USER GUIDE

applied biosystems®
by life technologies™

High Resolution Melt Module for QuantStudio™ 6 and 7 Flex Real-Time PCR System Software

Getting Started Guide

for use with:
QuantStudio™ 6 and 7 Flex Real-Time PCR Systems

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Revision A

life technologies™

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About This Guide

CAUTION! ABBREVIATED SAFETY ALERTS. Hazard symbols and hazard types specified in procedures may be abbreviated in this document. For the complete safety information, refer to the instrument user guide.

IMPORTANT! Before using this product, read and understand the information in the instrument user guide.

Revision history

Revision	Date	Description
A	October 2013	New document

Purpose

The *High Resolution Melt Module for QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide* is designed to help you quickly learn to perform High Resolution Melt experiments with the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software.

This guide provides step-by-step procedures for:

- Calibrating the QuantStudio™ 6 and 7 Flex Instruments for High Resolution Melt experiments
- Performing High Resolution Melt (HRM) experiments: Designing the experiment, preparing the reactions, running the reactions, and reviewing and analyzing the HRM data using the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software

Prerequisites

This getting started guide is intended for personnel who have been specifically trained by Life Technologies. The manufacturer is not liable for damage or injury that results from use of this manual by unauthorized or untrained parties.

This guide assumes that you have working knowledge of the:

- Microsoft® Windows® operating system
- Software for your QuantStudio™ 6 and 7 Flex Instruments
- General techniques for handling DNA samples and preparing them for PCR

Note: Ensure that you have activated and installed the HRM license in the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software. In the absence of the license, the HRM module will not be visible in the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software. For instructions on activating and installing the HRM license, see "Activate the HRM license" on page 14.

How to use this guide

This guide functions as both a tutorial and a guide for performing an HRM experiment. It contains:

