.**

Note: If no data are displayed, click **Analyze**.

1. Display all 384 wells in the Presence/Absence Plot screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.

2. Enter the Plot Settings:

Menu	Selection
Target Reporter	TGFB
Control Reporter	IPC
Show Calls	All Calls
(This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.)	Check (default)

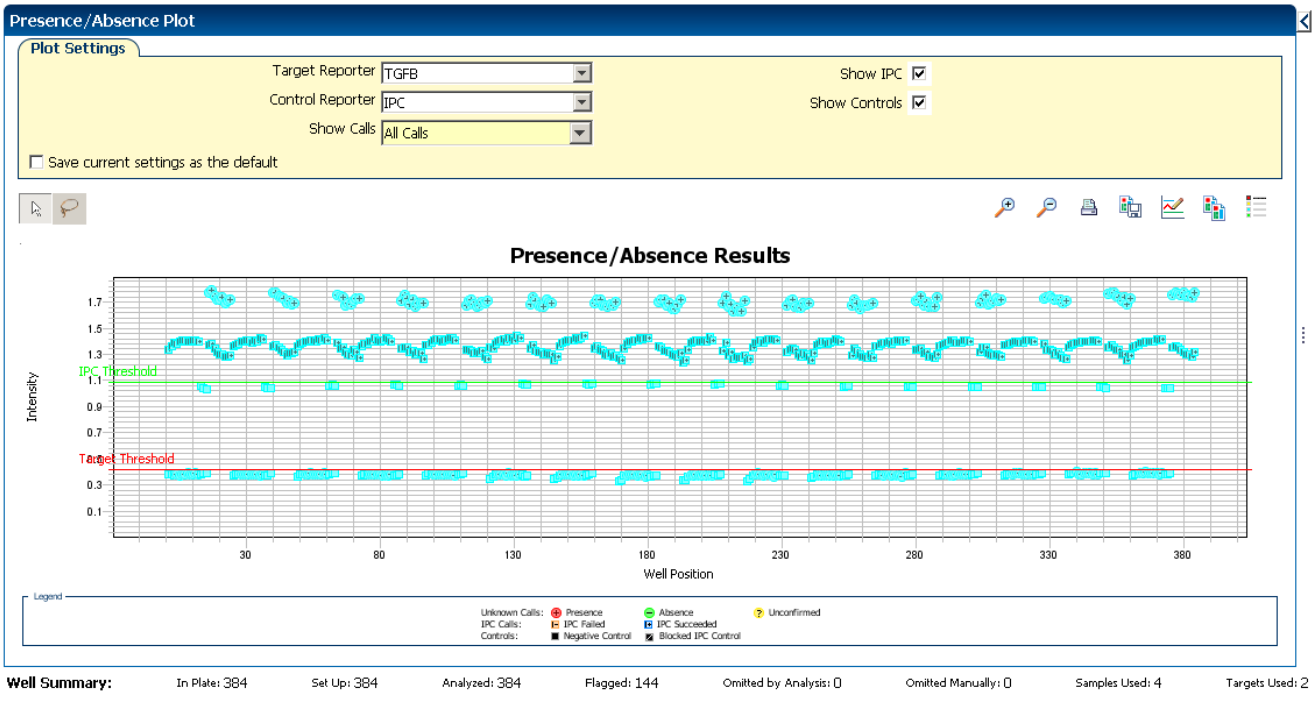
3. Click the **Show IPC** check box to view the fluorescence intensity of the IPC target in the Unknown-IPC wells.

4. Click the **Show Controls** check box to view the fluorescence intensity of the IPC target in the Negative Control-IPC wells and the Negative Control-Blocked IPC wells.

5. To view the fluorescence intensity of:
   - Presence calls– select **Presence** from the Show Calls drop-down menu.
   - Absence calls– select **Absence** from the Show Calls drop-down menu.
   - Unconfirmed calls– select **Unconfirmed** from the Show Calls drop-down menu.

     Note: The Presence/Absence example experiment does not contain any unconfirmed calls.

The Presence/Absence Plot for the example experiment looks like this:



Tips for viewing presence/absence plots in your own experiments

- The **IPC threshold** is calculated from the Negative Control- Blocked IPC reactions.
- **The Target Threshold** is calculated from the Negative Control- IPC reactions. If the target's intensity is:
  - Above the target threshold, the call is present (regardless of the intensity of the IPC).
  - Below the target threshold, and the IPC's intensity is above the IPC threshold, the call is absent.
  - Below the target threshold, and the IPC's intensity is below the IPC threshold, the call is unconfirmed.
- T**arget Calls**:
  - Presence
  - Absence
  - Unconfirmed
- **IPC Calls**:
  - IPC Succeeded
  - IPC Failed
- **Control Well Calls**:
  - Negative Control - IPC
  - Negative Control - Blocked IPC

Assess amplification results using the Amplification Plot

IMPORTANT! Amplification plots are not used to make presence/absence calls. Examine the plots to help with troubleshooting and quality control.

Amplification plots available for viewing

The Amplification Plot displays amplification of all samples in the selected wells. There are three amplification plot views available:

- **ΔRn vs Cycle** – ΔRn is the difference in normalized fluorescence signal generated by the reporter between the pre-PCR read and the post-PCR read. This plot displays ΔRn as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle** – Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays Rn as a function of cycle number. You can use this plot to identify and examine irregular amplification.
- **C_T vs Well** – C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. You can use this plot to locate outlying amplification (outliers).

Each plot can be viewed as a linear or log10 graph type.

Purpose

The purpose of viewing the amplification plot for the example experiment is to review the target to identify:

- Correct baseline and threshold values
- Irregular amplification
- Outliers

View the Amplification Plot

1. From the Experiment menu pane, select **Analysis ▸ Amplification Plot.**

   Note: If no data are displayed, click **Analyze**.

2. Display all 384 wells in the amplification plot by clicking the upper left corner of the plate layout in the Plate Layout tab.

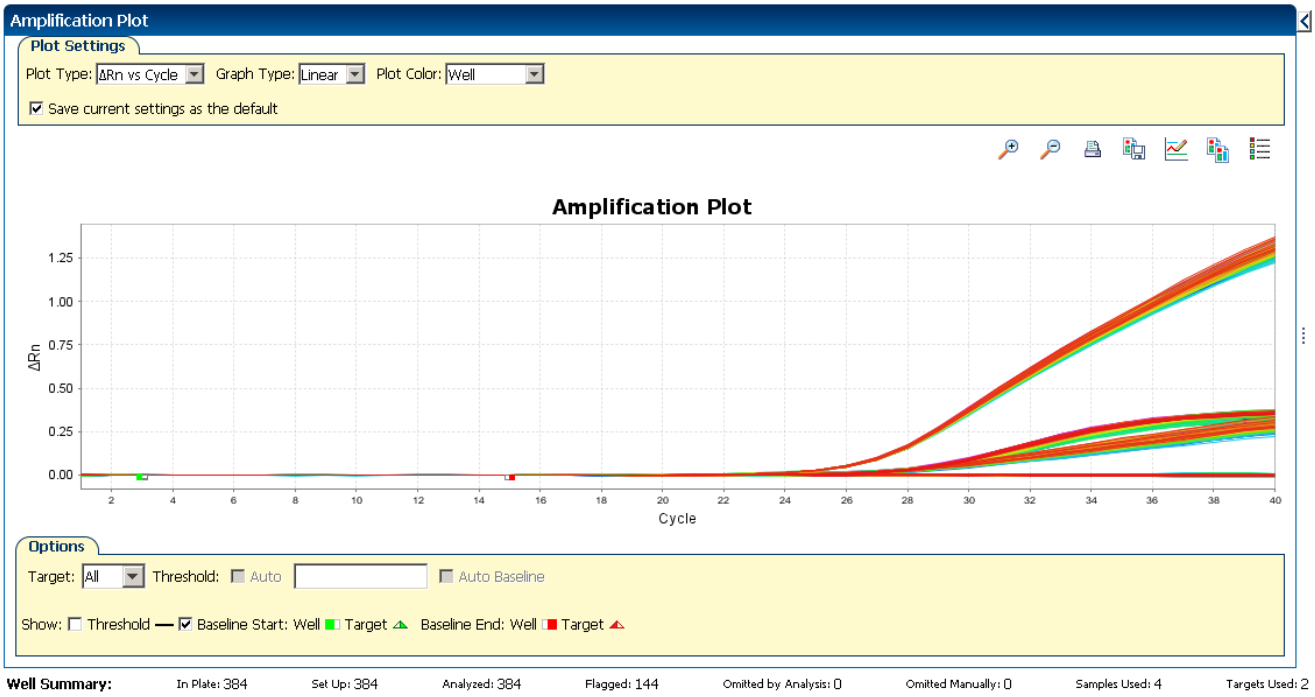
3. Expand the Plate Layout tab by clicking the left facing arrow that is left of the tab.

4. In the Amplification Plot screen, enter:

Menu	Selection
Plot Type	ΔRn vs Cycle
Plot Color	Well
(This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.)	Check (default)

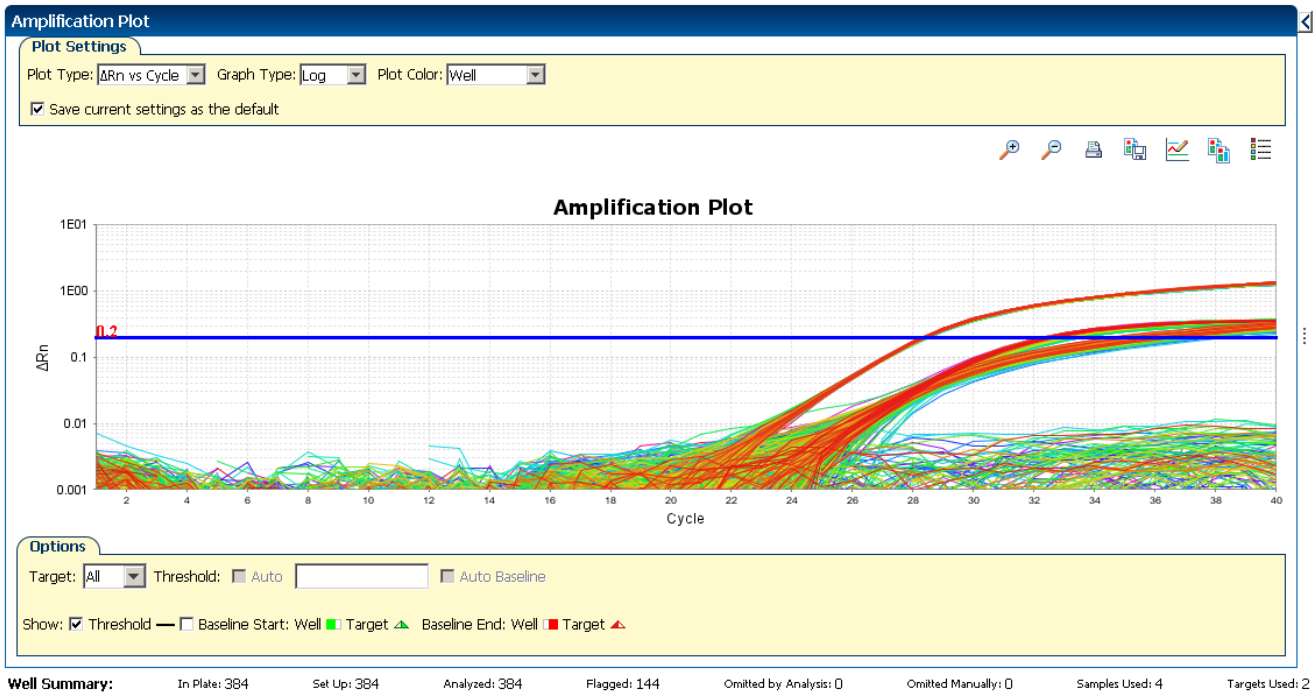
5. View the baseline values:
   a. From the Graph Type drop-down menu, select **Linear**.
   b. Select the **Baseline** check box to show the start cycle and end cycle.
   c. Verify that the baseline is set correctly: The end cycle should be set a few cycles before the cycle number where significant fluorescent signal is detected. In the example experiment, the baseline is set correctly.

Your screen should look like this:



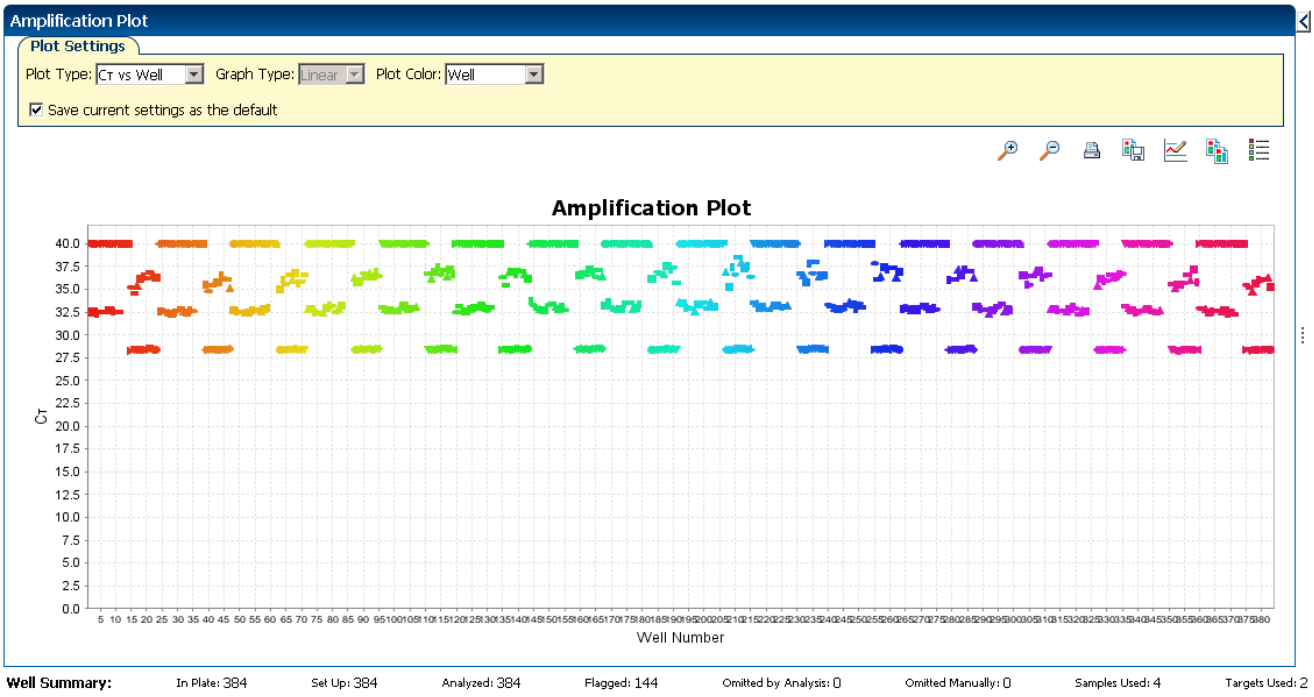
6. View the threshold values:
   a. From the Graph Type drop-down menu, select **Log**.
   b. Select the **Threshold** check box to show the threshold.
   c. Verify that the threshold is set correctly.

Your screen should look like this:



7. Locate any outliers:
   a. From the Plot Type drop-down menu, select **C_T vs Well**.
   b. Look for outliers from the amplification plot. In the example experiment, there are no outliers for IPC.

Your screen should look like this:

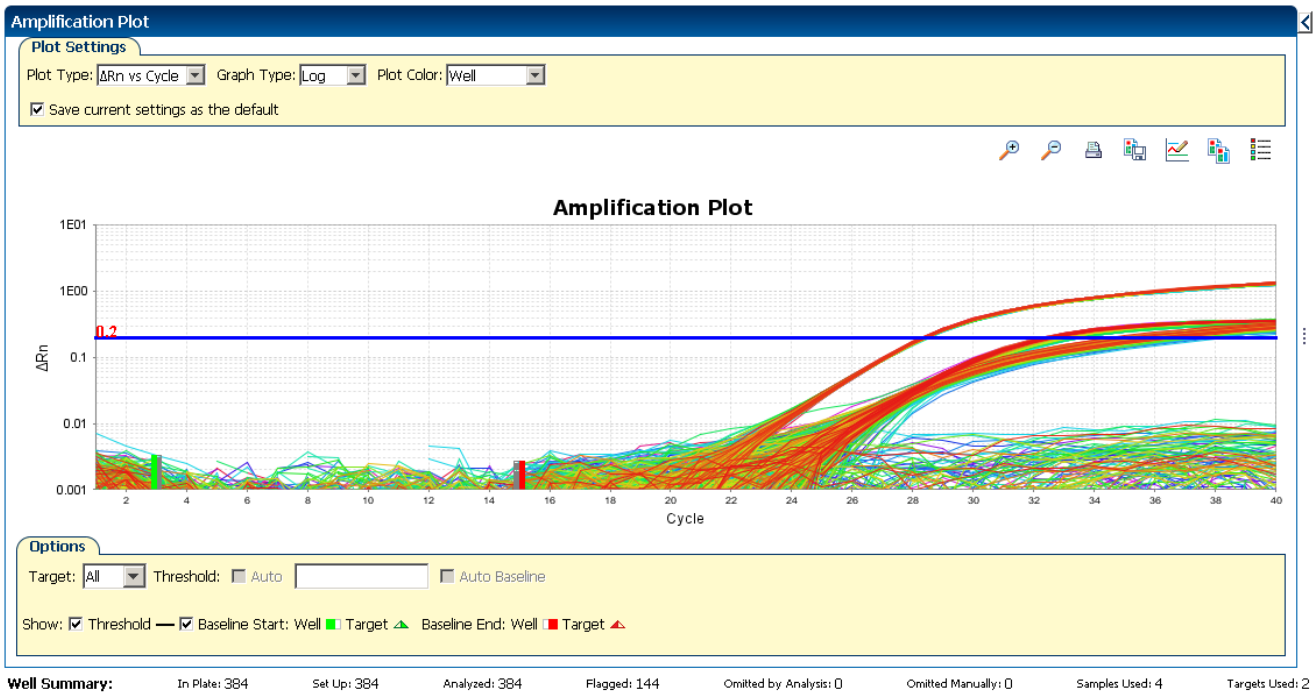


Tips for viewing amplification plots in your own experiments

When you analyze your own Presence/ Absence experiment, look for:

- **Outliers**
- **A typical amplification plot** – The ViiA™ 7 Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear phase
  - Exponential (geometric phase)
  - Baseline

A typical amplification plot should look like this:



IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the ViiA™ 7 Software. Applied Biosystems recommends that you examine the Amplification Plot screen and review the assigned baseline and threshold values for each well after analysis.

View the Well Table

The well table displays results data for each well in the reaction plate, including:

- The well number, sample name, target name, task, and dyes
- The calculated values: ΔRn, ΔRn mean, and ΔRn SD

  Note: ΔRn, ΔRn mean, and ΔRn SD are calculated only when the analysis call settings specify to analyze data from the pre-PCR read and the post-PCR read.

- Target and IPC thresholds, Call, Comments
- Flags

Purpose

In the Presence/Absence example experiment, you review the well table for:

- Call
- ΔRn
- Flag

To view the Well Table

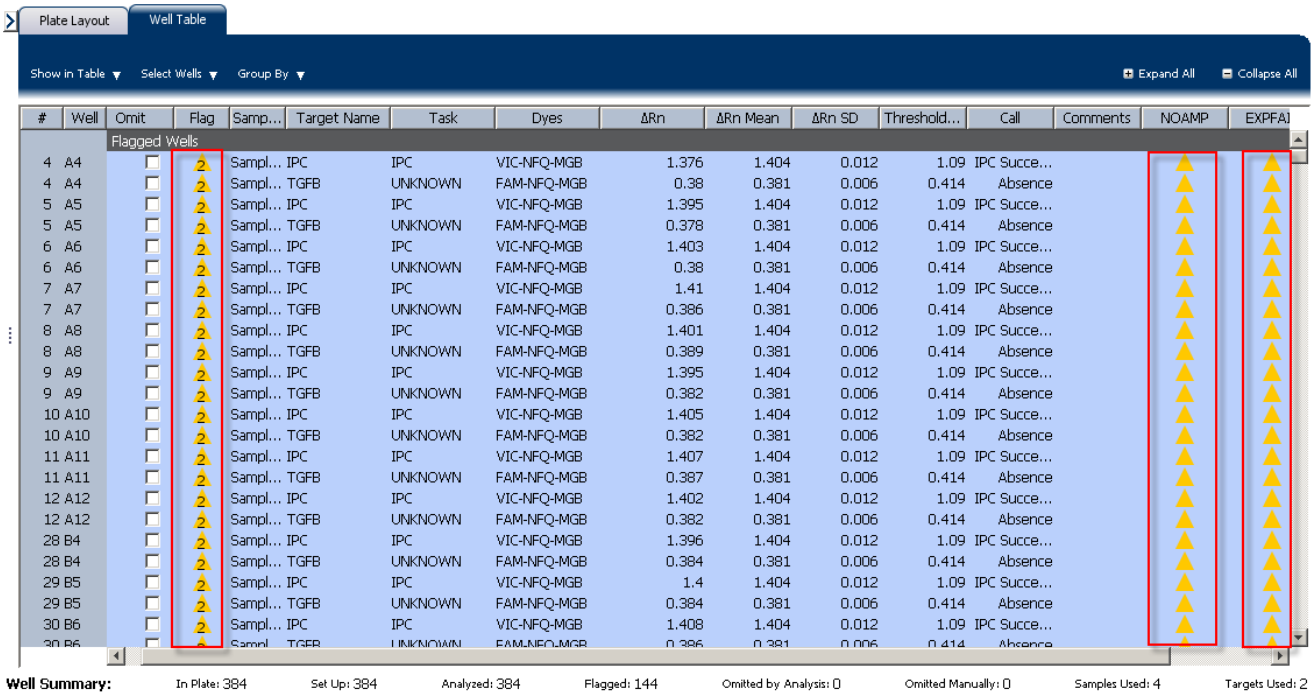
1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**, then select the **Well Table** tab.

   Note: If no data are displayed, click **Analyze**.

2. Use the Group By drop-down menu to group wells by a specific category. For the example experiment, group the wells by flag, call, and ΔRn value.

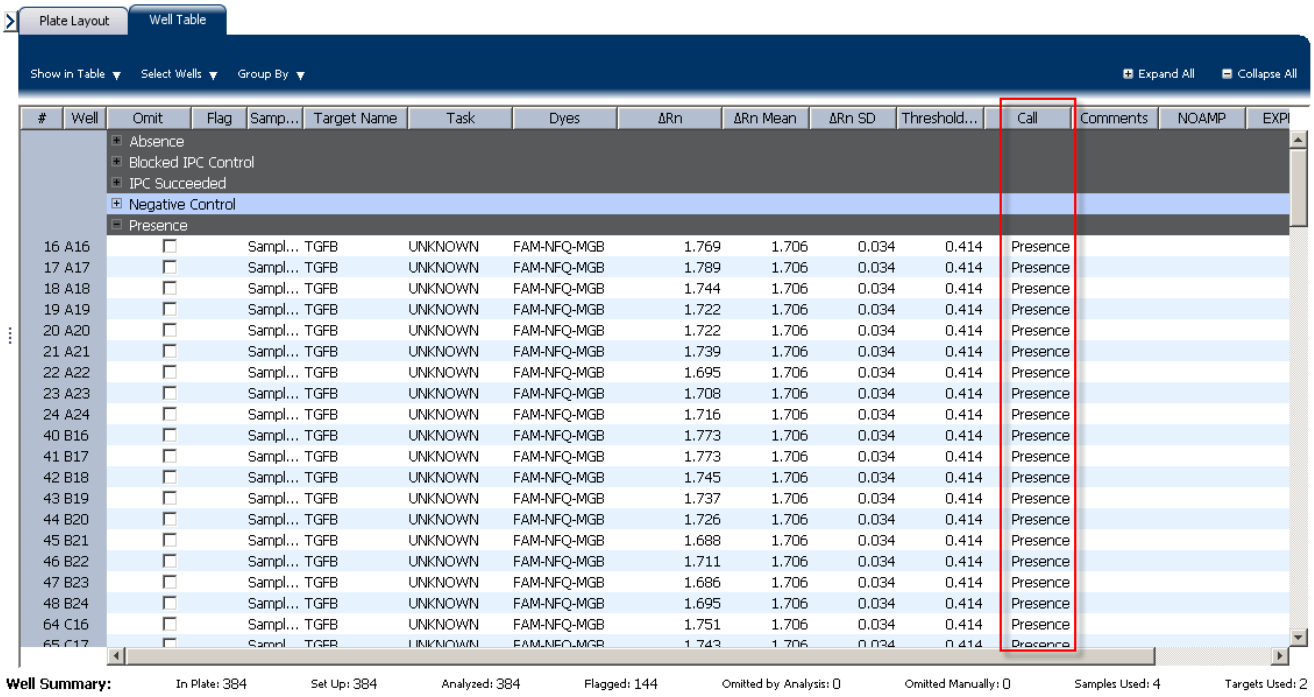
   Note: You can select only one category at a time.

   a. From the Group By drop-down menu, select **Flag**:
      - 144 wells are listed under Flagged.
      - 240 wells are listed under Unflagged.

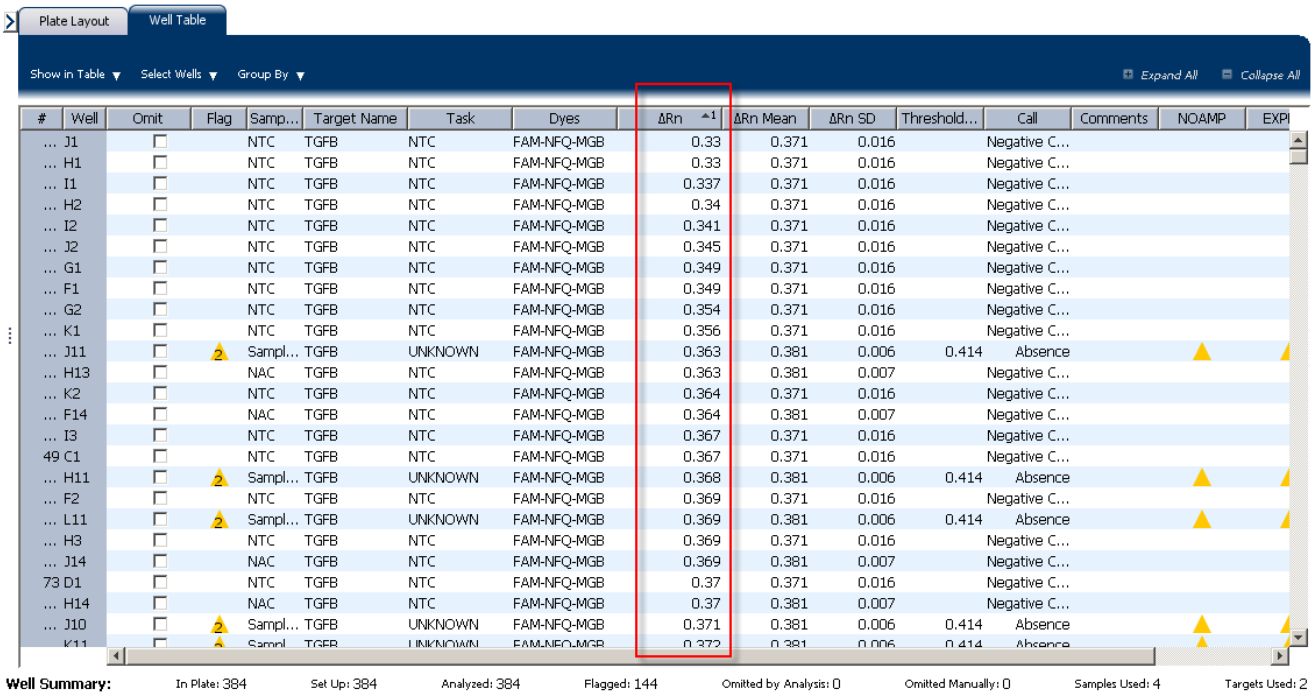


   b. From the Group By drop-down menu, select **Call**. Wells are listed in the order:
      - Absence
      - Blocked IPC Control
      - IPC Succeeded

- Negative Control
- Presence



c. From the Group By drop-down menu, select **None**. In the table, click the column heading **ΔRn**. Wells are listed in order of increasing ΔRn. Click the column heading again to reverse the sort order.



Tips for analyzing your own experiments

When you analyze your own Presence/Absence experiment, group the wells by:

- **Flag** –The software groups the flagged and unflagged wells. A flag indicates that the software has found an error in the flagged well. For a description of the ViiA™ 7 Software flags, see "Review the flags in the QC Summary" on page 43.
- **Call** – The software groups the wells by call: Negative Control, Blocked-IPC, Presence, Absence, Unconfirmed, IPC Succeeded, and IPC Failed.

Confirm accurate dye signal using the Multicomponent Plot

The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the Presence/Absence example experiment, you review the Multicomponent Plot screen for:

- ROX™ dye (passive reference)
- FAM™ dye (reporter)
- VIC® dye (reporter)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

View the Multicomponent Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Multicomponent Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the wells **one at a time** in the Multicomponent Plot screen:
   a. Click the **Plate Layout** tab.
   b. Select one well in the plate layout; the well is shown in the Multicomponent Plot screen.

      Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

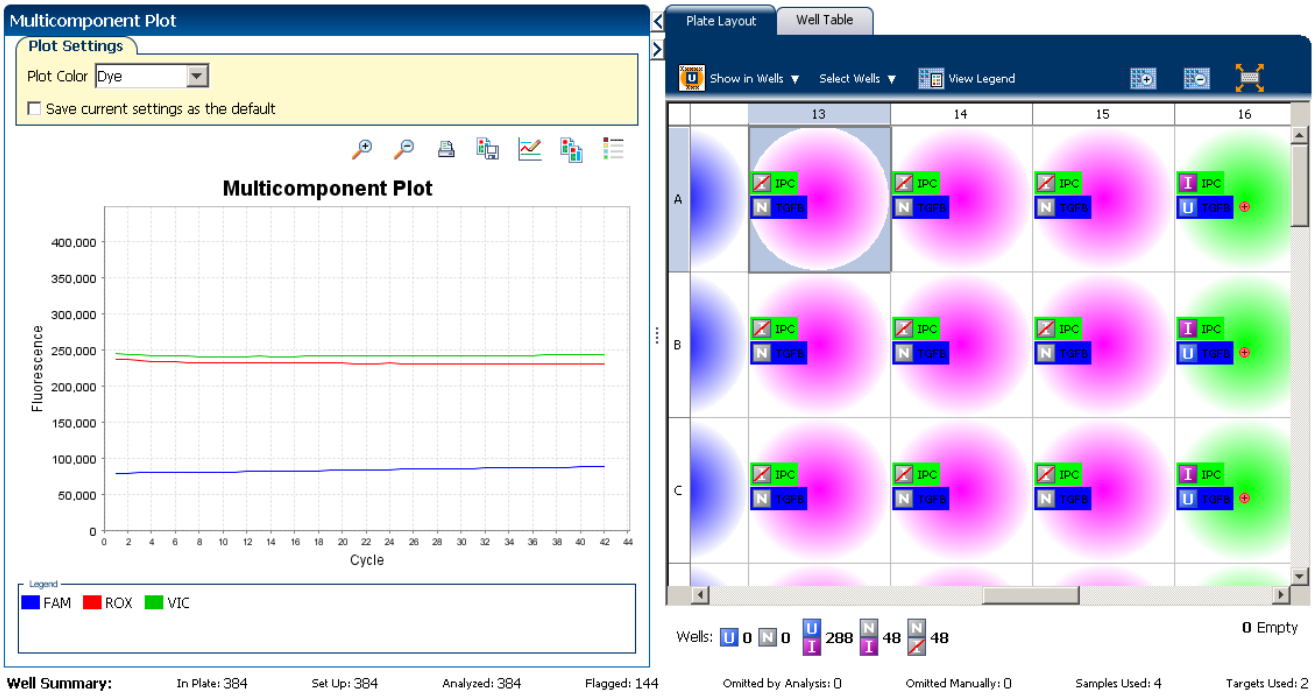
3. From the Plot Color drop-down menu, select **Dye**.

4. Click **Show a legend for the plot** (default).

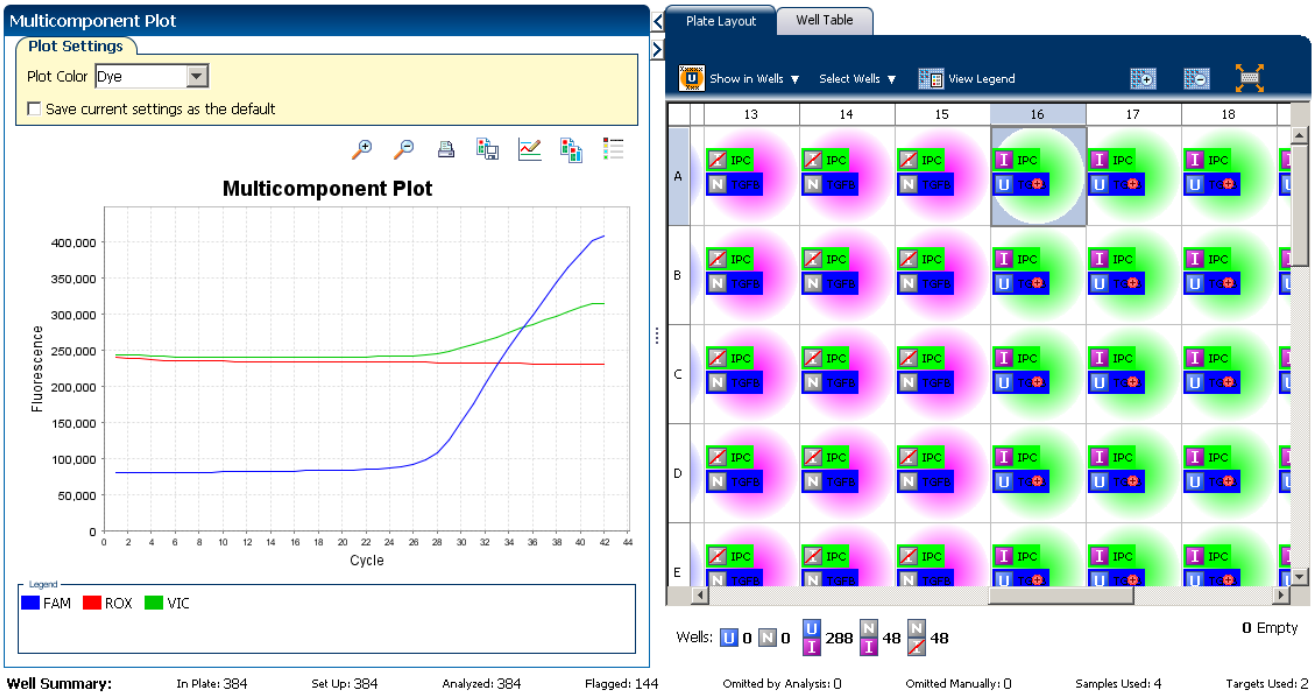
   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

5. Check the ROX dye signal. In the example experiment, the ROX dye signal remains constant throughout the PCR process; a constant ROX dye signal indicates typical data.

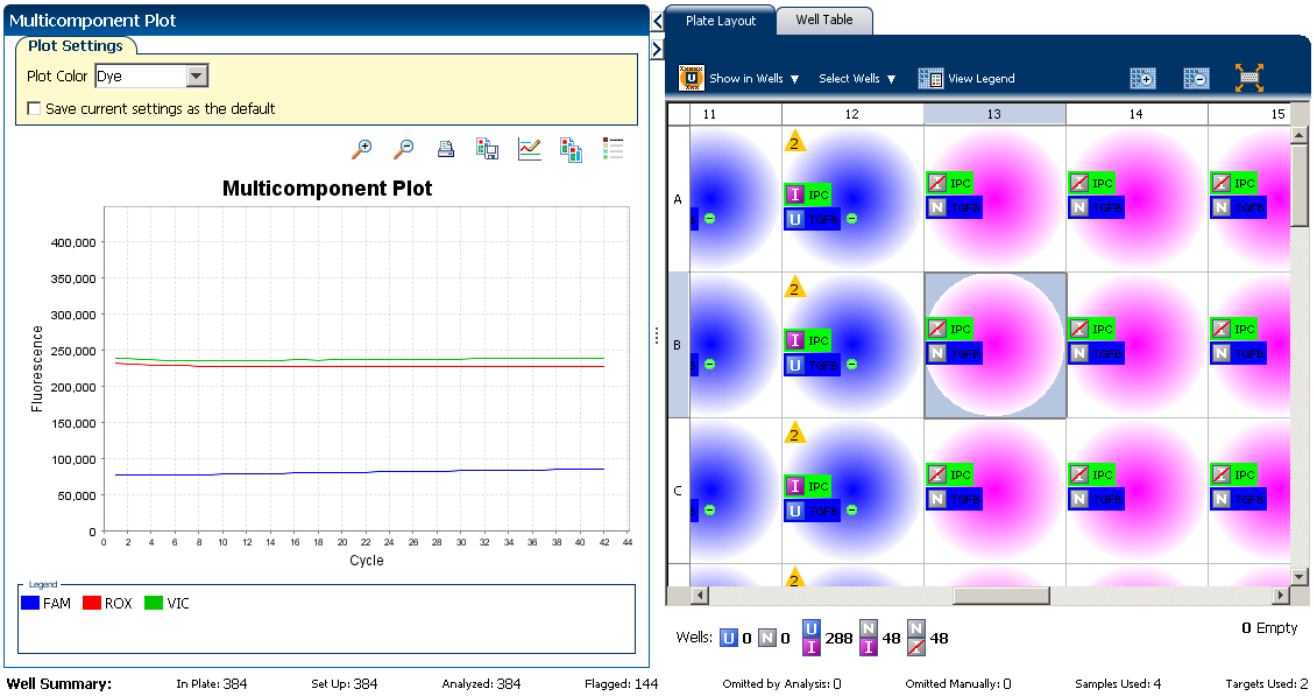
6. Check the VIC dye signal. In the example experiment the VIC dye signal should not amplify for NC-Blocked IPC wells or if the IPC call for the Unknown-IPC well is IPC Failed.



7. Check the FAM dye signal. In the example experiment, for the Sample (+), the FAM dye signal increases throughout the PCR process; increase in FAM dye signal indicates normal amplification.



8. Select the negative control (NAC) wells one at time and check for amplification. In the example experiment, there is no amplification in the negative control wells.



Tips for confirming dye accuracy in your own experiment

When you analyze your own Presence/Absence experiment, look for:

- **Passive reference (ROX)**– The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye (FAM)** – The reporter dye fluorescence level should display a flat region corresponding to the baseline. If target is present in the sample (a Presence call is made), the baseline will be followed by a rapid rise in fluorescence as the amplification proceeds.
- **Irregularities in the signal** – There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative control wells** – There should not be any amplification in the negative control wells.

Determine signal accuracy using the Raw Data Plot

The Raw Data Plot screen displays the raw fluorescence signal (not normalized) for each optical filter for the selected wells during each cycle of the real-time PCR.

Purpose

In the Presence/Absence example experiment, review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the Raw Data Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Raw Data Plot**.

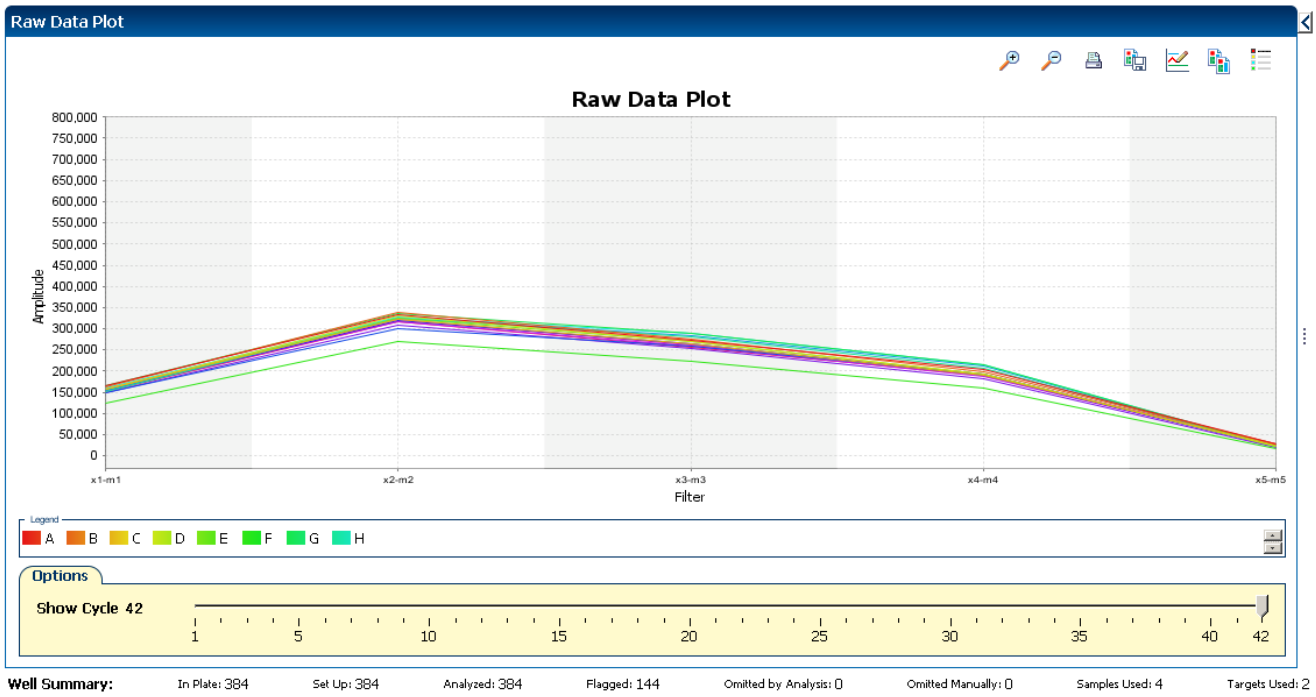
   Note: If no data are displayed, click **Analyze**.

2. Click **Show a legend for the plot** (default). The legend displays the color code for each row of the reaction plate (see the legend in the Raw Data Plot shown below).

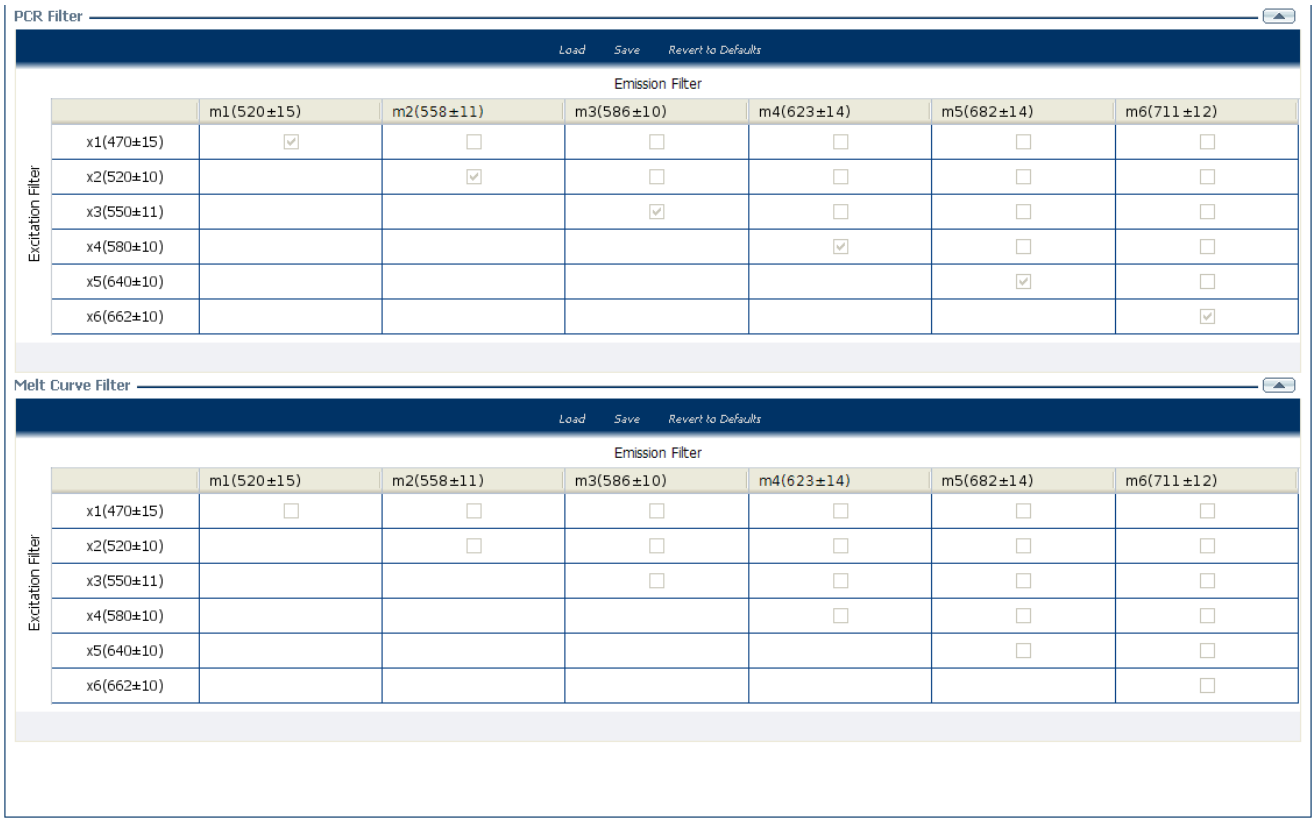
3. Display all 384 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the Plate Layout tab.

4. Select wells corresponding to a replicate group:
   - Sample (-) wells: From the Select Wells with drop-down menus, select Sample (-).
   - Sample (+) wells: From the Select Wells with drop-down menus, select Sample (+).
   - Negative control-IPC wells: Select wells A1 - P1, A2 - P2, and A3 - P3.
   - Negative control-blocked IPC wells: Select wells A13 - P13, A14 -P14, and A15 - P15.

5. Click and drag the Show Cycle pointer from cycle 1 to cycle 42. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.



The filters used for the example experiment are:



Tips for determining signal accuracy in your own experiments

When you analyze your own Presence/ Absence experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

Review the flags in the QC Summary

The QC Summary screen displays a list of the ViiA™ 7 Software flags, including the flag frequency and location for the open experiment. In the example experiment, 144 flags have been triggered.

Note: The flags triggered in the example experiment are seen in the Sample (-) wells. The flags, NOAMP and EXPFAIL indicate that the wells containing the Sample (-) did not amplify and that the software could not identify the exponential region of the amplification plot (as amplification did not take place). The occurrence of these flags in the example experiment is valid because it indicates the absence of the target in the sample.

View the QC Summary

1. From the Experiment Menu pane, select **Analysis ▸ QC Summary**.

   Note: If no data are displayed, click **Analyze**.

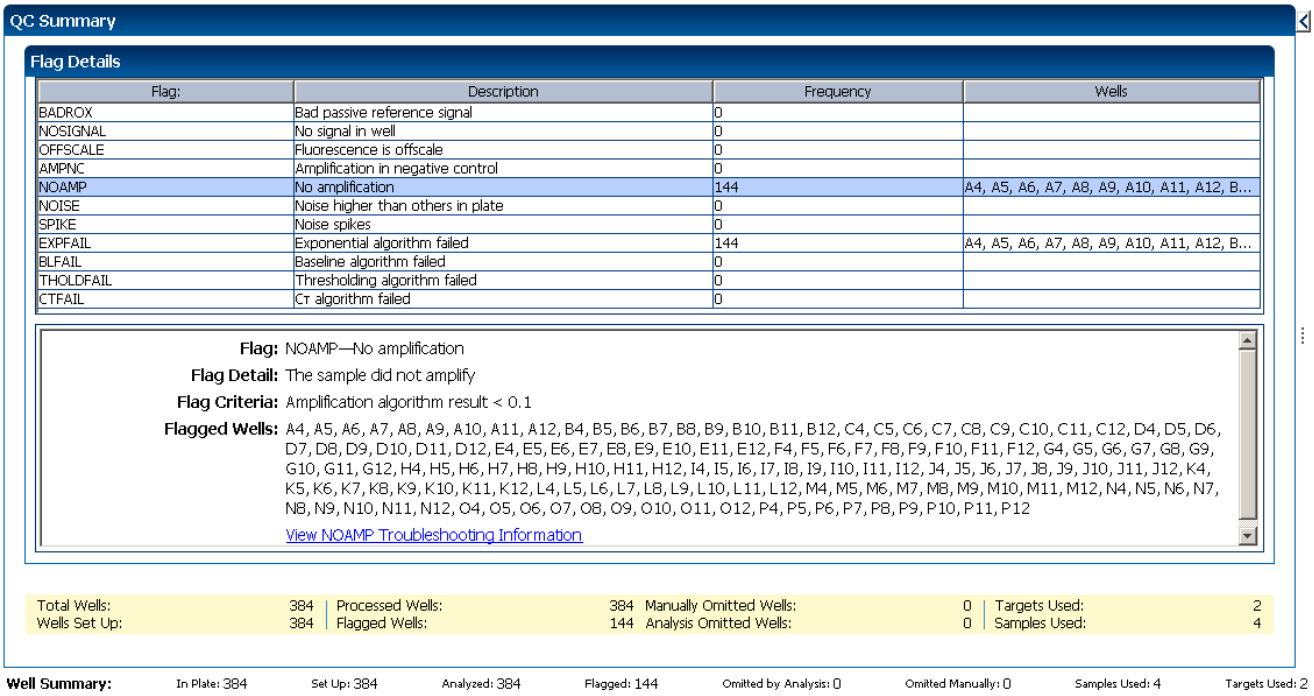
2. Review the Flags Summary

   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment. If the frequency is >0, the flag appears somewhere in the experiment; the well position is listed in the Wells column.

   In the example experiment, there are 144 flagged wells.

3. In the Flag Details table, click each flag with a frequency >0 to display detailed information about the flag. In the example experiment:
   - The NOAMP flag appears 144 times, in the wells A4 - P4, A5 - P5, A6 - P6, A7 - P7, A8 - P8, A9 - P9, A10 - P10, A11 - P11, and A12 - P12.
   - The EXPFAIL flag appears 144 times, in the same wells as the NOAMP flag, that is, A4 - P4, A5 - P5, A6 - P6, A7 - P7, A8 - P8, A9 - P9, A10 - P10, A11 - P11, and A12 - P12.

4. *(Optional)* For those flags with frequency >0, click the troubleshooting link to view information on correcting the flag.



Possible flags

For Presence/Absence experiments, the flags listed below may be triggered by the experiment data.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed

Flag	Description
Secondary analysis flags	
AMPNC	Amplification in negative control

Note: If the experiment does not include amplification, then the only flags are BADROX, NOSIGNAL, and OFFSCALE.

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the Frequency column for each flag).

For more information

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

5.2 Section 5.2 Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

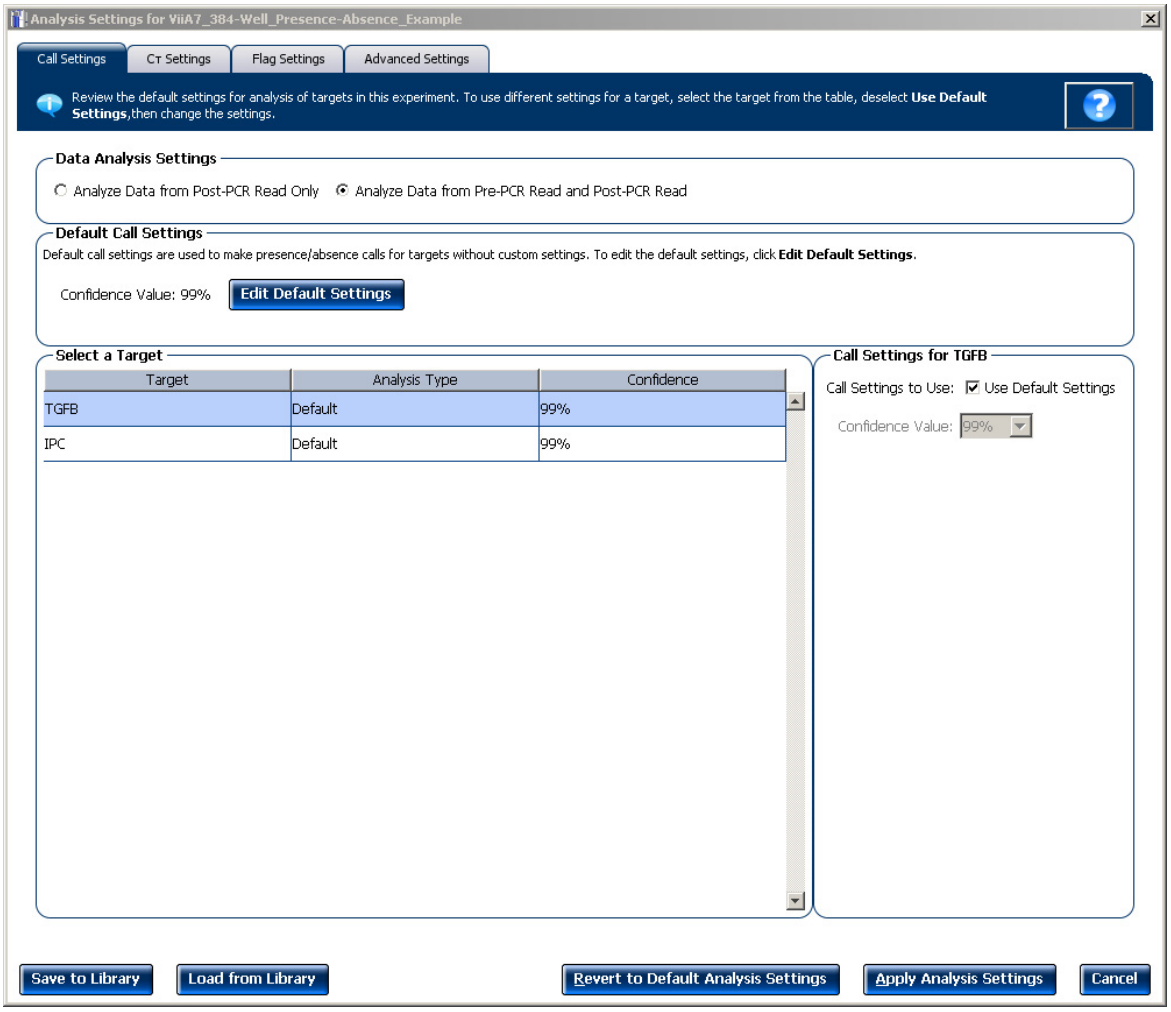
The Analysis Settings dialog box displays the analysis settings for the call, threshold cycle (C_T), flags, and advanced options.

If the default analysis settings in the ViiA™ 7 Software are not suitable for your own experiment, you can change the settings in the Analysis Settings dialog box, then reanalyze your experiment.

View the analysis settings

1. From the Experiment Menu pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the Analysis Settings dialog box.
   In the example experiment, the default analysis settings are used for each tab:
   - Call Settings
   - C_T Settings
   - Flag Settings
   - Advanced Settings

The Analysis Settings dialog box for a Presence/Absence experiment looks like this:



3. View and, if necessary, change the analysis settings (see "Adjust analysis settings" below).

   Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see About the Analysis Settings Library in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*.

4. Click **Apply Analysis Settings** to apply the current analysis settings.

   Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

Call Settings

Use the Call Settings tab to:

- Change the default data analysis settings. You can select from:
  - Analyze data from Post-PCR Read only
  - Analyze data from Pre-PCR Read and Post-PCR Read
- Edit the default call settings.
  - Click **Edit Default Settings**, then select the confidence value to use to make presence/absence calls. If the confidence value is less than the call setting, the call is unconfirmed.
  - Click **Save Changes**.
- Use custom call settings for a target.
  - Select one or more targets in the table, then deselect the **Default Settings** checkbox.
  - Select the confidence value to use to make presence/absence calls for the selected target(s).

C_T Settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method.
- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

  The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for gene quantification.

  The Relative Threshold algorithm lets you compare the data on a per-well or per-target basis. This setting is ideal for analyzing a single gene across samples or a single sample across genes with no dependence on targets, thereby reducing variability. Any settings for baseline or threshold do not affect the analysis when you use the Relative Threshold algorithm.
- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the targets that do not have custom settings. To edit the default settings, click **Edit Default Settings**.
- **C_T Settings for Target**

  When you manually set the threshold and baseline, Applied Biosystems recommends:

Setting	Recommendation
Threshold	Enter a value for the threshold so that the threshold is: • Above the background. • Below the plateau and linear regions of the amplification curve. • Within the exponential phase of the amplification curve.
Baseline	Select the Start Cycle and End Cycle values so that the baseline ends before significant fluorescent signal is detected.

Note: This setting is applicable only to the Baseline Threshold algorithm.

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag Settings

Use the Flag Settings tab to:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the ViiA™ 7 Software.

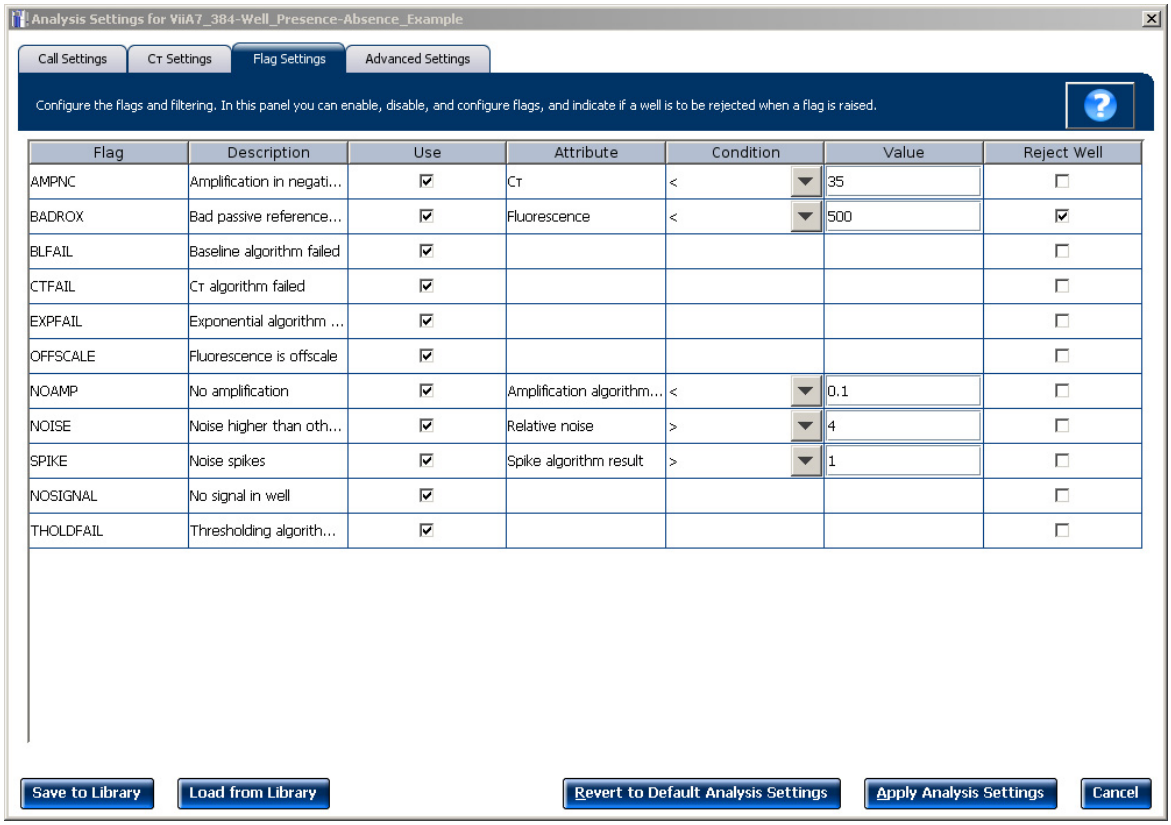
To adjust the flag settings:

1. In the Use column, select the check boxes for flags to apply during analysis.
2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.
3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations may change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.
4. Click **Apply Analysis Settings** in the Analysis Settings dialog box. If the run status is complete, the data are reanalyzed.

The Flag Settings tab looks like this:



Advanced Settings

Use the Advanced Settings tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold setting.

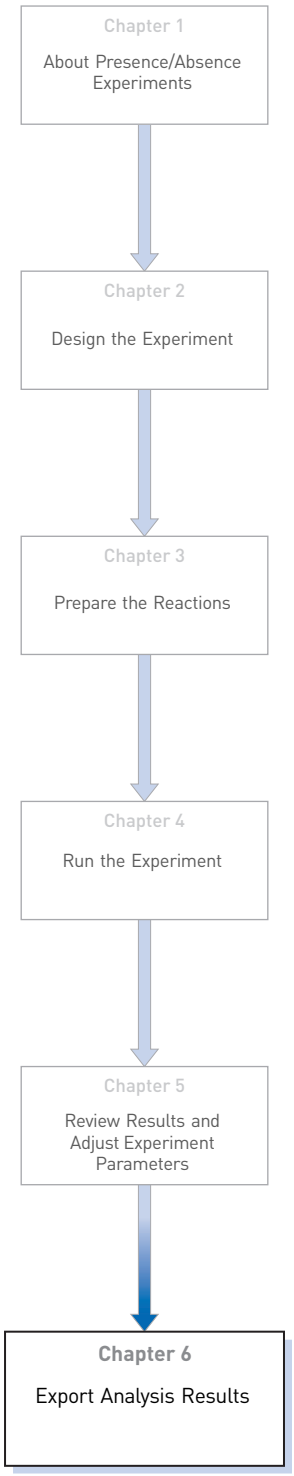
To use custom baseline settings for a well-target combination:

1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** check box.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, then enter the baseline start cycle and baseline end cycle.

For more information

For more information on...	Refer to	Part number
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*.	127AP05-03

6 Export Analysis Results

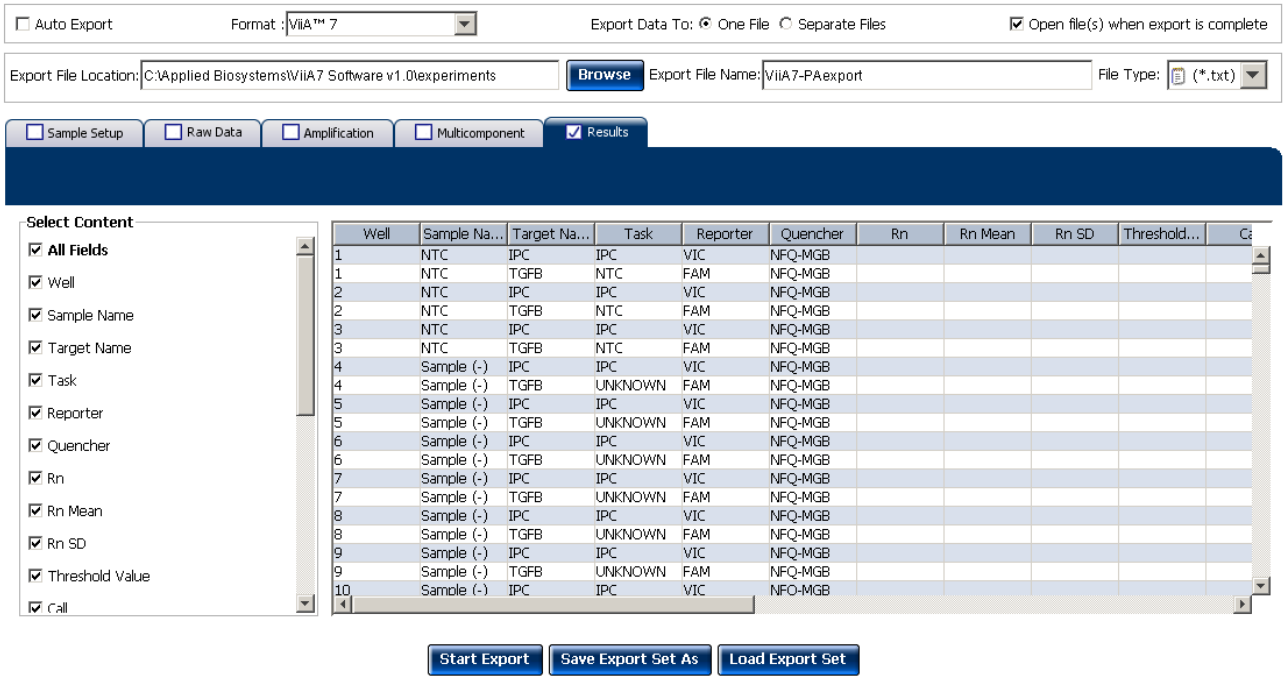


1. Open the Presence/Absence example experiment file that you analyzed in Chapter 5.
2. In the Experiment Menu, click **Export.**

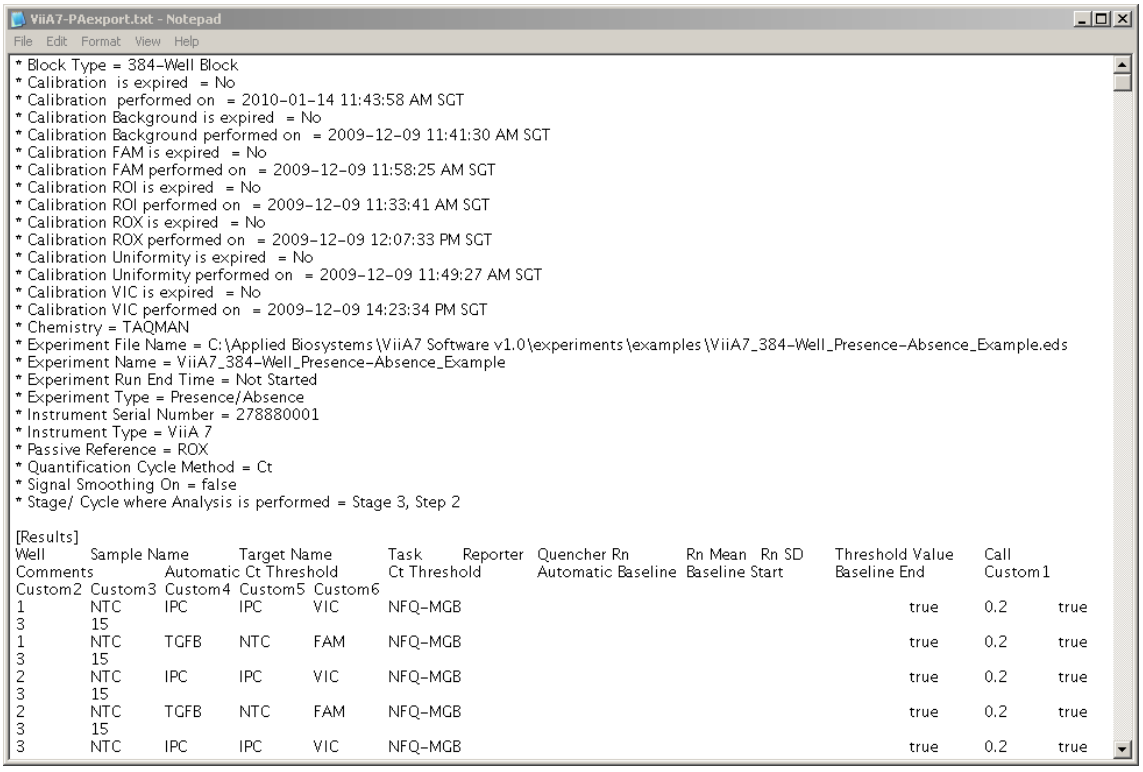
   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.
3. Select **ViiA™7 Format**.
4. Complete the Export dialog box as shown below:

Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	ViiA7PAexport
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\ViiA7 Software v1.1\experiments

Your Export screen should look like this:



Your exported file when opened in Notepad should look like this:



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BOOKLET 6

Running Melt Curve Experiments

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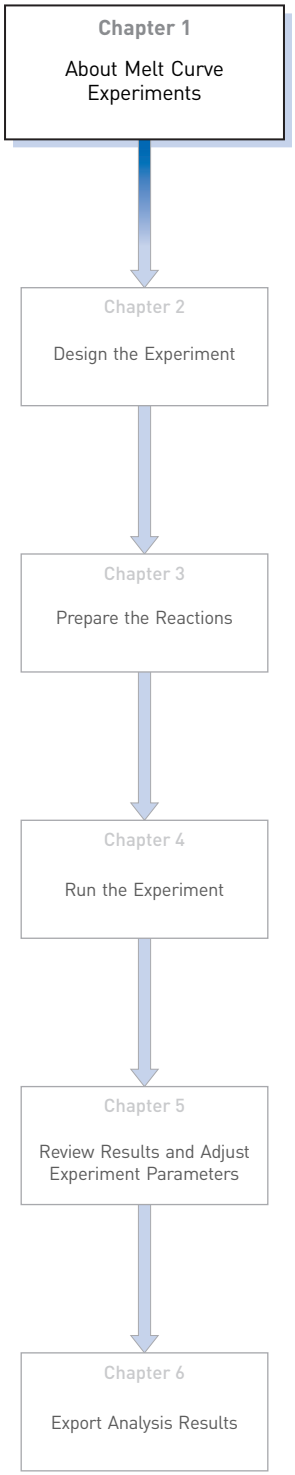
1 About Melt Curve Experiments



This chapter covers:

- Overview 6
- About the Melt Curve reactions 6
- About the example experiment 6

IMPORTANT! First-time users of the ViiA™ 7 System, please read Booklet 1, *Getting Started with ViiA™ 7 System Experiments* and Booklet 7, *ViiA™ 7 System Experiments - Appendixes* of this binder thoroughly. The booklet provides information and general instructions that are applicable to all the experiments described in this binder.

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems ViiA™ 7 Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ ViiA™ 7 Software Help**.

Overview

A Melt Curve, also known as dissociation curve, is a plot of data collected during the Melt Curve stage of an experiment. Melt Curve experiments are performed to determine the melting temperature (Tm) of a target nucleic acid sequence or to identify nonspecific PCR amplification.

Melting temperature (Tm) is the temperature at which 50% of the target DNA is double-stranded and 50% is dissociated into single-stranded DNA.

The melting temperature and non-specific PCR amplification can be identified as peaks in the melt curve stage of an experiment.

About the Melt Curve reactions

With Melt Curve experiments, the reactions consist of completed PCR reactions that contain amplified products and SYBR® Green dye to detect double-stranded DNA.

The ViiA™ 7 Software detects the number of fluorescence peaks, determines the melting temperature (Tm) for each peak, and plots the results in a melt curve.

The fluorescence data collected during the ViiA™ 7 Instrument run are stored in an experiment data file (*.eds).

There are two types of reactions in a Melt Curve experiment:

- **Unknowns** - Wells containing PCR product with unknown melting temperature(s).
- **Negative controls** - Wells containing buffer or water instead of sample. Negative controls should contain no double-stranded DNA.

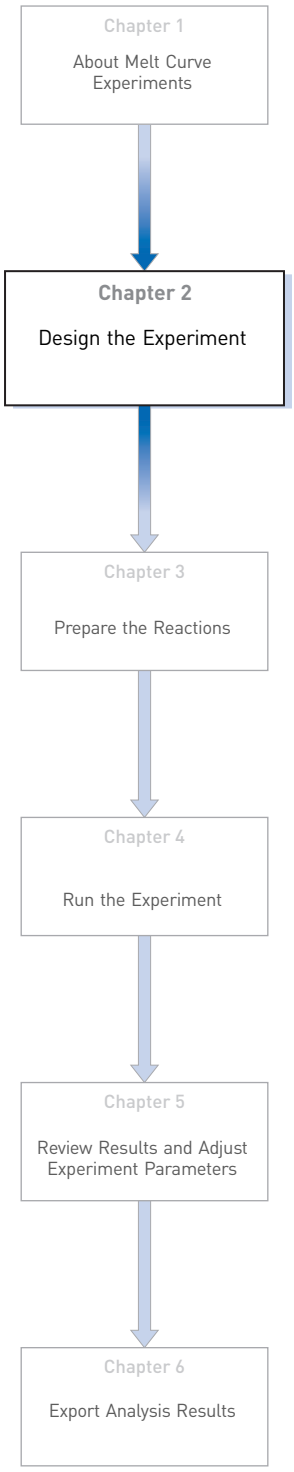
About the example experiment

To illustrate how to perform Melt Curve experiments, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with a ViiA™ 7 System.

The objective of the example Melt Curve experiment is to investigate the melting temperature of Target 1, and verify that no extraneous peaks appear. The SYBR Green reagent is used to detect the melting temperature stage.

Note: The example experiment performs a melt curve analysis on PCR products from a PCR performed on the ViiA™ 7 System or on another thermal cycler.

2 Design the Experiment



This chapter explains how to design the example experiment from the Experiment Setup menu.

This chapter covers:

- Define the experiment properties. 8
- Define targets and samples 9
- Assign targets and samples. 10
- Set up the run method 10
- Order materials for the experiment 12
- For more information. 13

Note: To automatically export the analyzed data to a specified location, select the Auto Export task from the Export screen, before running the experiment. For more information on Auto Export, refer to Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*.

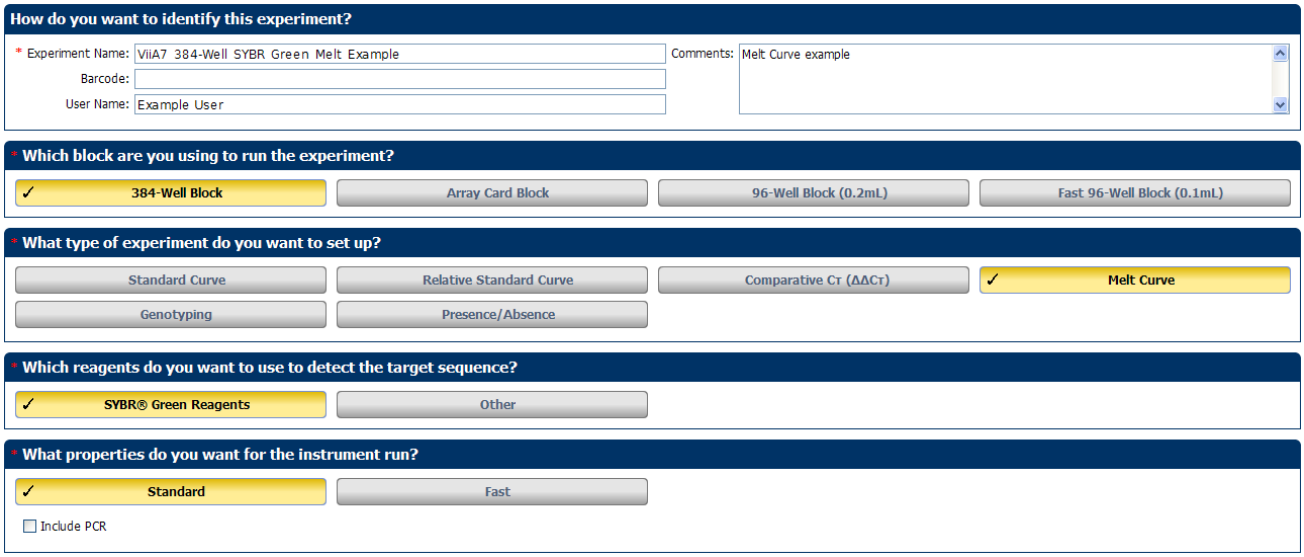
Define the experiment properties

Click **Experiment Setup** ▸ **Experiment Properties** to create a new experiment in the ViiA™ 7 Software. Enter:

Field or Selection	Entry
Experiment Name	ViiA7_384-Well_SYBR_Green_Melt_Example
Barcode	Leave field empty
User Name	Example User
Comments	Melt Curve example
Block	384-Well Block
Experiment Type	Melt Curve
Reagents	SYBR® Green Reagents
Ramp speed	Standard
Include PCR	Unchecked

Save the experiment.

Your Experiment Properties screen should look like this:



Define targets and samples

Click **Define** to access the Define screen. Enter:

1. Targets

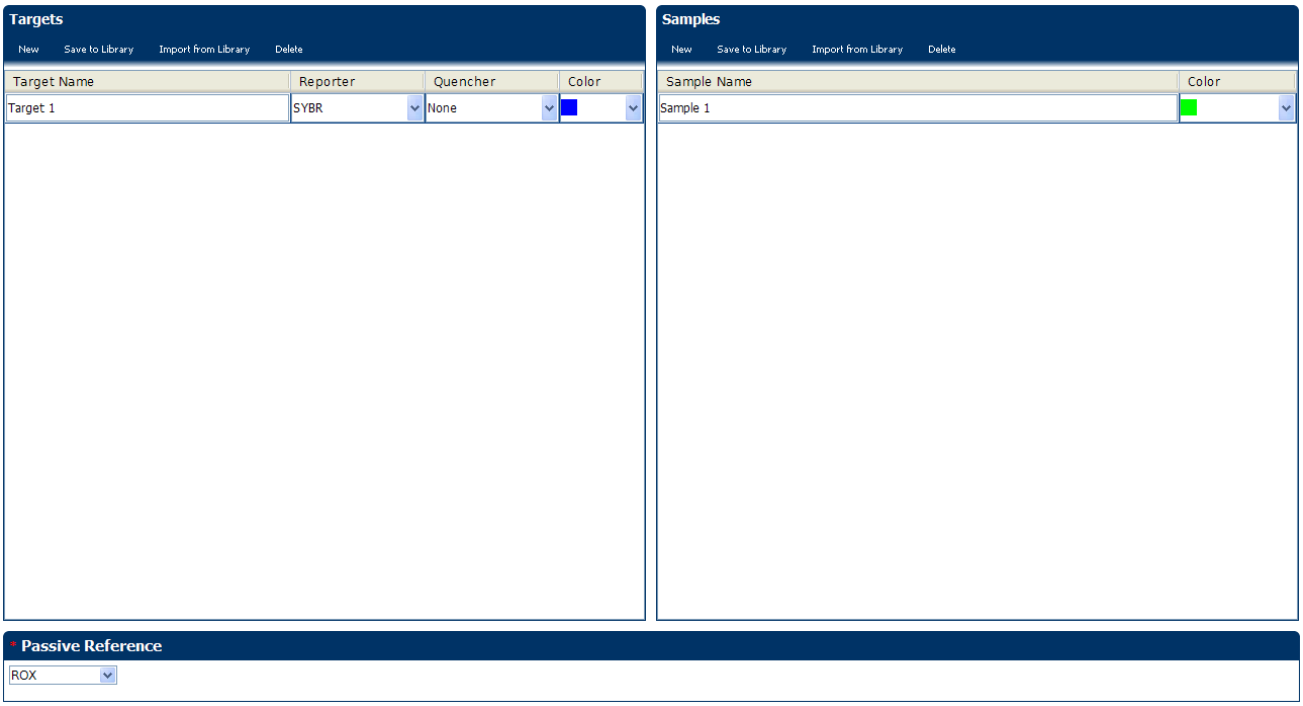
Target Name	Reporter	Quencher	Color
Target 1	SYBR	None	

2. Samples

Sample Name	Color
Sample 1	

3. Dye to be used as a Passive Reference
   ROX

Your Define screen should look like this:

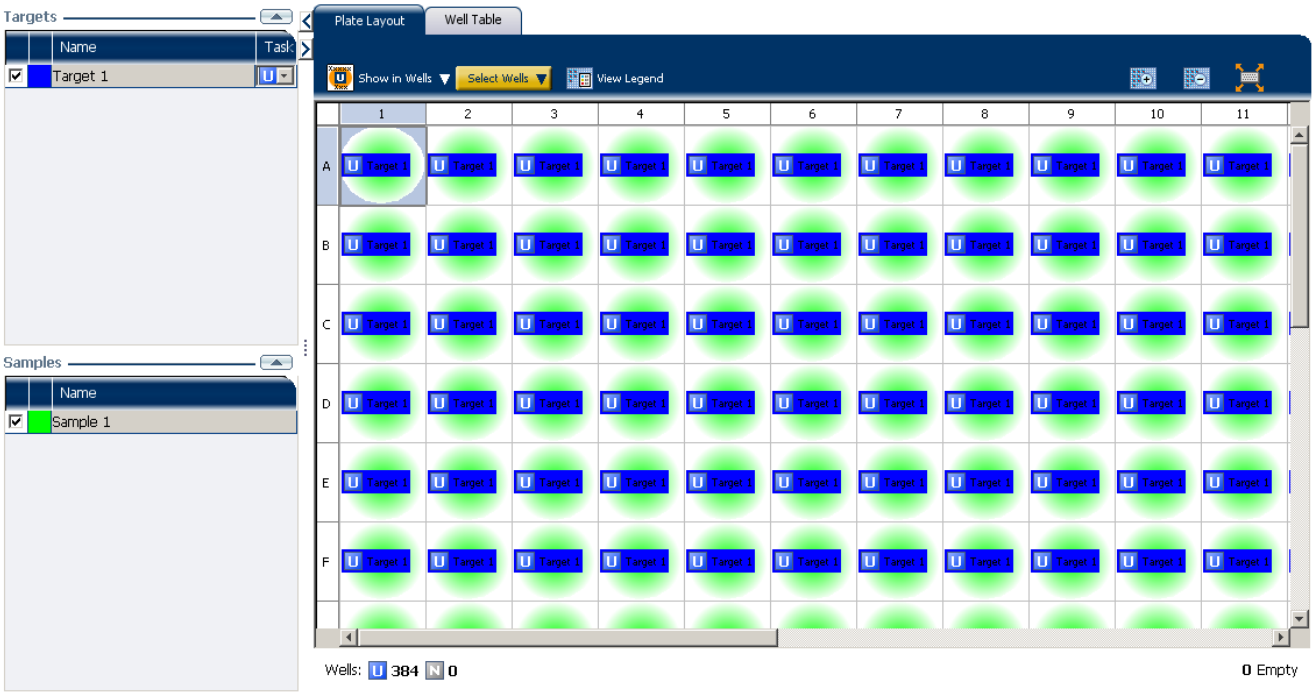


Assign targets and samples

Click **Assign** to access the Assign screen. Enter the targets and samples:

Target Name	Sample	Well Number	Task
SYBR	Sample 1	A1 - P24 (Columns 1 -24)	Unknown

Your Assign screen should look like this:



Set up the run method

Set the thermal profile

Click **Run Method** to access the Run Method screen. Set the thermal profile under the Graphical View tab. Enter:

- Reaction Volume Per Well: 20µl
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Melt Curve Stage	Step 1	1.6 °C/s	95ºC	15 seconds
	Step 2	1.6 °C/s	60ºC	1 minute
	Step 3 (Dissociation)	0.05 °C/s	95ºC	15 seconds

Edit the ramp increment

Edit the ramp increment for a melt curve (dissociation) step.

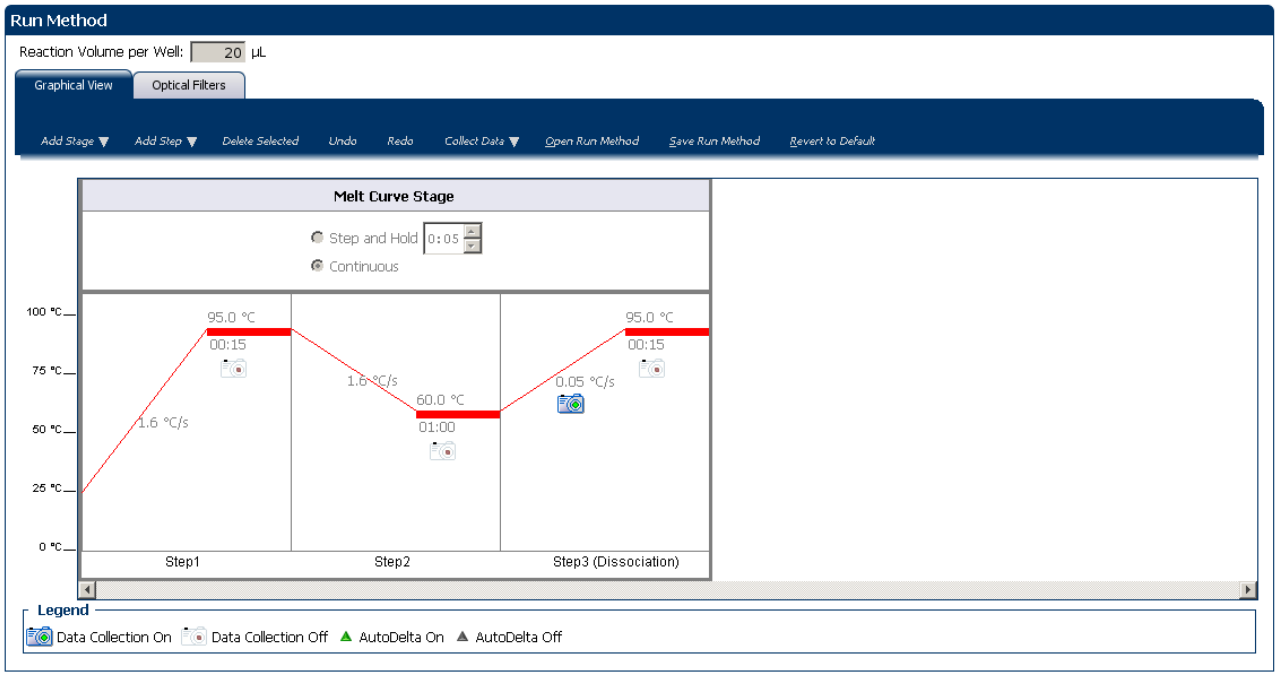
1. Select a melt curve ramp increment method:
   - **Step and Hold** – Increases or decreases the ramp temperature in 0.1 °C increments over the time (duration) for the melt curve ramp.
   - **Continuous (default)** – Increases or decreases the ramp rate in 0.005 °C per second increments.

2. If you selected the Step and Hold ramp increment method, edit the melt curve ramp time:
   - To increase or decrease the time in 1-minute or 1-second increments, click the **Step and Hold** field, select the minutes or seconds, then use the up or down arrow keys or click the up or down buttons in the field until you reach the desired time.
   - To enter the desired time, click the **Step and Hold** field, select the minutes or seconds, then enter the desired time.

3. Edit the melt curve ramp increment:
   - To increase or decrease the ramp increment, click the melt curve (dissociation) ramp increment in the thermal profile, then use the up or down arrow keys or click the up or down buttons in the field until you reach the desired value.
   - To enter the desired ramp increment, click the melt curve (dissociation) ramp increment in the thermal profile, select the value in the field, then enter the desired value.

     Note: To view the maximum and minimum allowed values, place the cursor over melt curve (dissociation) ramp increment in the thermal profile and wait for the tooltip to pop up.

Your Run Method screen should look like this:

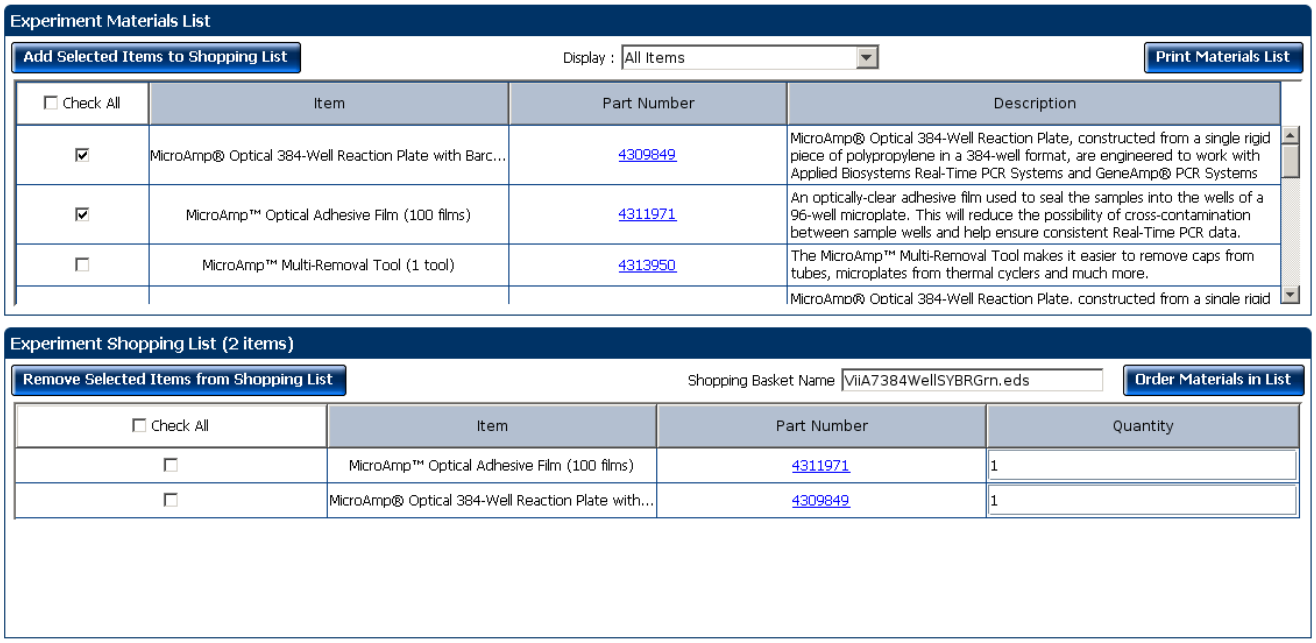


Order materials for the experiment

The recommended materials are:

- MicroAmp® Optical 384-Well Reaction Plate
- MicroAmp® Optical Adhesive Film
- Power SYBR® Master Mix
- Two target-specific primers (one forward, one reverse)

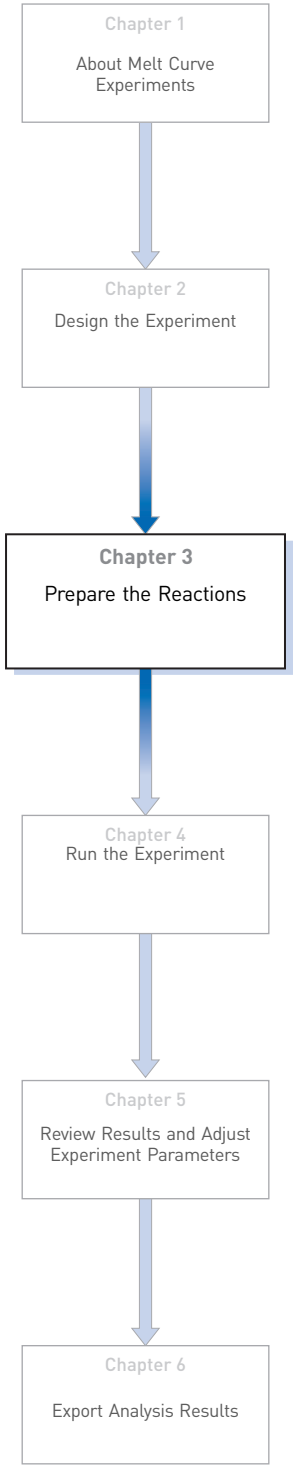
Your Materials List screen should look like this:



For more information

For more information on...	Refer to	Part number
Consumables	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments* Appendix A in Booklet 7, *ViiA™ 7 System Experiments - Appendixes*	4441434
Using Alternative Setup	Chapter 2 in Booklet 1, *Getting Started with ViiA™ 7 Systems Experiments*	4441434

3 Prepare the Reactions



This chapter explains how to prepare the reactions for running a PCR prior to running a Melt Curve.

To perform a Melt Curve experiment without running a PCR, use the reaction plate containing the PCR product.

Note: The example experiment performs a melt curve analysis on PCR products from a PCR performed on the ViiA™ 7 System or on another thermal cycler.

This chapter covers:

- Assemble required materials 16
- Prepare the sample dilutions 16
- Prepare the reaction mix (“cocktail mix”). 16
- Prepare the reaction plate 17
- For more information. 18

Assemble required materials

- Items listed in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*
- Sample 1
- Example experiment reaction mix components:
  - Power SYBR® Master Mix
  - Target - Assay Mix Forward primer (10µM)
  - Target - Assay Mix Reverse primer (10µM)

Prepare the sample dilutions

The stock concentration of each sample is 100 ng/µL. After you dilute the sample according to the Sample Dilutions Calculations table, the sample will have a concentration of 10 ng/µL. Add 2µL to each reaction.

Sample name	Stock concentration (ng/µL)	Sample volume (µL)	Diluent volume (µL)	Total volume of diluted sample (µL)
Sample 1	100.0	2	18	20

Prepare the reaction mix ("cocktail mix")

The following table lists the universal assay conditions [volume and final concentration for using the Power SYBR Master Mix (2X)].

Reaction Component	Volume for 1 reaction (µL)	Volume for 384 reactions + 10% excess (µL) = 424 reactions
Power SYBR®Green PCR Master Mix (2X)	10	4240
Forward primer (10µM)	0.1	42.4
Reverse primer (10µM)	0.1	42.4
Water	7.8	3307.2
Total reaction mix volume	18	7632

Procedure

1. Label an appropriately sized tube for the reaction mix: Power SYBR Reaction Mix.
2. Add the required volume of each cocktail mix component to the tube.
3. Mix the cocktail thoroughly by gently pipetting up and down several times, then cap the tube.

4. Centrifuge the tube briefly to remove air bubbles.
5. Place the cocktail mix on ice until you prepare the reaction plate.

Calculations

Determine the quantity of primer to be added to the reaction mix by performing the following calculation:

Concentration (initial) C1 x Volume (primer stock) V1 = Concentration (final) C2 x Volume (final reaction) V2

(10µM) x (V1) = (0.05µM) (20µL)

V1 = (0.05 x 20) / 10 = 0.1

Prepare the reaction plate

1. Add reaction mix and sample to a tube.
   a. To an appropriately sized tube, add the volumes of reaction mix and sample listed below.

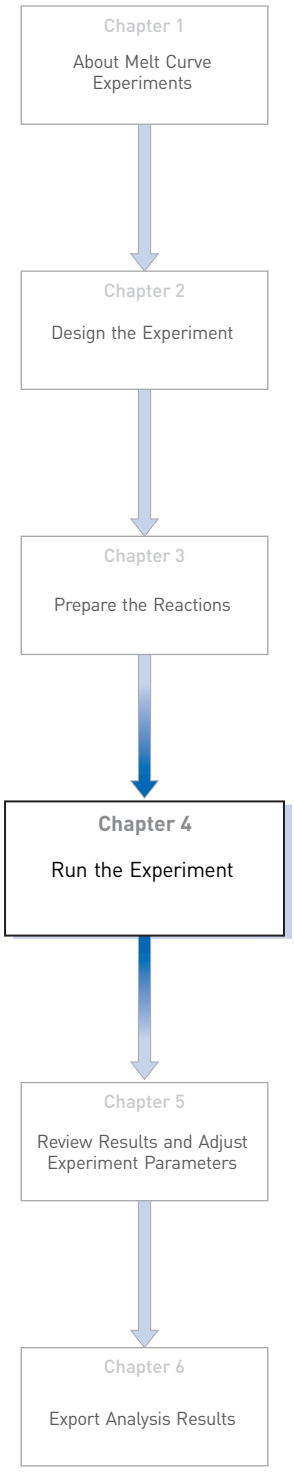
Tube	Unknown reaction	Reaction mix	Reaction mix volume (µL)	Sample	Sample volume (µL)
1	Target 1	Power SYBR reaction mix	7632	Sample 1	848

   b. Mix the reactions by gently pipetting up and down, then cap the tubes.
   c. Centrifuge the tubes briefly to remove air bubbles.
2. Pipette 20 µL of the unknown (sample) reaction to each well in the reaction plate.
3. Seal the reaction plate with optical adhesive film.
4. Centrifuge the reaction plate briefly to remove air bubbles.
5. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.
6. Until you are ready to perform the PCR run, place the reaction plate at 4 °C, in the dark.
7. Run the PCR.
8. After the PCR is completed, use the same reaction plate containing the PCR product to run the Melt Curve as described in Chapter 4.

For more information

For more information on...	Refer to...	Part number
Assigning the reaction plate components	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

4 Run the Experiment



This chapter explains how run the example experiment on the ViiA™ 7 Instrument.

This chapter covers:

- Start the run 20
- Monitor the run. 20

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the ViiA™ 7 Instrument is in operation.

Start the run

1. Open the Melt Curve example file that you created using instructions in Chapter 2.

   IMPORTANT! The example experiment includes the melt curve analysis of a PCR product from PCR on ViiA™ 7 System or another thermal cycler. To run a Melt Curve on the example file you created in Chapter 2, ensure that PCR has already been performed on the reaction plate you load into the instrument. Absence of the PCR product will lead to no results in the Dissociation Step of the Melt Curve Stage.

2. Load the reaction plate, containing the PCR product, into the instrument.

3. Start the run.

Monitor the run

Monitor the example experiment run:

- From the ViiA™ 7 Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the ViiA™ 7 Software (to monitor an experiment started from another computer or from the ViiA™ 7 Instrument touchscreen).
- From the ViiA™ 7 Instrument touchscreen.

From the Instrument Console of the ViiA™ 7 Software

1. In the Instrument Console screen, select the instrument icon.

2. Click **Manage Instrument** or double-click on the instrument icon.

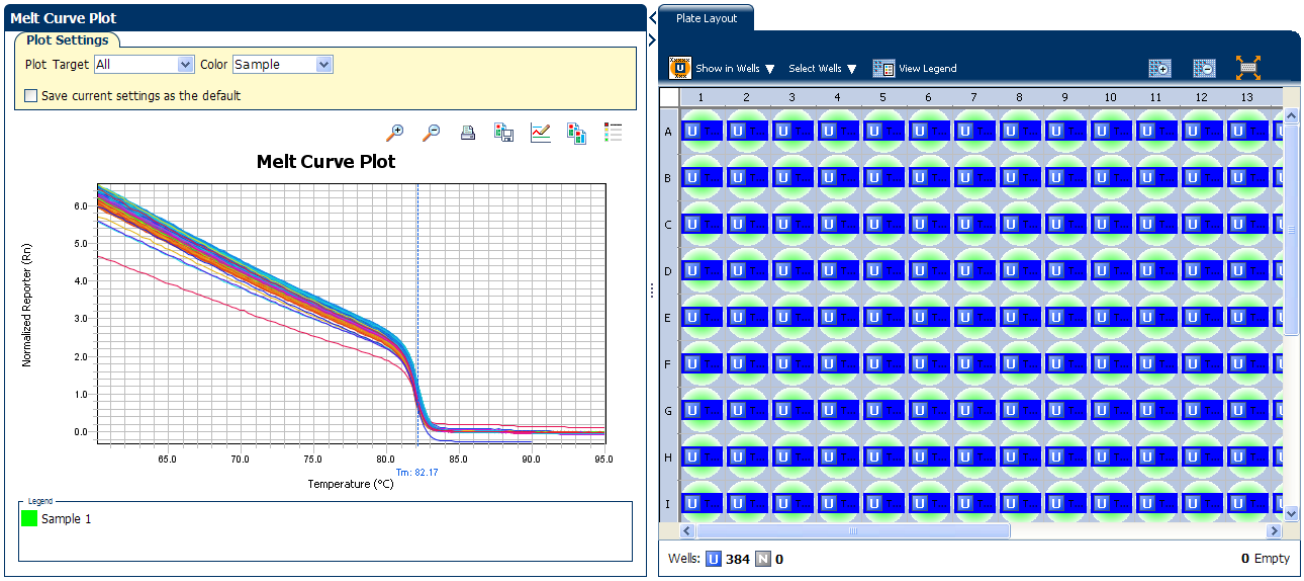
3. On the Manage Instrument screen, click **Monitor Running Instrument** to access the Run screen.

View the Melt Curve

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the ViiA™ 7 Software for potential problems.

Click **Melt Curve** from the Run Experiment Menu, select the Plate Layout tab, then select the wells to view.

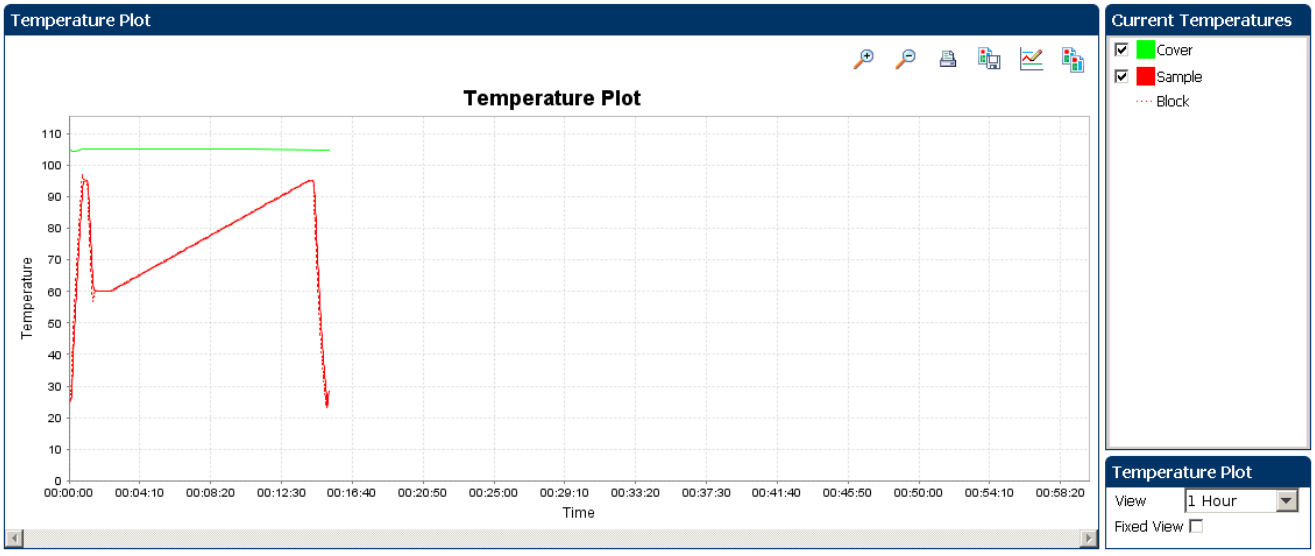
The figure below shows the Melt Curve as it appears at the end of the example experiment.



View the Temperature Plot

Click **Temperature Plot** from the Run Experiment Menu.

The figure below shows the Temperature Plot screen as it appears during the example experiment.

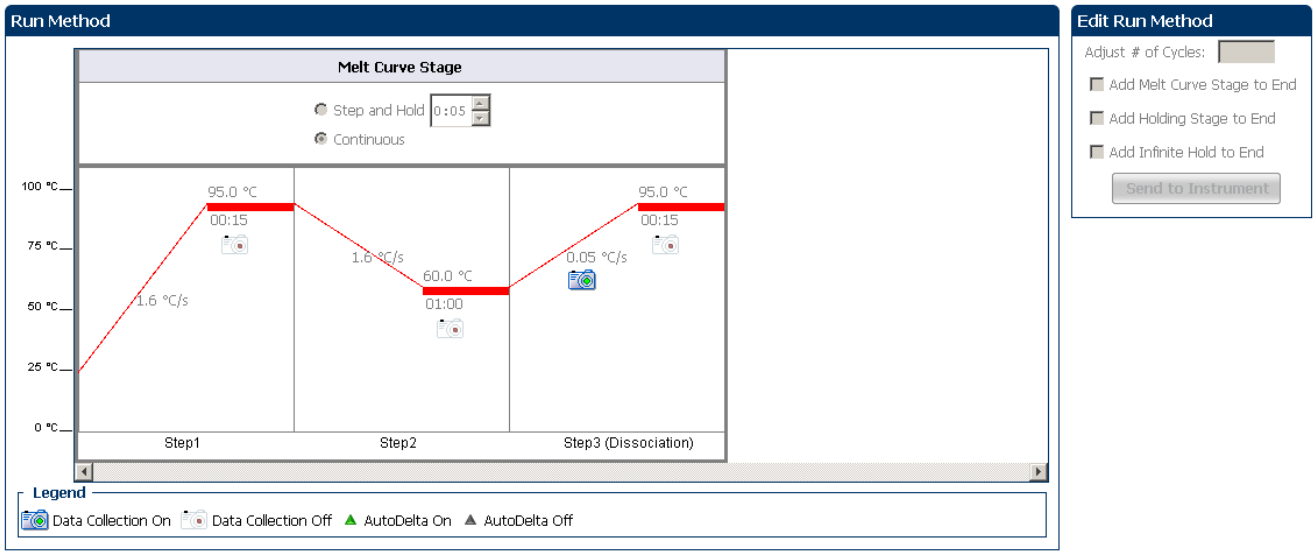


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

View the Run Method

Click **Run Method** from the Run Experiment Menu.

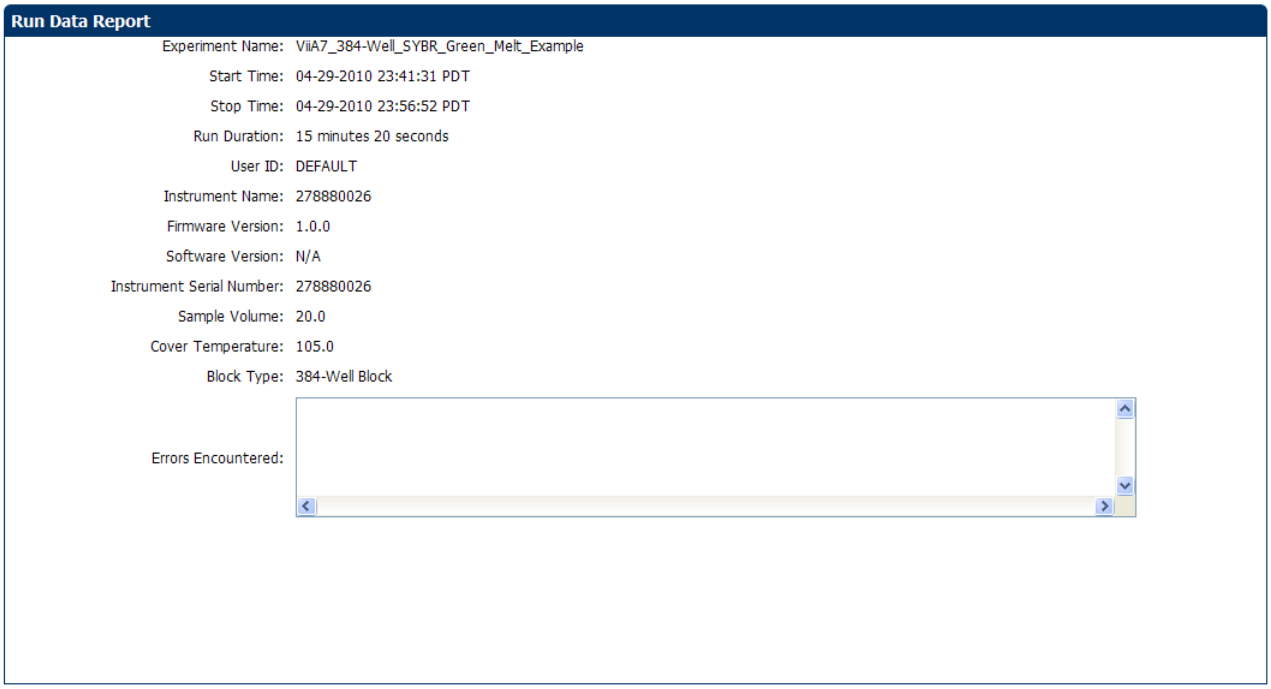
The figure below shows the Run Method screen as it appears in the example experiment.



View run data

Click **View Run Data** from the Run Experiment Menu.

The figure below shows the View Run Data screen as it appears in the example experiment.

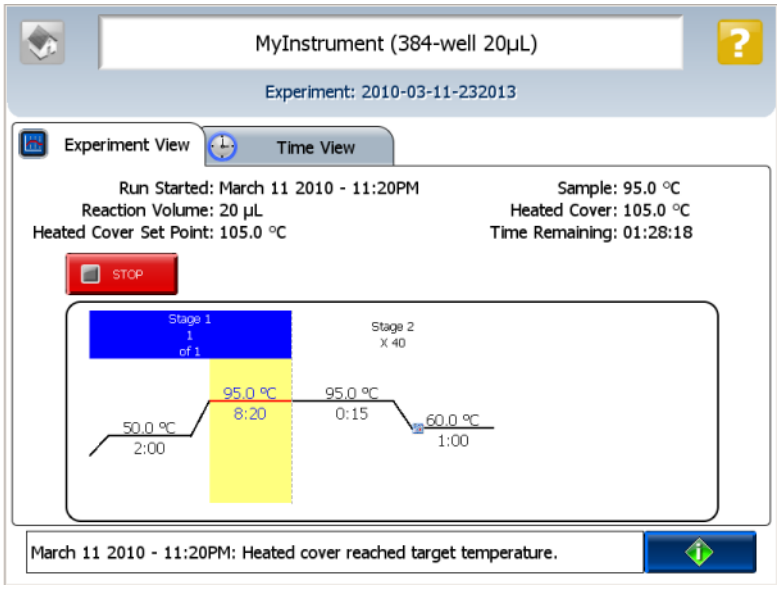


From the ViiA™ 7 Instrument touchscreen

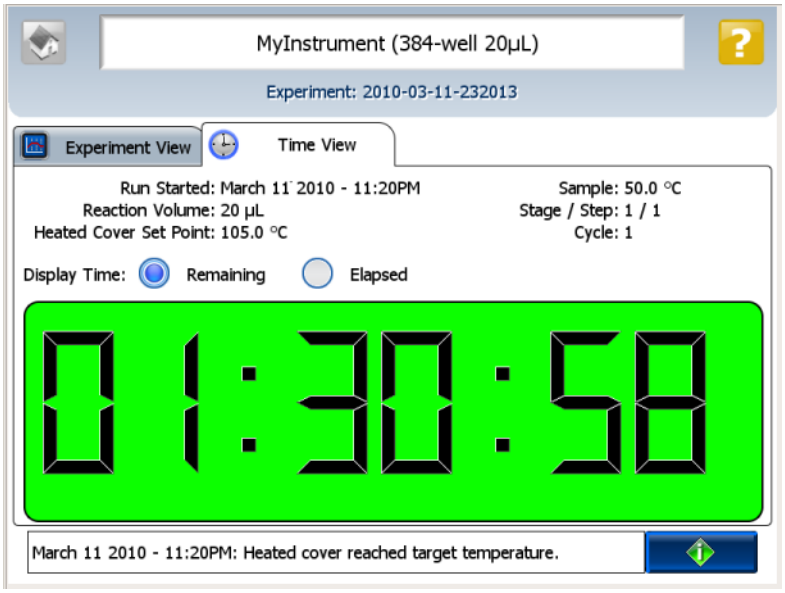
You can also view the progress of the run from the touchscreen of the ViiA™ 7 Instrument.

The Run Method screen on the **ViiA™ 7 Instrument** touchscreen looks like this:

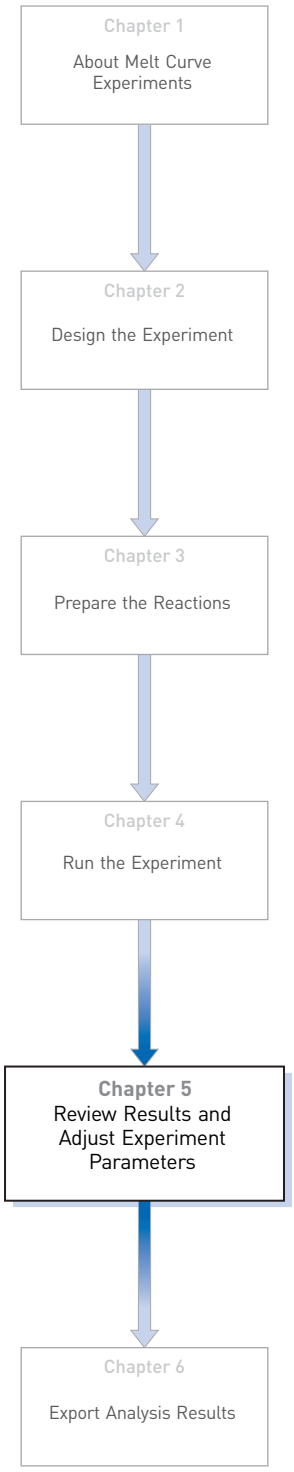
Experiment View



Time View



5 Review Results and Adjust Experiment Parameters



In Section 5.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 5.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

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- Analyze the example experiment. 27
- View the Melt Curve Plot 27
- Identify well problems using the Well Table 29
- Confirm accurate dye signal using the Multicomponent Plot 31
- Determine signal accuracy using the Raw Data Plot 32
- Review the flags in the QC Summary 34
- For more information. 36

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- Adjust analysis settings 37
- For more information. 42

5.1

Section 5.1 Review Results

Analyze the example experiment

1. Open the example experiment file that you ran in Chapter 4.
2. Click **Analyze**. The software analyzes the data using the default analysis settings.

   Note: You can also access the experiment to analyze from the Home screen.

View the Melt Curve Plot

View the Melt Curve Plot as the Derivative Reporter (-Rn) versus the Temperature Plot generated by the target.

The Melt Curve screen displays the melt curve of the targets in the selected wells. Use the Melt Curve plots to confirm the results of the experiment:

- **Normalized Reporter (Rn) vs. Temperature** – This plot displays the fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference. You can use this plot to see the change in Rn with change in the temperature. You cannot use this plot to determine the Tm of the target.
- **Derivative Reporter (-Rn) vs. Temperature** – This plot displays the derivative reporter signal in the y-axis. The peaks in the plot indicate significant decrease in SYBR Green signal, and therefore the Tm of the target.

Purpose

The purpose of viewing the Melt Curve Plot for the example experiment is to review the melting temperature of the target.

To view and assess the Melt Curve

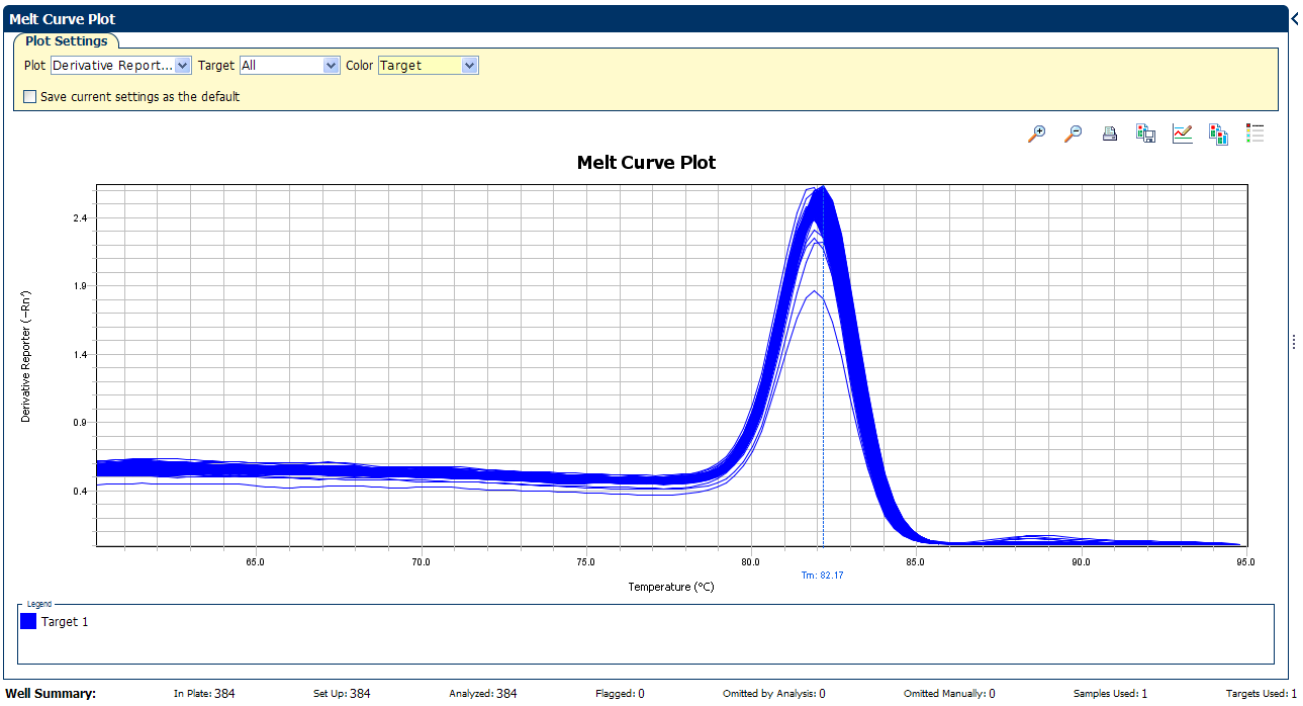
1. From the Experiment menu pane, select **Analysis ▸ Melt Curve Plot.**

   Note: If no data are displayed, click **Analyze**.
2. Enter the Plot Settings:

Menu	Selection
Plot	Derivative Reporter
Target	All
Plot Color	Target

Menu	Selection
(This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.)	Check (default)

The Melt Curve for the example experiment looks like this:



Tips for viewing melt curves in your own experiments

When you analyze your own Melt Curve experiment, look for wells with multiple peaks, indicating non-specific amplifications or primer dimer formation.

If your experiment does not amplify properly or indicates non-specific amplification, troubleshoot by manually adjusting the Melt Curve settings (see "Adjust analysis settings" on page 37).

Identify well problems using the Well Table

Review the details of the experiment results in the well table and identify any flagged wells. The well table displays the assay-specific setup and analysis properties for the experiment in a tabular format.

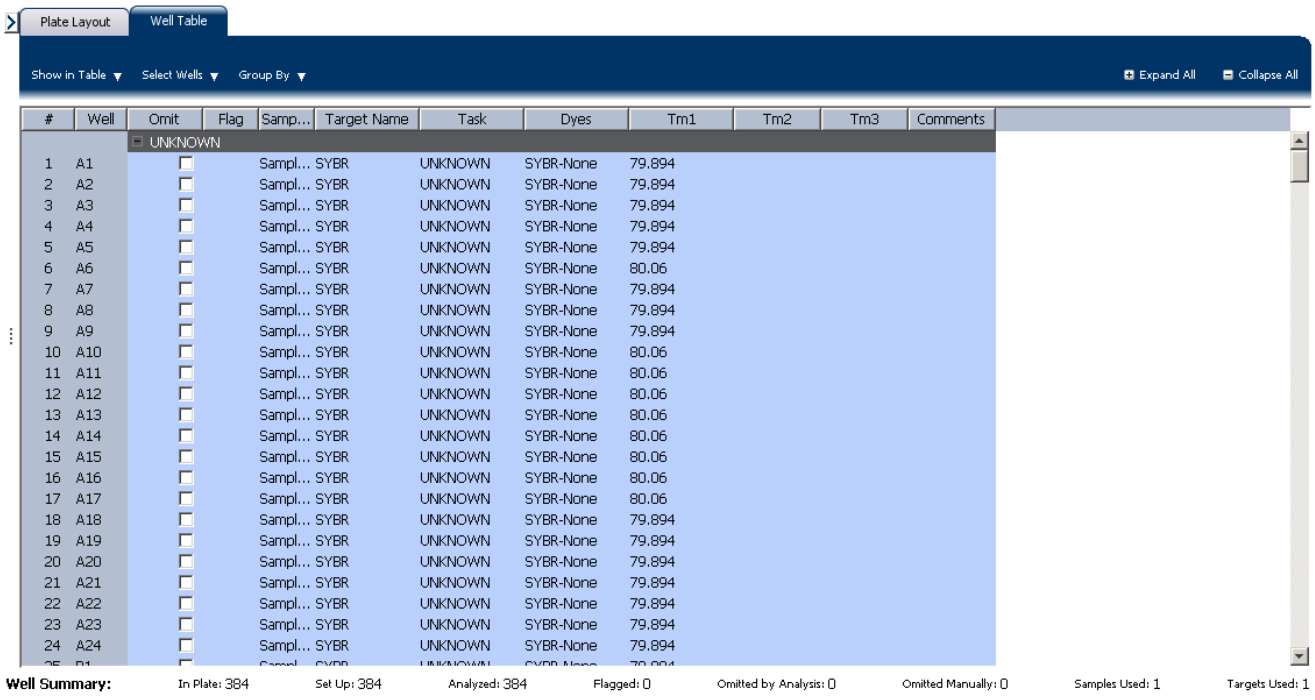
Example experiment values and flags

For the example experiment, confirm that no wells of the reaction plate triggered QC flags ▲.

View the well table

1. Select the **Well Table** tab.
2. Click the **Flag** column header to sort the data so that the wells that triggered flags appear at the top of the table.
3. Confirm the integrity of the controls:
   a. From the Group By menu, select **Task** to organize the table rows by their function on the reaction plate.
   b. Confirm that each of the controls do not display flags (▲1).

The figure below shows the well table of the example Melt Curve experiment.



The table below gives the description of each column in the well table.

Column	Description
Well	The position of the well on the reaction plate.
Omit	A check mark indicates that the well has been removed from the analysis.
Flag	A ⚠ indicates that the well triggered the number of flags listed inside the symbol.
Sample Name	The name of the sample.
Target Name	The name of the target evaluated by the well.
Task	The task assigned to the well (Unknown, Negative Control, or Positive Control).
Dyes	The name of the reporter and quencher dyes of the associated sample for the target evaluated by the well.
Tm1	The melting temperature of the target.
Tm2	The second melting temperature (for targets with multiple melting temperatures).
Tm3	The third melting temperature (for targets with multiple melting temperatures).

Tips for viewing well tables your own experiments

When you analyze your own experiment:

- Review the data for the Unknown samples. For each row that displays ⚠ in the Flag column, note the data and the flag(s) triggered by the associated well.
- Select areas of the table or wells of a specified type by:
  - Left-clicking the mouse and dragging across the area you want to select an area of the table.
  - Selecting **Sample**, **Target**, or **Task** from the Select Items menu in the Well Table tab, then selecting the sample, target, or task name from the second Select Items menu to select wells of a specific type using the well-selection tool.
- Group the rows of the plate layout by selecting an option from the Group By menu. You can then collapse or expand the lists either by clicking the **+/-** icon next to individual lists, or by clicking **Collapse All** or **Expand All**.
- Omit a well from the analysis by selecting the **Omit** check box for that well. To include the well in the analysis, deselect the **Omit** check box.

  Note: You must reanalyze the experiment each time you omit or include a well.

Confirm accurate dye signal using the Multicomponent Plot

The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the Melt Curve example experiment, you review the Multicomponent Plot screen for:

- ROX™ dye (passive reference)
- SYBR® dye (reporter)
- Spikes, dips, and/or sudden changes

View the Multicomponent Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Multicomponent Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the unknown wells in the plate layout to display the corresponding data in the Multicomponent Plot screen:
   a. Click the **Plate Layout** tab.
   b. Select one well in the plate layout; the well is shown in the Multicomponent Plot screen.

      Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

3. From the Plot Color drop-down menu, select **Dye**.

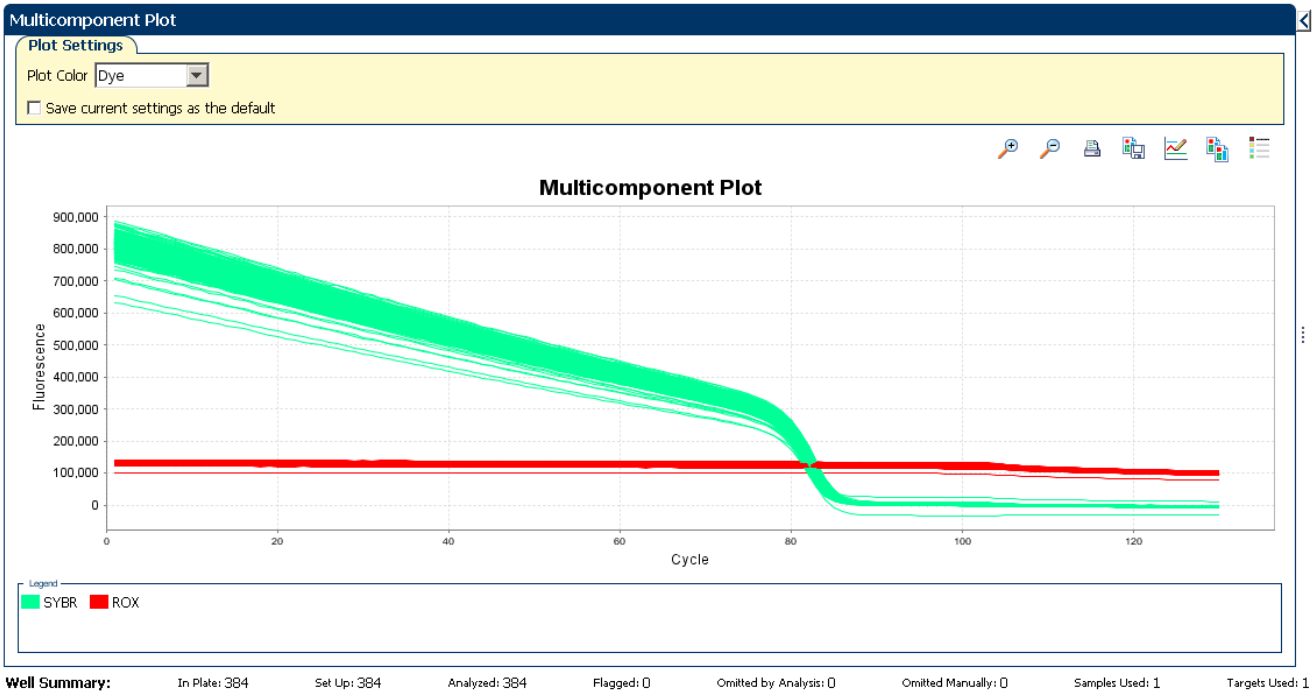
4. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

5. Check the ROX dye signal. In the example experiment, the ROX dye signal remains constant throughout the PCR process; a constant ROX dye signal indicates typical data.

6. Check the SYBR dye signal. In the example experiment, because the PCR run has already been completed, the SYBR dye signal shows gradual decrease throughout the run and a sudden dip in the fluorescence at one point; the sudden drop in the SYBR dye signal indicates the melting temperature of the target.

The Multicomponent Plot screen for the example experiment looks like this:



Tips for confirming dye accuracy in your own experiment

When you analyze your own Melt Curve experiment, look for:

- **Passive reference** – The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye** – The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds. If the Melt Curve is being performed post-PCR, then there should be a gradual decrease in fluorescence and a sudden dip indicating the melting temperature of the target.
- **Irregularities in the signal** – There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.

Determine signal accuracy using the Raw Data Plot

The Raw Data Plot screen displays the raw fluorescence signal (not normalized) for each optical filter for the selected wells during each cycle of the real-time PCR.

About the example experiment

In the Melt Curve example experiment, you review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the Raw Data Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Raw Data Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display all 384 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the Plate Layout tab.

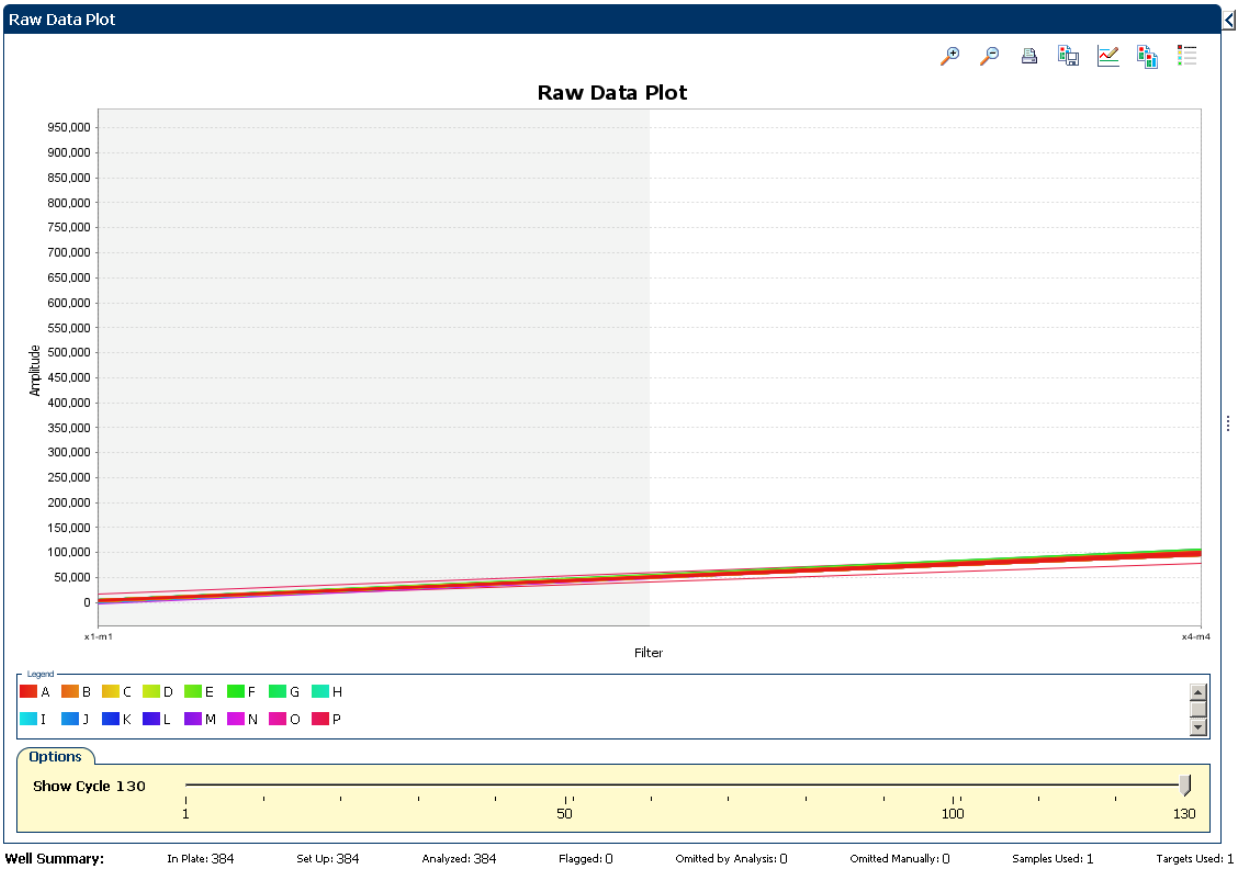
3. Click **Show a legend for the plot** (default). The legend displays the color code for each row of the reaction plate (see the legend in the Raw Data Plot shown below).

4. Click and drag the Show Cycle pointer from cycle 1 to cycle 130. In the example experiment, the signal from filter 1, which corresponds to the SYBR™ dye filter, is stable throughout.

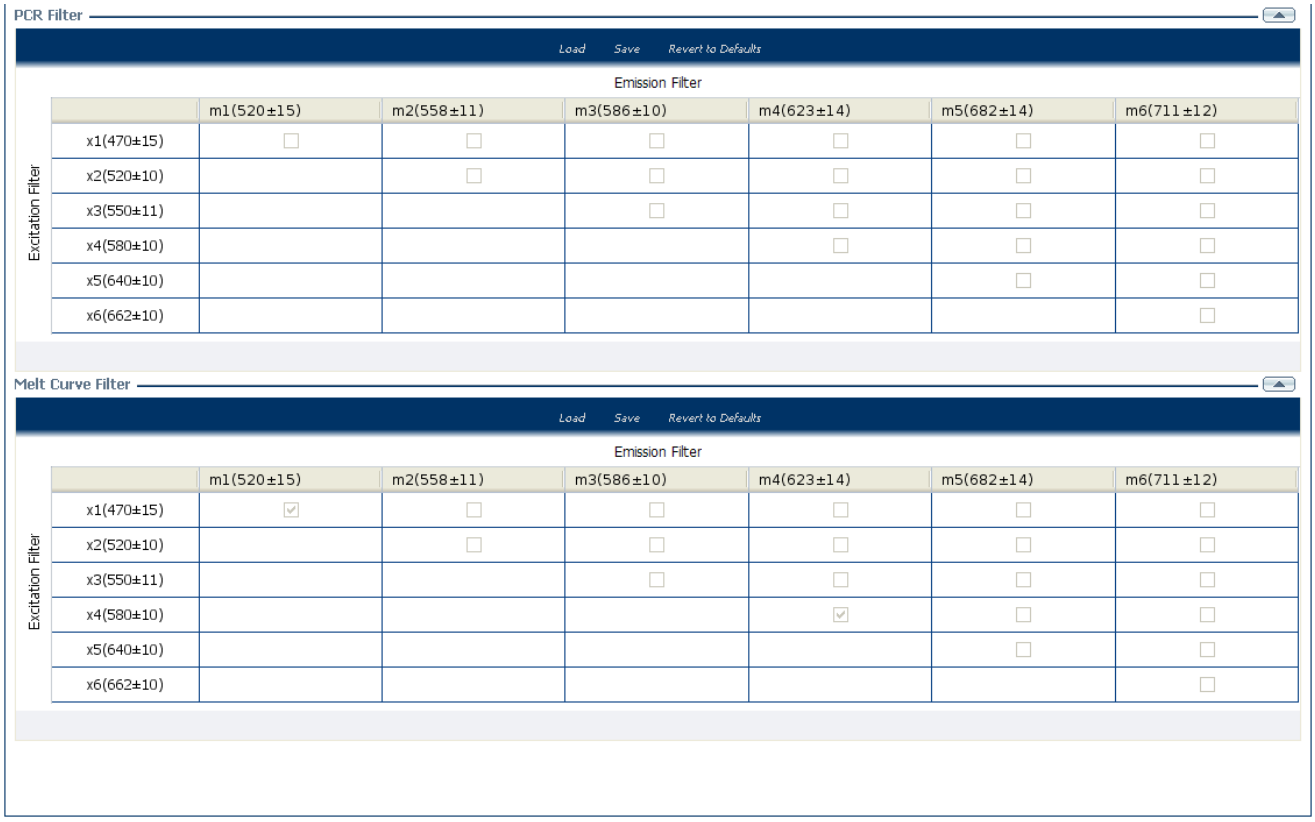
   Note: The readings shown below are from the example experiment. Actual results will vary with individual experiment setup.

   Note: The cycle number in the Melt Curve represents the number of data collection points for that experiment.

The Raw Data plot for the example experiment looks like this:



The filters used for the example experiment are:



Tips for determining signal accuracy in your own experiments

When you analyze your own Melt Curve experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

Review the flags in the QC Summary

The QC Summary screen displays a list of the ViiA™ 7 Software flags, including the flag frequency and location for the open experiment.

View the QC Summary

1. From the Experiment Menu pane, select **Analysis** ▸ **QC Summary**.

   Note: If no data are displayed, click **Analyze**.

2. Review the Flags Summary.

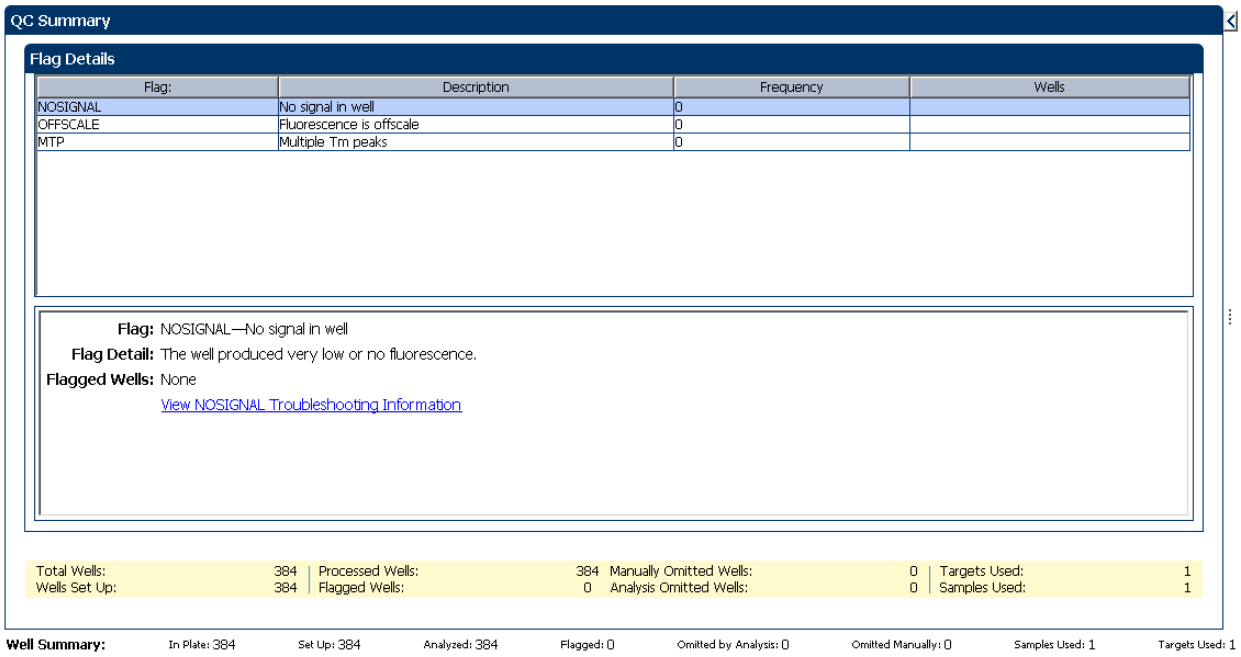
   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment. If the frequency is >0, the flag appears somewhere in the experiment; the well position is listed in the Wells column.

   In the example experiment, there are no flagged wells.

3. In the Flag Details table, click each flag with a frequency >0 to display detailed information about the flag. In the example experiment, the Frequency column displays 0 for the three flags NOSIGNAL, OFFSCALE, and MTP.

4. *(Optional)* For those flags with frequency >0, click the troubleshooting link to view information on correcting the flag.

   The QC Summary for the example experiment looks like this:



Possible flags

For Melt Curve experiments that do not include amplification, the flags listed below may be triggered by the experiment data.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flag	
NOSIGNAL	No signal in well
Secondary analysis flag	
MTP	Multiple Tm peaks

For Melt Curve experiments that include amplification, the flags listed below may be triggered by the experiment data.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
Secondary analysis flags	
MTP	Multiple Tm peaks
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the Frequency column for each flag).

For more information

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*	4441434

5.2 Section 5.2 Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

The Analysis Settings dialog box displays the analysis settings for the Melt Curve and flags.

If the default analysis settings in the ViiA™ 7 Software are not suitable for your own experiment, you can change the settings in the Analysis Settings dialog box, then reanalyze your experiment.

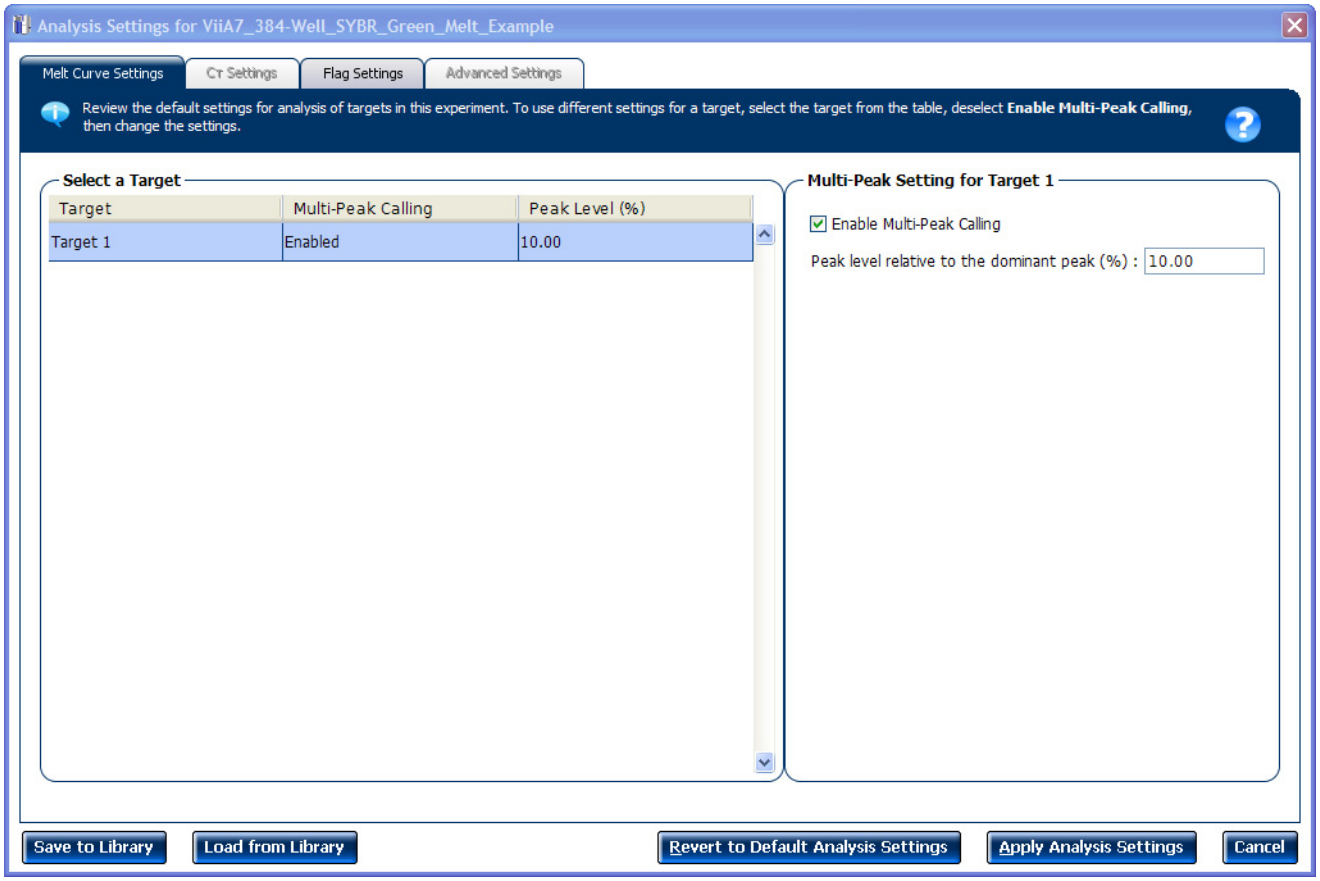
View the analysis settings

1. From the Experiment Menu pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the Analysis Settings dialog box.
   In the example experiment, the default analysis settings are used for each tab:
   - Melt Curve Settings
   - C_T Settings
   - Flag Settings
   - Advanced Settings

     Note: The C_T Settings and Advanced Settings tabs appear in the Analysis Settings dialog box only if the Melt Curve experiment you are performing includes the PCR process.

     Note: Select the **Include PCR** check box on the Experiment Properties screen to include amplification in your Melt Curve experiment.

The Analysis Settings dialog box for a Melt Curve experiment looks like this:



3. View and, if necessary, change the analysis settings (see "Adjust analysis settings" below).

   Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see About the Analysis Settings Library in Booklet 1, *Getting Started with ViiA™ 7 System Experiments*.

4. Click **Apply Analysis Settings** to apply the current analysis settings.

   Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

You may change the following settings:

Melt Curve Settings

Use this tab to:

- Enable or disable multi-peak calling.
  - Select the **Enable the Multi-Peak Calling** check box if you expect to amplify more than 1 PCR product and you want to determine the Tm for more than one peak.
  - Deselect the **Enable the Multi-Peak Calling** check box if you expect to amplify 1 PCR product and you do not want to determine the Tm for more than one peak.
- Enter a value (in percentage) for the peak level relative to the dominant peak.

  Specify a fractional level value as the peak detection threshold. The detected peaks are measured relative to the height of the tallest peak, which has a perfect fractional level 100%. The default value is initially set at 10%.

  For example, if you set a fractional level detection threshold value at 40, then only peaks above 40% of the tallest peak are reported and the peaks at lower height are regarded as noise.

C_T Settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method.
- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

  The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for gene quantification.

  The Relative Threshold algorithm lets you compare the data on a per-well or per-target basis. This setting is ideal for analyzing a single gene across samples or a single sample across genes with no dependence on targets, thereby reducing variability. Any settings for baseline or threshold do not affect the analysis when you use the Relative Threshold algorithm.
- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the targets that do not have custom settings. To edit the default settings, click **Edit Default Settings**.
- **C_T Settings for Target**

  When you manually set the threshold and baseline, Applied Biosystems recommends:

Setting	Recommendation
Threshold	Enter a value for the threshold so that the threshold is: • Above the background. • Below the plateau and linear regions of the amplification curve. • Within the exponential phase of the amplification curve.

Setting	Recommendation
Baseline	Select the Start Cycle and End Cycle values so that the baseline ends before significant fluorescent signal is detected.

Note: This setting is applicable only to the Baseline Threshold algorithm.

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag Settings

Use the Flag Settings tab to:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the ViiA™ 7 Software.

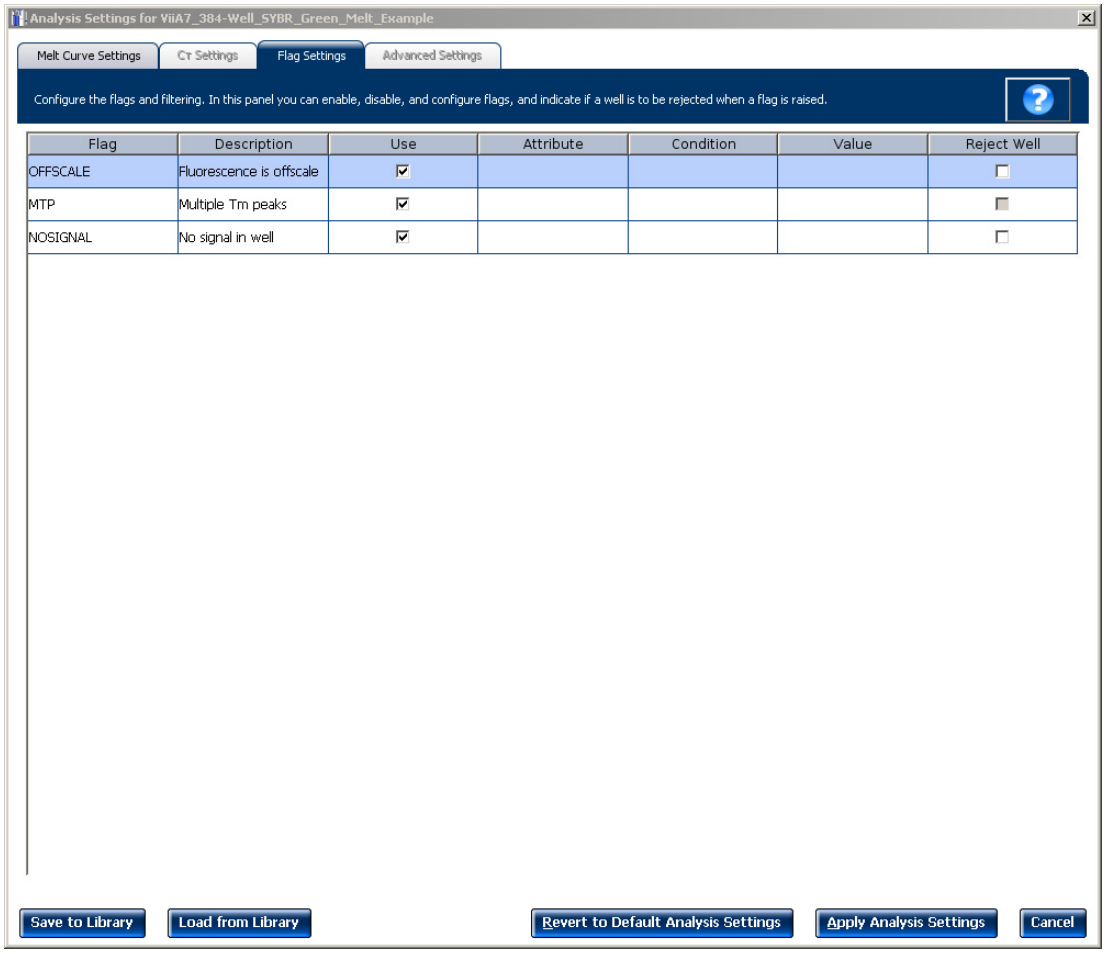
To adjust the flag settings:

1. In the Use column, select the check boxes for flags to apply during analysis.
2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.
3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations may change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.
4. Click **Apply Analysis Settings** in the Analysis Settings dialog box. If the run status is complete, the data are reanalyzed.

The Flag Settings tab looks like this:



Advanced Settings

Use the Advanced Settings tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold setting.

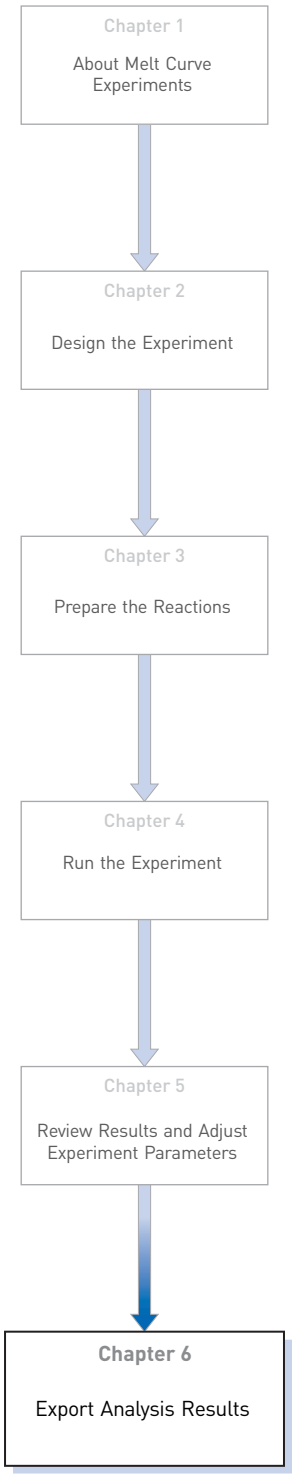
To use custom baseline settings for a well-target combination:

1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** check box.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, then enter the baseline start cycle and baseline end cycle.

For more information

For more information on...	Refer to	Part number
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*.	127AP05-03

6 Export Analysis Results

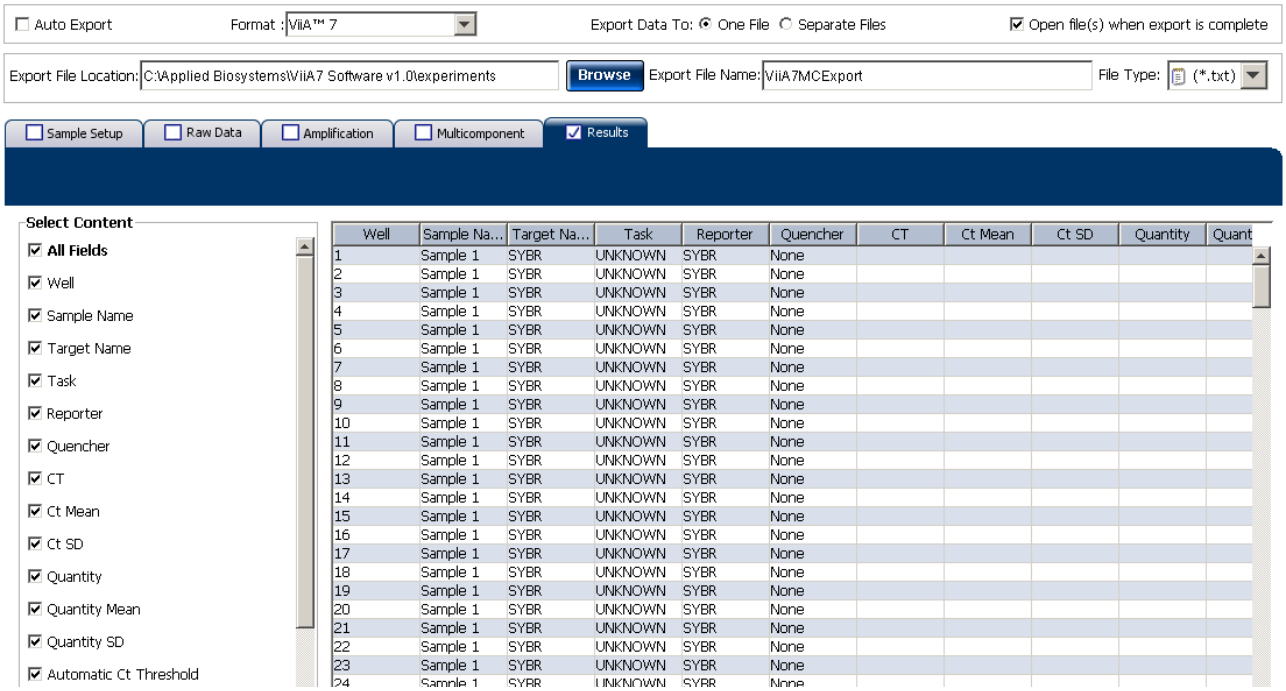


1. Open the Melt Curve example experiment file that you analyzed in Chapter 5.
2. In the Experiment Menu, click **Export.**

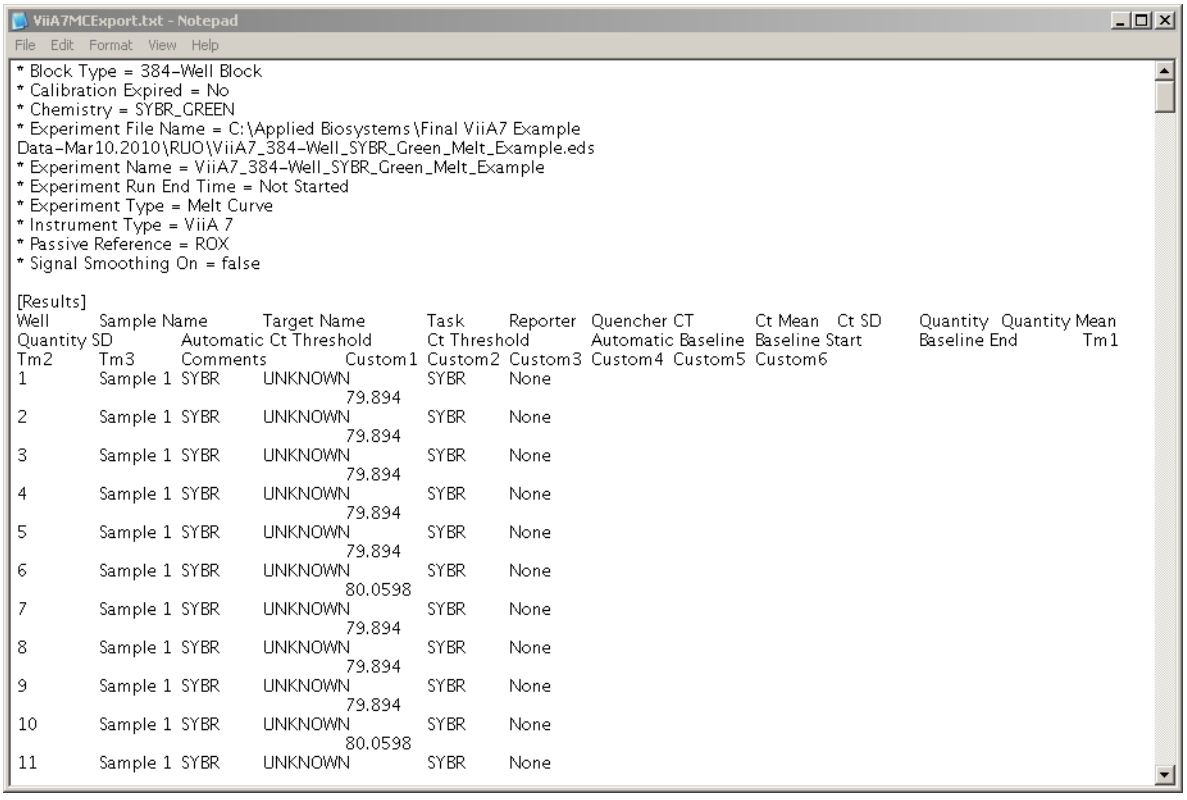
   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.
3. Select **ViiA™7 Format**.
4. Complete the Export dialog box as shown below:

Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	ViiA7MCExport
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\ViiA7 Software v1.1\experiments

Your Export screen should look like this:



Your exported file when opened in Notepad should look like this:



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BOOKLET 7

ViiA™ 7 System Experiments - Appendixes

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

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A Ordering Information

This appendix covers:

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Consumables

The consumables listed below are required for calibrating the ViiA™ 7 Instrument and for performing experiments with the ViiA™ 7 System.

Note: For reagent or consumable shelf-life expiration date, see the package label.

Calibration and verification consumables

The following table shows the reagents and consumables required to calibrate the ViiA™ 7 Instrument.

384-well sample block

Consumable	Part number	Shelf-life at environmental temperature	Storage conditions (°C)
384-Well Spectral Calibration Plate with FAM™ Dye	4432271	Use the consumable by the expiration date mentioned on the package	–15 to –25
384-Well Spectral Calibration Plate with VIC® Dye	4432278		
384-Well Spectral Calibration Plate with ROX™ Dye	4432284		
384-Well Spectral Calibration Plate with SYBR® Green Dye	4432290		
384-Well Spectral Calibration Plate with TAMRA™ Dye	4432296		
384-Well Spectral Calibration Plate with NED™ Dye	4432302		
384-Well Region of Interest (ROI) and Background Plates	4432320		
384-Well Normalization Plates with FAM™/ROX™ and VIC®/ROX™ Dyes	4432308		
TaqMan® RNase P Fast 384-Well Instrument Verification Plate	4455280		

96-well (0.2µL) sample block

Consumable	Part number	Shelf-life at environmental temperature	Storage conditions (°C)
96-Well Spectral Calibration Plate with FAM™ Dye	4432327	Use the consumable by the expiration date mentioned on the package	–15 to –25
96-Well Spectral Calibration Plate with VIC® Dye	4432334		
96-Well Spectral Calibration Plate with ROX™ Dye	4432340		
96-Well Spectral Calibration Plate with SYBR® Green Dye	4432346		
96-Well Spectral Calibration Plate with TAMRA™ Dye	4432352		
96-Well Spectral Calibration Plate with NED™ Dye	4432358		
TaqMan® RNase P 96-Well Instrument Verification Plate	4432382		
96-Well Region of Interest (ROI) and Background Plates	4432364		
96-Well Normalization Plates with FAM™/ROX™ and VIC®/ROX™ Dyes	4432370		
TaqMan® RNase P 96-Well Instrument Verification Plate	4432382		

Fast 96-well (0.1µL) sample block

Consumable	Part number	Shelf-life at environmental temperature	Storage conditions (°C)
Fast 96-Well Spectral Calibration Plate with FAM™ Dye	4432389	Use the consumable by the expiration date mentioned on the package	–15 to –25
Fast 96-Well Spectral Calibration Plate with VIC® Dye	4432396		
Fast 96-Well Spectral Calibration Plate with ROX™ Dye	4432402		
Fast 96-Well Spectral Calibration Plate with SYBR® Green Dye	4432408		
Fast 96-Well Spectral Calibration Plate with TAMRA™ Dye	4432414		
Fast 96-Well Spectral Calibration Plate with NED™ Dye	4432420		
Fast 96-Well Region of Interest (ROI) and Background Plates	4432426		
Fast 96-Well Normalization Plates with FAM™/ROX™ and VIC®/ROX™ Dyes	4432432		
TaqMan® RNase P Fast 96-Well Instrument Verification Plate	4351979		

Array card sample block

Consumable	Part number	Shelf-life at environmental temperature	Storage conditions (°C)
ViiA™ 7 Array Card Spectral Dye Calibration Kit	4432314	Use the consumable by the expiration date mentioned on the package	–15 to –25
ViiA™ 7 Array Card RNase P Instrument Verification Kit	4432265		

Experiment consumables

The following table shows the reagents and consumables required to perform experiments with the ViiA™ 7 System.

Consumable		Part number	Shelf-life at environmental temperature	Storage conditions
Array Card Buckets/Clip Set	1st Generation	4337762	Use the consumable by the expiration date mentioned on the package	Room temperature
	2nd Generation	4442571		
TaqMan® Array Micro Fluidic Card Sealer		4331770		
MicroAmp® Optical 384-Well Reaction Plate with Barcode	1000 plates	4343814		
	500 plates	4326270		
	50 plates	4309849		
MicroAmp® Optical 384-Well Reaction Plate, 1000 plates		4343370		
MicroAmp® Optical 96-Well Reaction Plate (0.2µL)	500 plates	4316813		
	10 plates	N8010560		
MicroAmp® Optical 96-Well Reaction Plate with Barcode (0.2µL	500 plates	4326659		
	20 plates	4306737		
MicroAmp® Fast Optical 96-Well Reaction Plate (0.1µL)	10 plates	4346907		
MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1µL)	200 plates	4366932		
	20 plates	4346906		
Centrifuge Buckets, Array Card	1st Generation	4337230		
	2nd Generation	4442573		
Clip, Array Card Centrifuge Adaptor		4334682		
MicroAmp® Optical Adhesive Film	100 films	4311971		
	25 films	4360954		
MicroAmp® Multi-Removal Tool (1 tool)		4313950		
RT-PCR Grade Water		AM9935		
MicroAmp® Optical 8-Cap Strip	300 strips	4323032		
MicroAmp® Optical 8-Tube Strip (0.2µL)	125 strips	4316567		
MicroAmp® Fast 8-Tube Strip (0.1µL)	125 strips	4358293		
MicroAmp® Optical Tube without cap (0.2µL)	2000 tubes	N8010933		
MicroAmp® Fast Reaction Tube with cap (0.1µL)	1000 tubes	4358297		
MicroAmp® 96-Well Tray/Retainer Set (Blue) (for 0.2µL)	10 pairs	4381850		
MicroAmp® 96-Well Tray (Black) (for 0.1µL)	10 plates	4379983		

Consumable		Part number	Shelf-life at environmental temperature	Storage conditions
ViiA™ 7 System 96-Well Plate Adapter (0.2µL)	1 piece	4459845	Use the consumable by the expiration date mentioned on the package	Room temperature
ViiA™ 7 System Fast 96-Well Plate Adapter (0.1µL)	1 piece	4459846		
ViiA™ 7 System 96-Well Tube Adapter (0.2µL)	1 piece	4462077		
ViiA™ 7 System 96-Well Tube Adapter (0.1µL)	1 piece	4462078		
ViiA™ 7 System 384-Well Plate Adapter	1 piece	4457087		
ViiA™ 7 System Array Card Adapter	1 piece	4454166		
MicroAmp® Cap Installing Tool (Handle) (1 tool)		4330015		
MicroAmp® Adhesive Film Applicator (5 applicators)		4333183		

Reagents

The following table lists the reagents that can be ordered for performing experiments with the ViiA™ 7 System.

Recommended reagent kits

To perform	Reagent	Part Number
Reverse Transcription	SuperScript® VILO™ cDNA Synthesis Kit	4453650
TaqMan® PCR	TaqMan® Fast Advanced Master Mix	4444557
	TaqMan® GTXpress™ Master Mix	4401892
	TaqMan® Fast Virus 1-Step Master Mix	4444432
	TaqMan® Gene Expression Master Mix	4369016
	TaqMan® Genotyping Master Mix	4371355
	TaqMan® Universal Master Mix II, with UNG	4440038
	TaqMan® RNA-to-CT™ 1-Step Kit	4392938
SYBR® Green PCR	Fast SYBR® Green Master Mix	4385612
	Power SYBR® Green PCR Master Mix	4367659
	Power SYBR® Green RNA-to-CT™ 1-Step Kit	4389986

General-use materials and consumables

The following general-use materials and consumables are required to calibrate, maintain, and perform experiments with the ViiA™ 7 System. Unless indicated otherwise, all materials shown below are available from major laboratory suppliers (MLS). The materials are applicable to all sample blocks.

Material/Consumable	Source
Bleach, 10% solution	MLS
Lint-free lab tissues	MLS
Cotton or nylon swabs and lint-free cloths	MLS
Centrifuge with buckets appropriate for your consumable type	MLS
Ethanol, 95% solution	MLS
Glasses, safety	MLS
Gloves, powder-free	MLS
Permanent marker or pen	MLS
Pipettors: 100-µL and 200-µL (with pipette tips)	MLS
Screwdriver, flathead	MLS

B Safety

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Instrumentation safety

Symbols on instruments

Electrical symbols on instruments

The following table describes the electrical symbols that may be displayed on Applied Biosystems instruments.

Symbol	Description	Symbol	Description
	Indicates the **On** position of the main power switch.		Indicates a protective grounding terminal that must be connected to earth ground before any other electrical connections are made to the instrument.
	Indicates the **Off** position of the main power switch.		
	Indicates a terminal that may be connected to the signal ground reference of another instrument. This is not a protected ground terminal.		Indicates a terminal that can receive or supply alternating current or voltage.
			Indicates that the device receives or supplies direct current or voltage.

Safety symbols

The following table describes the safety symbols that may be displayed on Applied Biosystems devices. Each symbol may appear by itself or with text that explains the relevant hazard. These safety symbols may also appear next to DANGERS, WARNINGS, and CAUTIONS that occur in the text of this and other product-support documents.

Symbol	Description	Symbol	Description
	Indicates that you should proceed with appropriate caution and consult the product insert for further information. If a product insert does not exist, or if the product insert does not contain the symbol or the required information, consult the user manual.		Indicates the presence of a pinching hazard and to proceed with appropriate caution.
			Indicates the presence of moving parts and to proceed with appropriate caution.
			Indicates the presence of a biological hazard and to proceed with appropriate caution.
	Indicates the presence of an electrical shock hazard and to proceed with appropriate caution.		Indicates the presence of a laser light in the instrument and to proceed with appropriate caution.
	Indicates the presence of a hot surface or other high-temperature hazard and to proceed with appropriate caution.		Indicates the presence of an ultraviolet light and to proceed with appropriate caution.

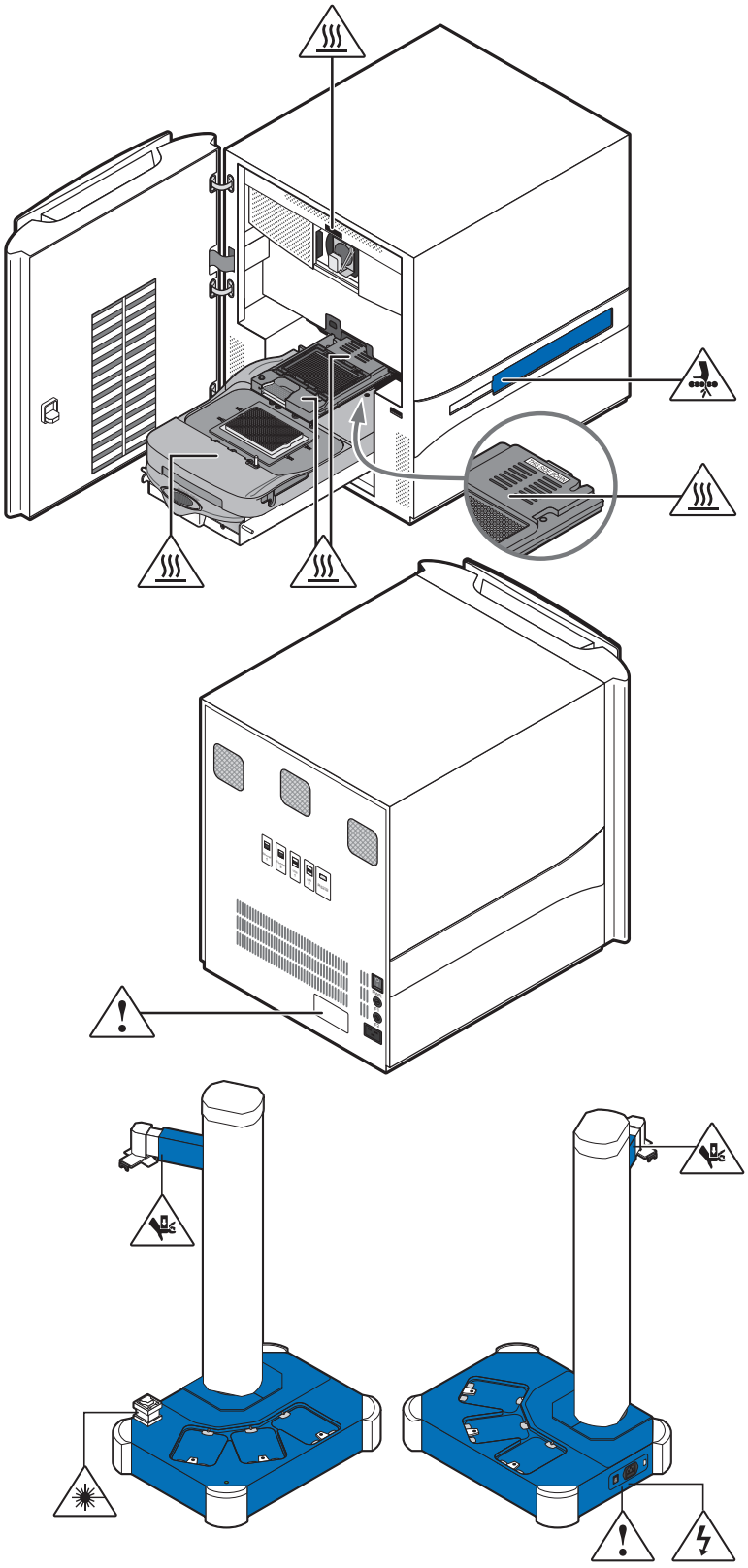
Environmental symbols on instruments

The following symbol applies to all Applied Biosystems electrical and electronic products placed on the European market after August 13, 2005.

Symbol	Description
	Do not dispose of this product as unsorted municipal waste. Follow local municipal waste ordinances for proper disposal provisions to reduce the environmental impact of waste electrical and electronic equipment (WEEE). **European Union customers:** Call your local Applied Biosystems Customer Service office for equipment pick-up and recycling. See **www.appliedbiosystems.com** for a list of customer service offices in the European Union.

Locations of safety labels on instruments

The ViiA™ 7 Instrument contains warnings at the locations shown below:



General instrument safety

WARNING! PHYSICAL INJURY HAZARD. Use this product only as specified in this document. Using this instrument in a manner not specified by Applied Biosystems may result in personal injury or damage to the instrument.

Moving and lifting the instrument

CAUTION! PHYSICAL INJURY HAZARD. The instrument is to be moved and positioned only by the personnel or vendor specified in the applicable site preparation guide. If you decide to lift or move the instrument after it has been installed, do not attempt to lift or move the instrument without the assistance of others, the use of appropriate moving equipment, and proper lifting techniques. Improper lifting can cause painful and permanent back injury. Depending on the weight, moving or lifting an instrument may require two or more persons.

Moving and lifting stand-alone computers and monitors

WARNING! Do not attempt to lift or move the computer or the monitor without the assistance of others. Depending on the weight of the computer and/or the monitor, moving them may require two or more people.

Things to consider before lifting the computer and/or the monitor:

- Make sure that you have a secure, comfortable grip on the computer or the monitor when lifting.
- Make sure that the path from where the object is to where it is being moved is clear of obstructions.
- Do not lift an object and twist your torso at the same time.
- Keep your spine in a good neutral position while lifting with your legs.
- Participants should coordinate lift and move intentions with each other before lifting and carrying.
- Instead of lifting the object from the packing box, carefully tilt the box on its side and hold it stationary while someone slides the contents out of the box.

Operating the instrument

Ensure that anyone who operates the instrument has:

- Received instructions in both general safety practices for laboratories and specific safety practices for the instrument.
- Read and understood all applicable Safety Data Sheets (SDSs). See "About SDSs" on page 20.

Cleaning or decontaminating the instrument

CAUTION! Before using a cleaning or decontamination method other than those recommended by the manufacturer, verify with the manufacturer that the proposed method will not damage the equipment.

Physical hazard safety

Ultraviolet light

WARNING! ULTRAVIOLET LIGHT HAZARD. Looking directly at a UV light source can cause serious eye damage. Never look directly at a UV light source and always prevent others from UV exposure. Follow the manufacturer's recommendations for appropriate protective eyewear and clothing.

Moving parts

WARNING! PHYSICAL INJURY HAZARD. Moving parts can crush and cut. Keep hands clear of moving parts while operating the instrument. Disconnect power before servicing the instrument.

Electrical safety

WARNING! ELECTRICAL SHOCK HAZARD. Severe electrical shock can result from operating the ViiA™ 7 Instrument without its instrument panels in place. Do not remove instrument panels. High-voltage contacts are exposed when instrument panels are removed from the instrument.

Fuses

WARNING! FIRE HAZARD. Improper fuses or high-voltage supply can damage the instrument wiring system and cause a fire. Before turning on the instrument, verify that the fuses are properly installed and that the instrument voltage matches the power supply in your laboratory.

WARNING! FIRE HAZARD. For continued protection against the risk of fire, replace fuses only with fuses of the type and rating specified for the instrument.

Power

WARNING! ELECTRICAL HAZARD. Grounding circuit continuity is required for the safe operation of equipment. Never operate equipment with the grounding conductor disconnected.

WARNING! ELECTRICAL HAZARD. Use properly configured and approved line cords for the voltage supply in your facility.

WARNING! ELECTRICAL HAZARD. Plug the system into a properly grounded receptacle with adequate current capacity.

Overvoltage rating

The ViiA™ 7 Instrument has an installation (overvoltage) category of II, and is classified as portable equipment.

Bar code scanner laser safety

Laser classification

The bar code scanners included with the ViiA™ 7 Instrument are categorized as Class 2 (II) lasers.

Laser safety requirements

Class 2 (II) lasers are low-power, visible-light lasers that can damage the eyes. Never look directly into the laser beam. The scanner is designed to prevent human access to harmful levels of laser light during normal operation, user maintenance, or during prescribed service operations.

WARNING! LASER HAZARD. Class 2 (II) lasers can cause damage to eyes. Avoid looking into a Class 2 (II) laser beam or pointing a Class 2 (II) laser beam into another person's eyes.

Workstation safety

Correct ergonomic configuration of your workstation can reduce or prevent effects such as fatigue, pain, and strain. Minimize or eliminate these effects by configuring your workstation to promote neutral or relaxed working positions.

CAUTION! MUSCULOSKELETAL AND REPETITIVE MOTION HAZARD. These hazards are caused by potential risk factors that include but are not limited to repetitive motion, awkward posture, forceful exertion, holding static unhealthy positions, contact pressure, and other workstation environmental factors.

To minimize musculoskeletal and repetitive motion risks:

- Use equipment that comfortably supports you in neutral working positions and allows adequate accessibility to the keyboard, monitor, and mouse.
- Position the keyboard, mouse, and monitor to promote relaxed body and head postures.

Safety and electromagnetic compatibility (EMC) standards

This section provides information on:

- U.S. and Canadian safety standards
- Canadian EMC standard
- European safety and EMC standards
- Australia and New Zealand EMC standards

U.S. and Canadian safety standards

The instrument has been tested to and complies with standard:

UL 61010-1:2nd Edition/CSA C22.2 No. 61010-1, "Safety Requirements for Electrical Equipment for Measurement, Control, and Laboratory Use, Part 1: General Requirements."

UL 61010-2-010, "Particular Requirements for Laboratory Equipment for the Heating of Materials."

Canadian EMC standard

This instrument has been tested to and complies with standard:

ICES-001, Issue 3: "Industrial, Scientific, and Medical Radio Frequency Generators."
Cet appareil numerique de la classe B est conforme a la norme NMB-001 du Canada.

European safety and EMC standards

Safety

This instrument meets European requirements for safety (Low Voltage Directive 2006/95/EC). This instrument has been tested to and complies with standards:

EN 61010-1:2001, "Safety Requirements for Electrical Equipment for Measurement, Control and Laboratory Use, Part 1: General Requirements."

EN 61010-2-010:2003, "Particular Requirements for Laboratory Equipment for the Heating of Materials."

EN 61010-2-081:2002+A1:2003, "Particular Requirements for Automatic and Semi-Automatic Laboratory Equipment for Analysis and Other Purposes."

EMC

The ViiA™ 7 Real-Time PCR System meets European requirements for emission and immunity (EMC Directive 2004/108/EC).

EN 61326-1:2006 "Electrical equipment for measurement, control and laboratory use-Part 1 General EMC requirements." (Group 1, Class B)

Australia and New Zealand EMC standards

This instrument has been tested to and complies with standard AS/NZS 2064, "Limits and Methods Measurement of Electromagnetic Disturbance Characteristics of Industrial, Scientific, and Medical (ISM) Radio-frequency Equipment."

Chemical safety

General chemical safety

Chemical hazard warning

WARNING! CHEMICAL HAZARD. Before handling any chemicals, refer to the Safety Data Sheet (SDS) provided by the manufacturer, and observe all relevant precautions.

WARNING! CHEMICAL HAZARD. All chemicals in the instrument are potentially hazardous. Always determine what chemicals have been used in the instrument before changing reagents or instrument components. Wear appropriate eyewear, protective clothing, and gloves when working on the instrument.

WARNING! CHEMICAL HAZARD. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

WARNING! CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About SDSs" on page 20.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

SDSs

About SDSs

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

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

Obtaining SDSs

The SDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain SDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **SDS**.
2. In the Keyword Search field, enter the chemical name, product name, SDS part number, or other information that appears in the SDS 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

Note: For the SDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Chemical waste safety

Chemical waste hazards

CAUTION! HAZARDOUS WASTE. Refer to Safety Data Sheets and local regulations for handling and disposal.

WARNING! CHEMICAL WASTE HAZARD. Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

WARNING! CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical waste safety guidelines

To minimize the hazards of chemical waste:

- Read and understand the Safety Data Sheets (SDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Handle chemical wastes in a fume hood.
- After emptying a waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste disposal

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

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

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

Biological hazard safety

General biohazard

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (**www.cdc.gov/biosafety/publications/index.htm**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **www.access.gpo.gov/ nara/cfr/waisidx_01/29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at **www.cdc.gov**.

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition **http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Safety alerts

For the definitions of the alert words **IMPORTANT**, **CAUTION**, **WARNING**, and **DANGER**, see "Safety alert words" on page 8.

General alerts for all chemicals

Avoid contact with (skin, eyes, and/or clothing). Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

General alerts for instrumentation

CAUTION! Before using a cleaning or decontamination method other than those recommended by the Applied Biosystems, verify with Applied Biosystems that the proposed method will not damage the equipment.

WARNING! This instrument is designed for 12 V, 75 W halogen lamps only.

Specific alerts for instrumentation

CAUTION! FIRE HAZARD. For continued protection against the risk of fire, replace fuses only with listed and certified fuses of the same type and rating as those currently in the instrument.

CAUTION! PHYSICAL INJURY HAZARD. Do not attempt to lift the instrument or any other heavy objects unless you have received related training. Incorrect lifting can cause painful and sometimes permanent back injury. Use proper lifting techniques when lifting or moving the instrument. At least two people are required to lift the instrument.

CAUTION! PHYSICAL INJURY HAZARD. Do not remove the instrument cover. There are no components inside the instrument that you can safely service yourself. If you suspect a problem, contact an Applied Biosystems Service Representative.

WARNING! PHYSICAL INJURY HAZARD. The ViiA™ 7 Instrument and lamp are hot! The lamp can become very hot while in use. Allow the lamp to cool for 15 minutes and put on protective, powder-free gloves before handling it.

CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the sample block can be heated to 100 °C. Before performing the following procedure, be sure to wait until the sample block reaches room temperature.

CAUTION! PHYSICAL INJURY HAZARD. Wear disposable, powder-free gloves when handling the lamp to prevent burns and to prevent shortening the life of the replacement lamp.

Documentation and Support

Related documentation

The following related documents are shipped with the system:

Document	PN	Description
Applied Biosystems ViiA™ 7 Real-Time PCR System Calibration, Maintenance, Networking, and Security User Guide	4442661	Explains how to use and maintain the Applied Biosystems ViiA™ 7 Real-Time PCR System Intended for laboratory staff responsible for the use and maintenance of the ViiA™ 7 Real-Time PCR Instrument.
Applied Biosystems ViiA™ 7 Real-Time PCR System Getting Started Guide	4441434	Contains five individual booklets that explain how to perform the six different experiments on the ViiA™ 7 Real-Time PCR Instrument The experiments include Standard Curve, Relative Standard Curve and Comparative C_T, Genotyping, Presence/ Absence and Melt Curve. Each Getting Started Guide booklet functions as both: • A tutorial, using example experiment data provided with the ViiA™ 7 Software. • A guide for your own experiments. Intended for laboratory staff and principal investigators who perform experiments using the ViiA™ 7 System.
Applied Biosystems ViiA™ 7 Real-Time PCR System Quick Reference Guide	4448987	Explains how to install and maintain the ViiA™ 7 Real-Time PCR Instrument Intended for laboratory staff responsible for the use and maintenance of the ViiA™ 7 Real-Time PCR Instrument.
Applied Biosystems ViiA™ 7 Real-Time PCR System Site Preparation Guide	4445302	Explains how to prepare your site to receive and install the ViiA™ 7 Real-Time PCR Instrument Intended for personnel who schedule, manage, and perform the tasks required to prepare your site for installation of the ViiA™ 7 Real-Time PCR Instrument.

Document	PN	Description
ViiA™ 7 Software Help	NA	Explains how to use the ViiA™ 7 Software to: • Set up, run, and analyze experiments. • Monitor a networked ViiA™ 7 Real-Time PCR Instrument. • Calibrate the ViiA™ 7 Real-Time PCR Instrument. • Verify the performance of ViiA™ 7 Real-Time PCR Instrument with an RNase P run. • Intended for: – Laboratory staff and principal investigators who perform experiments using the ViiA™ 7 System. – Laboratory staff responsible for the installation and maintenance of the ViiA™ 7 Real-Time PCR Instrument.

Note: For additional documentation, see "How to obtain support" on page 27.

Other related documents

Documents related to Genotyping experiments

Document	PN
Allelic Discrimination Pre-Developed TaqMan® Assay Reagents Quick Reference Card	4312212
Custom TaqMan® Genomic Assays Protocol	4367671
Custom TaqMan® SNP Genotyping Assays Protocol	4334431
Ordering TaqMan® SNP Genotyping Assays Quick Reference Card	4374204
Pre-Developed TaqMan® Assay Reagents Allelic Discrimination Protocol	4312214
TaqMan® Drug Metabolism Genotyping Assays Protocol	4362038
TaqMan® SNP Genotyping Assays Protocol	4332856

Documents related to Presence/ Absence experiments

Document	PN
DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol	4343586
NucPrep® Chemistry: Isolation of Genomic DNA from Animal and Plant Tissue Protocol	4333959
PrepMan® Ultra Sample Preparation Reagent Protocol	4318925

Documents related to Relative Standard Curve and Comparative C_T experiments

Document	PN
Amplification Efficiency of TaqMan® Gene Expression Assays Application Note	127AP05
Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol	4375575
Custom TaqMan® Gene Expression Assays Protocol	4334429
Primer Express® Software Version 3.0 Getting Started Guide	4362460
TaqMan® Gene Expression Assays Protocol	4333458
User Bulletin #2: Relative Quantitation of Gene Expression	4303859

Documents related to Standard Curve experiments

Document	PN
Amplification Efficiency of TaqMan® Gene Expression Assays Application Note	127AP05
Custom TaqMan® Gene Expression Assays Protocol	4334429
Primer Express® Software Version 3.0 Getting Started Guide	4362460
TaqMan® Gene Expression Assays Protocol	4333458
User Bulletin #2: Relative Quantitation of Gene Expression	4303859

How to obtain support

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

- Access 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, SDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

Obtaining information from the Help system

The ViiA™ 7 Software has a Help system that describes how to use each feature of the user interface. Access the Help system by doing one of the following:

- Click in the toolbar of the ViiA™ 7 Software window.
- Select **Help ▸ ViiA™ 7 Software Help**.
- Press **F1**.

You can use the Help system to find topics of interest by:

- Reviewing the table of contents
- Searching for a specific topic
- Searching an alphabetized index

You can also access PDF versions of all documents in theViiA™ 7 Software document set from the Help system.

Glossary

AIF	See assay information file (AIF).
AIX	XML version of the assay information file. See also assay information file (AIF).
allele	In a diploid organism, one of two DNA sequences found at the same locus (for example, a particular gene), but located on homologous chromosomes. Two corresponding alleles may have the identical sequence, or they may differ somewhat, often at one or more single-base sites (SNPs).
allelic discrimination plot	Display of genotyping data collected during the post-PCR read. The allelic discrimination plot is a graph of the normalized reporter signal from the allele 1 probe, plotted against the normalized reporter signal from the allele 2 probe.
amplicon	A segment of DNA amplified during PCR.
amplification	Part of the instrument run in which PCR amplifies the target. Fluorescence data collected during amplification are displayed in an amplification plot, and the data are used to calculate results. **Note:** Only quantitative real-time PCR experiments, not end-point experiments, take amplification data into account.
amplification efficiency (EFF%)	Calculation of the efficiency of the PCR amplification in an experiment. EFF% is calculated using the slope of the regression line in the standard curve. A slope close to –3.32 indicates optimal, 100% PCR amplification efficiency.
amplification plot	Display of data collected during the cycling stage of PCR amplification. The amplification plot can be viewed as: • Baseline-corrected normalized reporter (ΔRn) vs. cycle • Normalized reporter (Rn) vs. cycle • Threshold cycle (C_T) vs. well
amplification stage	Part of the instrument run in which PCR amplifies the target. The amplification stage, called a cycling stage in the thermal profile, consists of denaturing, primer annealing, and extension steps that are repeated. Fluorescence data collected during the extension stage are displayed in an amplification plot, and the data are used to calculate results. With TaqMan chemistry, the last two steps of a PCR stage are typically combined. See also cycling stage.
Analysis Settings Library	In the software, a collection of analysis settings to use in experiments. You can save settings and reuse them. You cannot edit or import settings into the library.

assay	In a PCR reaction mix, two target-specific primers or two primers and a probe used to amplify a target.
Assay ID	Identifier assigned by Applied Biosystems to TaqMan® assays.
assay information file (AIF)	Tab-delimited data file on a CD shipped with each assay order. The AIF contains technical details about all assays in the shipment. It includes information about assay concentrations; reporters and quenchers used; part and lot numbers; and assay, vial, and plate ID numbers. The file name includes the number from the bar code on the plate.
assay mix	PCR reaction component in Applied Biosystems TaqMan® assays. The assay mix contains primers designed to amplify a target and a TaqMan® probe designed to detect amplification of the target.
AutoDelta	In the run method, a setting to increase or decrease the temperature and/or time for a step with each subsequent cycle in a cycling stage. When AutoDelta is enabled for a cycling stage, the settings are indicated by an icon in the thermal profile: • AutoDelta on: ▲ • AutoDelta off: ▲
automatic baseline	An analysis setting in which the software calculates the baseline start and end cycles for the amplification plot. See also baseline.
automatic threshold	An analysis setting in which the software calculates the baseline start and end cycles and the threshold in the amplification plot. The software uses the baseline and threshold to calculate the threshold cycle (C_T). See also threshold cycle (C_T).
background calibration	Type of calibration in which the instrument performs reads of a background plate, averages the spectra recorded during the run, and extracts the resulting spectral component to a calibration file. The software then uses the calibration file during subsequent runs to remove the background fluorescence from the run data.
baseline	In the amplification plot, a cycle-to-cycle range that defines background fluorescence. This range can be set manually on an assay-by-assay basis, or automatically to set each individual well.
baseline-corrected normalized reporter (ΔRn)	The magnitude of normalized fluorescence signal generated by the reporter. In experiments that contain data from real-time PCR, the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. In the ΔRn vs Cycle amplification plot, ΔRn is calculated at each cycle as: ΔRn (cycle) = Rn (cycle) –Rn (baseline), where Rn = normalized reporter In genotyping experiments and presence/absence experiments, the difference in normalized fluorescence signal generated by the reporter between the pre-PCR read and the post-PCR read. In the allelic discrimination plot (genotyping experiments) and the presence/absence plot (presence/absence experiments), ΔRn is calculated as:

	ΔRn = Rn (post-PCR read) –Rn (pre-PCR read), where Rn = normalized reporter See also normalized reporter (Rn).
baseline threshold algorithm	Expression estimation algorithm (C_T) which subtracts a baseline component and sets a fluorescent threshold in the exponential region for gene quantification.
biological replicates	Reactions that contain identical components and volumes, but evaluate separate samples of the same biological source (for example, samples from three different mice of the same strain, or separate extractions of the same cell line or tissue sample). When an experiments uses biological replicate groups in a gene expression study, the values displayed in the Biological Replicates tab are calculated by combining the results of the separate biological samples and treating this collection as a single population (that is, as one sample). For ΔC_T computations (normalizing by the endogenous control) in a singleplex experiment, the software treats separate biological samples as unpaired data when computing variability estimates of the single biological replicate. Individual contributions of the separate biological samples to the single biological replicate results are observed in the Technical Replicates tab. See also technical replicates.
blocked IPC	In presence/absence experiments, a reaction that contains IPC blocking agent, which blocks amplification of the internal positive control (IPC). In ViiA™ 7 Software, also the name of the task for the IPC target in wells that contain IPC blocking agent. See also negative control-blocked IPC wells.
calibrator	See reference sample.
chemistry	See reagents.
comparative C_T ($\Delta\Delta C_T$) method	Method for determining relative target quantity in samples. The software measures amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the endogenous control. The software determines the relative quantity of target in each sample by comparing normalized target quantity in each sample to normalized target quantity in the reference sample.
C_{RT}	See relative threshold cycle (C_{RT}).
C_{RT} algorithm	See Relative Threshold algorithm.
C_T	See threshold cycle (C_T).
C_T algorithm	Algorithm used to determine the threshold cycle. The software provides two C_T algorithms: Baseline Threshold and Relative Threshold.
cycle threshold	See threshold cycle (C_T).
cycling stage	In the thermal profile, a stage that is repeated. A cycling stage is also called an amplification stage.

	See also amplification stage.
C_q	See quantification cycle (C_q).
data collection	During the instrument run, a process in which an instrument detects fluorescence data from each well of the reaction plate. The instrument transforms the signal to electronic data and saves the data in the experiment file. In the ViiA™ 7 Software, a data collection point is indicated by an icon in the thermal profile: • Data collection on: • Data collection off:
delta Rn (ΔRn)	See baseline-corrected normalized reporter (ΔRn).
diluent	A reagent used to dilute a sample or standard before it is added to the PCR reaction.
dilution factor	See serial factor.
dye calibration	Type of calibration in which the software collects spectral data from a series of dye standards and stores the spectral information for the dye standards in a pure spectra calibration file. This file is used during experiment runs to characterize and distinguish the individual contribution of each dye in the total fluorescence collected by the instrument.
Dye Library	In the software, a collection of dyes to use in experiments. Custom dyes can be added to the library, but system dyes cannot be removed. Before using a dye, make sure that the dye calibration is current in the Instrument Console.
EFF%	See amplification efficiency (EFF%).
efficiency correction	In Comparative C_T experiments, a feature that allows you to manually enter previously-determined amplification efficiencies for each experiment, following the experimental run. The real-time software mathematically compensates for differences in efficiency between each target assay and the endogenous control when calculating sample-to-sample relative quantities. This method can be employed as a substitute for the Relative Standard Curve Method.
endogenous control	A gene that is used to normalize template differences and sample-to-sample or run-to-run variation.
endpoint read	See post-PCR read.
error	The standard error of the slope of the regression line in the standard curve. The error can be used to calculate a confidence interval (CI) for the slope. Because the amplification efficiency (EFF%) is calculated from the slope, knowing the error allows a CI for the amplification efficiency to be calculated.

experiment	Refers to the entire process of performing a run, including setup, run, and analysis. You can perform the following types of experiments: • Quantification - Standard curve • Quantification - Relative standard curve • Quantification - Comparative C_T ($\Delta\Delta C_T$) • Melt Curve • Genotyping • Presence/absence
experiment document	The Applied Biosystems name for the electronic records that comprise all information about a particular plate or array card consumable, including metadata (name, bar code, comments), plate setup (well contents, assay definitions), run method (thermal cycling protocol), run results, analysis protocol, analysis results, audit records, and other plate-specific data. Experiment documents have the suffixes .eds (experiment document single), .edt (template), and .edm (multiple).
experiment name	Entered during experiment setup, the name that is used to identify the experiment.
Experiment Setup	A software feature that allows you to set up an experiment according to your experiment design. Experiment Setup provides you with maximum flexibility in the design and setup of your experiment.
experiment type	The type of experiment to perform: • Standard curve • Comparative C_T ($\Delta\Delta C_T$) • Relative standard curve • Genotyping • Presence/absence • Melt curve The experiment type that you select affects setup, run, and analysis.
export	A software feature that allows you to export experiment setup files, experiment results, instrument information, and security and auditing settings to spreadsheet, presentation, or text files. You can edit the default location of the exported file.
filter	Dye excitation and emission filter combination that you select for an experiment. The ViiA™ 7 System includes a six-color filter set that supports FAM™, ROX™, TAMRA™, and VIC® dyes.
flag	A quality control (QC) indicator which, when applied by the software to a well during analysis, indicates a possible issue with that reaction. For example, a flag may be issued if no amplification is detected in a well. Flags indicating potential problems are displayed in the Quality Control tab of the plate layout, well table, and QC Summary screens.
forward primer	Oligonucleotide that flanks the 5′ end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.

genotyping experiment	An experiment used to identify known mutations in a DNA sample. With this experiment type, you can determine if a DNA sample is: • Homozygous (samples having only allele 1). Also called wild type homozygote. • Homozygous (samples having only allele 2). Also called variant homozygote. • Heterozygous (samples having both allele 1 and allele 2).
heterozygote	Samples having both allele 1 and allele 2. See also genotyping experiment.
holding stage	In the thermal profile, the stage that holds the temperature constant for a defined period of time. A stage that includes one or more steps. You can add a holding stage to the thermal profile to activate enzymes, to inactivate enzymes, or to incubate a reaction.
homozygote	Samples having only allele 1 or only allele 2. See also genotyping experiment.
housekeeping gene	A gene that is involved in basic cellular functions and that may be constitutively expressed. Housekeeping genes may be candidates for use as endogenous controls; however, their constancy should always be validated experimentally. See also endogenous control.
import	A software feature that allows you to import plate setup information or security settings before an experiment run. You can also import information into some libraries in the system.
Instrument Console	A software feature that allows you to view information about instruments on the network. In the Instrument Console, you can monitor the status of any instrument on the network; view calibration, maintenance, and instrument properties for a selected instrument; and open and close the instrument drawer.
Instrument Manager	A software feature that allows you to view information about instrument available on the network. In the Instrument Manager, you can monitor the status of an instrument; monitor amplification plots and temperature plots in real time; view the calibration status, perform calibrations and manage files on the instrument, including downloading completed experiments to your computer.
internal positive control (IPC)	In presence/absence experiments, a short synthetic DNA template that is added to PCR reactions. The IPC can be used to distinguish between true negative results (the target is absent in the samples) and negative results caused by PCR inhibitors, incorrect assay setup, or reagent or instrument failure.
inventoried assays	TaqMan® Gene Expression Assays and TaqMan® SNP Genotyping Assays that have been previously manufactured, passed quality control specifications, and stored in inventory.
IPC	See internal positive control (IPC).
IPC blocking agent	Reagent added to PCR reactions to block amplification of the internal positive control (IPC).

IPC+	See negative control-IPC wells.
made-to-order assays	TaqMan® Gene Expression Assays that are manufactured at the time of order. Only assays that pass manufacturing quality control specifications are shipped.
manual baseline	An analysis setting for the Baseline Threshold algorithm. You enter the baseline start and end cycles for the amplification plot. See also baseline.
manual threshold	An analysis setting for the Baseline Threshold algorithm. You enter the threshold value and select whether to use automatic baseline or manual baseline values. The software uses the baseline and the threshold values to calculate the threshold cycle (C_T).
melt curve	A plot of data collected during the melt curve stage. Peaks in the melt curve can indicate the melting temperature (T_m) of the target, or they can identify nonspecific PCR amplification. In the software, you can view the melt curve as normalized reporter (R_n) vs. temperature or as derivative reporter ($-R_n'$) vs. temperature. In a high resolution melting experiment, you can view the melt curve as fluorescence vs. temperature. Also called dissociation curve.
melt curve characteristics	The melt curve shape and the difference in melting temperature (T_m) values.
melt curve stage	In the thermal profile, a stage with a temperature increment to generate a melt curve.
melting temperature (T_m)	The temperature at which 50% of the DNA is double-stranded and 50% of the DNA is dissociated into single-stranded DNA. In a melt curve experiment, the melt curve plot displays the melting temperature.
multicomponent plot	A plot of the complete spectral contribution of each dye for the selected well(s) over the duration of the PCR run.
negative control (NC)	The task for targets or SNP assays in wells that contain water or buffer instead of sample. No amplification of the target should occur in negative control wells. Previously called no template control (NTC).
negative control-blocked IPC wells	In presence/absence experiments, wells that contain IPC blocking agent instead of sample in the PCR reaction. No amplification should occur in negative control-blocked IPC wells because the reaction contains no sample and amplification of the IPC is blocked. Previously called no amplification control (NAC).
negative control-IPC wells	In presence/absence experiments, wells that contain IPC template and buffer or water instead of sample. Only the IPC template should amplify in negative control-IPC wells because the reaction contains no sample. Previously called IPC+.
no amplification control (NAC)	See negative control-blocked IPC wells.
no template control (NTC)	See negative control (NC).

nonfluorescent quencher-minor groove binder (NFQ-MGB)	Molecules that are attached to the 3' end of TaqMan® probes. When the probe is intact, the nonfluorescent quencher (NFQ) prevents the reporter dye from emitting fluorescence signal. Because the NFQ does not fluoresce, it produces lower background signals, resulting in improved precision in quantification. The minor groove binder (MGB) increases the melting temperature (T_m) of the probe without increasing its length, allowing for the design of shorter probes.
normalization calibration	Type of calibration in which the software collects data from the normalization standards, then stores it in a normalization calibration file. This file is used in comparisons of data from multiple instruments within a study.
normalized quantity	Either the C_T Avg. of the target gene minus the C_T Avg. of the endogenous control (Comparative C_T experiments), or the Q Avg. of the target divided by the Q Avg. of the endogenous control (Relative Standard Curve experiments).
normalized quantity mean	The relative standard curve equivalent of the ΔC_T mean value found in Comparative C_T experiments (computed as the geometric mean).
normalized quantity SE	The relative standard curve equivalent of the ΔC_T SE value found in Comparative C_T experiments (computed as the geometric standard error of the mean).
normalized reporter (Rn)	Fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference dye (usually ROX dye on Applied Biosystems instruments).
omit well	An action that you perform before reanalysis to omit one or more wells from analysis. Because no algorithms are applied to omitted wells, omitted wells contain no results. You can add wells back in to the analysis; no information is permanently discarded.
outlier	A measurement (such as a C_T) that deviates significantly from the measurement of the other replicates for that same sample.
passive reference	A dye that produces fluorescence signal independent of PCR amplification, and that is added to each reaction at a constant concentration. Because the passive reference signal should be consistent across all wells, it is used to normalize the reporter dye signal to account for non-PCR related fluorescence fluctuations caused by minor well-to-well differences in volume. Normalization to the passive reference signal generally results in data with noticeably high precision among technical replicates.
plate layout	An illustration of the grid of wells and assigned content in the reaction plate. The number of rows and columns in the grid depends on the sample block that you use. In the software, you can use the plate layout as a selection tool to assign well contents, to view well assignments, and to view results. The plate layout can be printed, included in a report, exported, and saved as a slide for a presentation.
plate setup file	A file (.txt, .csv, .xml, or .sds) that contains setup information such as the well number, sample name, sample color, target name, dyes, and other reaction plate contents.

point	One standard in a standard curve. The standard quantity for each point in a standard curve is calculated based on the starting quantity and serial factor.
positive control	In genotyping and presence/absence experiments, a DNA sample with a known genotype, homozygous or heterozygous. In the software, the task for the SNP assay in wells that contain a sample with a known genotype.
post-PCR read	In genotyping and presence/absence experiments, the part of the instrument run that occurs after amplification. In genotyping experiments, fluorescence data collected during the post-PCR read are displayed in the allelic discrimination plot and used to make allele calls. In presence/absence experiments, fluorescence data collected during the post-PCR read are displayed in the presence/absence plot and used to make detection calls. Also called endpoint read.
pre-PCR read	In genotyping and presence/absence experiments, the part of the instrument run that occurs before amplification. The pre-PCR read is optional but recommended. Fluorescence data collected during the pre-PCR read can be used to normalize fluorescence data collected during the post-PCR read.
primer mix	PCR reaction component that contains the forward primer and reverse primer designed to amplify the target.
primer/probe mix	PCR reaction component that contains the primers designed to amplify the target and a TaqMan® probe designed to detect amplification of the target.
pure dye	Fluorescent compound used to calibrate the instrument. See system dye.
quantification cycle (C_q)	The fractional PCR cycle used for quantification, according to the Real-time PCR Data Markup Language (RDML) data standard. C_T and C_{RT} are the algorithm-specific calculations of C_q.
quantification method	In quantification experiments, the method used to determine the quantity of target in the samples.
quantity	In quantification experiments, the amount of target in the samples. Absolute quantity can refer to copy number, mass, molarity, or viral load. Relative quantity refers to the fold-difference between normalized quantity of target in the sample and normalized quantity of target in the reference sample.
quencher	A molecule attached to the 3′ end of TaqMan® probes to prevent the reporter from emitting fluorescence signal while the probe is intact. With TaqMan® reagents, a nonfluorescent quencher-minor groove binder (NFQ-MGB) can be used as the quencher.
QuickStart	A feature that allows you to run an experiment without entering plate setup information, if your instrument and computer are in the same network. QuickStart requires an experiment template file.

R^2 value	Regression coefficient calculated from the regression line in the standard curve. An important quality value, the R^2 value indicates the closeness of fit between the standard curve regression line and the individual C_T data points from the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points.
ramp	The step at which the temperature changes during the instrument run. The ramp rate is defined as °C per second. In the graphical view of the thermal profile, the ramp rate is indicated by a diagonal line.
ramp speed	Speed at which the temperature ramp occurs during the instrument run. Available ramp speeds include fast and standard.
raw data plot	A plot of raw fluorescent signal as detected through each emission filter, used to view raw data for individual wells and at individual cycles.
reaction mix	A solution that contains all components to run the PCR reaction, except for the template (sample, standard, or control). Also called a "PCR cocktail".
reagents	The PCR reaction components used to amplify the target and to detect amplification.
real-time PCR	Process of collecting fluorescence data during PCR. Data from the real-time PCR are used to calculate results for quantification experiments or to troubleshoot results for genotyping or presence/absence experiments.
Real-time PCR Data Markup Language (RDML)	A reporting format that is compliant with the Minimum Information for Publication for Quantitative Real Time Experiments (MIQE) guidelines.
reference sample	In relative standard curve and Comparative C_T ($\Delta\Delta C_T$) experiments, the sample used as the basis for relative quantification results. Also called the calibrator.
refSNP ID	The reference SNP (refSNP) cluster ID. Generated by the Single Nucleotide Polymorphism Database of Nucleotide Sequence Variation (dbSNP) at the National Center for Biotechnology Information (NCBI). The refSNP ID can be used to search the Applied Biosystems Store for an Applied Biosystems SNP Genotyping Assay. Also called an rs number.
region of interest (ROI) calibration	Type of calibration in which the software maps the positions of the wells on the sample block of the instrument. The software uses the ROI calibration data to associate increases in fluorescence during a run with specific wells of the plate. A calibration image for each individual filter must be generated to account for minor differences in the optical path.
regression coefficients	Values calculated from the regression line in standard curves, including the R^2 value, slope, and y-intercept. You can use the regression coefficients to evaluate the quality of results from the standards. See also standard curve.
regression line	In standard curve and relative standard curve experiments, the best-fit line from the standard curve. Regression line formula:

	$C_T = m [\log (Qty)] + b$ where m is the slope, b is the y-intercept, and Qty is the standard quantity. See also regression coefficients.
reject well	An action that the software performs during analysis to remove one or more wells from further analysis if a specific flag is applied to the well.
relative standard curve method	An experimental method to determine relative quantities. This method compensates for target and endogenous control efficiency differences within each run. In all experiments, unknown samples and dilution series of template (such as cDNA) are amplified. Following a run, the instrument software interpolates relative quantities for each unknown sample from the appropriate dilution curve, then normalizes the data for each sample (or set of replicates) as follows: target QAvg. ÷ endogenous control QAvg.
Relative Threshold algorithm	Expression estimation algorithm (C_{RT}) which calculates a relative threshold from a fitted efficiency model for gene quantification.
relative threshold cycle (C_{RT})	The PCR cycle number for the threshold calculated from the modeled amplification efficiency profile.
replicate group	A user-defined biological grouping. A replicate group may be a set of identical reactions in an experiment.
replicates	Total number of identical reactions containing identical components and identical volumes.
reporter	A fluorescent dye used to detect amplification. With TaqMan® reagents, the reporter dye is attached to the 5′ end. With SYBR® Green reagents, the reporter dye is SYBR® Green dye. SYBR® and HRM-specific dyes are DNA-binding dyes.
reverse primer	An oligonucleotide that flanks the 3′ end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.
reverse transcriptase	An enzyme that converts RNA to cDNA.
R_n	See normalized reporter (Rn).
ROX™ dye	A dye supplied by Applied Biosystems and precalibrated on the instrument. ROX dye is used as the passive reference.
rs number	See refSNP ID.
run method	Definition of the reaction volume and the thermal profile for the instrument run. The run method specifies the temperature, time, ramp, and data collection points for all steps and stages of the instrument run.
sample	The biological tissue or specimen that you are testing for a target gene.

sample definition file	A tab-delimited file (*.txt or *.csv) that contains the following setup information: well number, sample name, and custom sample properties.
Sample Library	In the software, an editable collection of sample names to use in experiments. The samples in the library contain the sample name and the sample color. The samples in the library may also contain comments about the sample.
sample/SNP assay reaction	In genotyping experiments, the combination of the sample to test and the SNP assay to perform in one PCR reaction. Each PCR reaction can contain only one sample and one SNP assay.
sample/target reaction	In quantification experiments, the combination of the sample to test and the target to detect and quantify in one PCR reaction.
security, auditing and eSignature	An optional software module that provides: • **System Security** – Controls user access to the software. A default Administrator user account is provided, and you can define additional user accounts and permissions. • **Auditing** – Tracks changes made to library items, actions performed by users, and changes to the Security and Audit settings. The software automatically audits some actions silently. You can select other items for auditing and specify the audit mode. Provides reports for audited library items, Security and Audit changes, and actions. • **Electronic Signature (eSignature) –** Controls whether users are permitted, prompted, or required to provide a user name and password when accessing certain software features. You can select which features are controlled and the number of signatures required for access. When authorized persons use this feature, they are creating a legally binding signature.
serial factor	In the software, a numeric value that defines the sequence of quantities in the standard curve. The serial factor and the starting quantity are used to calculate the standard quantity for each point in the standard curve. For example, if the standard curve is defined with a serial factor of 1:10 or 10×, the difference between any 2 adjacent points in the curve is 10-fold.
slope	Regression coefficient calculated from the regression line in the standard curve. The slope indicates the PCR amplification efficiency for the assay. A slope of -3.32 indicates 100% amplification efficiency. See also amplification efficiency (EFF%) and regression line.
SNP	Single nucleotide polymorphism. The SNP can consist of a base difference or an insertion or deletion of one base.
SNP assay	Used in genotyping experiments, a PCR reaction that contains primers to amplify the SNP and two probes to detect different alleles.
SNP Assay Library	In the software, an editable collection of SNP assays to add to genotyping experiments. The SNP assays in the library contain the SNP assay name; SNP assay color; and for each allele, the allele name or base(s), reporter, quencher, and allele colors. The SNP assays in the library may also contain the assay ID and comments about the SNP assay.

stage	In the thermal profile, a group of one or more steps. Examples: PCR stage, cycling stage (also called amplification stage), and hold stage.
standard	A sample that you dilute and amplify along with unknown samples. This dilution series can contain known starting quantities of the target of interest (absolute standard curve) or it can be of known dilution factor (relative standard curve). Following the run, the software interpolates the C_T values of the unknowns to this curve, yielding either specific quantities of the target (for absolute curves) or relative quantities (for relative dilution curves). See also standard curve.
standard curve	In standard curve and relative standard curve experiments: • The best-fit line in a plot of the C_T values from the standard reactions plotted against standard quantities. See also regression line. • A set of standards containing a range of known quantities. Results from the standard curve reactions are used to generate the standard curve. The standard curve is defined by the number of points in the dilution series, the number of standard replicates, the starting quantity, and the serial factor.
standard curve method	Method for determining absolute target quantity in samples. With the standard curve method, the software measures amplification of the target in samples and in a standard dilution series. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates the absolute quantity of target in the samples. See also standard and standard curve.
standard dilution series	In standard curve and relative standard curve experiments, a set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards. For example, the standard stock is used to prepare the first dilution point, the first dilution point is used to prepare the second dilution point, and so on. In the software, the volumes needed to prepare a standard dilution series are calculated by the number of dilution points, the number of standard replicates, the starting quantity, the serial factor, and the standard concentration in the stock. See also standard curve.
standard quantity	In the PCR reaction, a known quantity. In standard curve experiments, the quantity of target in the standard. In the software, the units for standard quantity can be for mass, copy number, viral load, or other units for measuring the quantity of target. Standard quantity can also refer to dilution factor.
starting quantity	When defining a standard curve in the software, the highest quantity.
step	A component of the thermal profile. For each step in the thermal profile, you can set the ramp rate (ramp increment for melt curve steps), hold temperature, and hold time (duration).You can turn data collection on or off for the ramp or the hold parts of the step. For cycling stages, a step is also defined by the AutoDelta status.
SYBR® Green reagents	PCR reaction components that consist of two primers designed to amplify the target and SYBR® Green dye to facilitate detection of the PCR product.

system dye

Dye supplied by Applied Biosystems and precalibrated on the ViiA™ 7 System. Before you use system dyes in your experiments, make sure the system dye calibration is current in the Instrument Console.

The system dyes are:

- FAM™ dye
- JOE™ dye
- ROX™ dye
- NED™ dye
- SYBR® Green dye
- TAMRA™ dye
- VIC® dye

TaqMan® reagents

PCR reaction components that consist of primers designed to amplify the target and a TaqMan® probe designed to detect amplification of the target.

target

The nucleic acid sequence to amplify and detect.

target color

In the software, a color assigned to a target to identify the target in the plate layout and analysis plots.

Target Library

In the software, an editable collection of targets to use in experiments. Targets in the library contain the target name, reporter, quencher, and target color. The targets in the library may also contain comments about the target.

task

In the software, the type of reaction performed in the well for the target or SNP assay. Available tasks:

- Unknown
- Negative Control
- Standard (standard curve and relative standard curve experiments)
- Positive control (genotyping experiments)
- IPC (presence/absence experiments)
- Blocked IPC (presence/absence experiments)

technical replicates

Wells containing identical reaction components, including sample; important for evaluating precision.

temperature plot

In the software, a display of temperatures for the instrument cover and instrument block during the instrument run.

template

The type of nucleic acid to add to the PCR reaction.

template file

A user-created file that contains experiment setup information (experiment type, sample names, target name, and thermal conditions) to be used as a starting point for new experiment setup. Template files have an .edt extension.

thermal profile

Part of the run method that specifies the temperature, time, ramp, and data collection points for all steps and stages of the instrument run.

threshold	• In amplification plots, the level of fluorescence above the baseline and within the exponential growth region. For the Baseline Threshold algorithm, the threshold can be determined automatically (see automatic threshold) or can be set manually (see manual threshold). • In presence/absence experiments, the level of fluorescence above which the software assigns a presence call.
threshold cycle (C_T)	The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.
Tm	See melting temperature (T_m).
touchscreen	Instrument display that you touch to control the instrument.
uniformity calibration	Type of calibration in which the software measures sample block uniformity. The calibration generates data that compensate for the physical effects of the ViiA™ 7 System filters on data collected during an experiment.
unknown	In the software, the task for the target or SNP assay in wells that contain the sample being tested. In quantification experiments, the task for the target in wells that contain a sample with unknown target quantities. In genotyping experiments, the task for the SNP assay in wells that contain a sample with an unknown genotype. In presence/absence experiments, the task for the target in wells that contain a sample in which the presence of the target is not known. In melt curve experiments, the task for the target in wells that contain a sample with an unknown melt curve profile.
unknown-IPC wells	In presence/absence experiments, wells that contain a sample and internal positive control (IPC).
y-intercept	In the standard curve, the value of y where the regression line crosses the y-axis. The y-intercept indicates the expected threshold cycle (C_T) for a sample with quantity equal to 1.

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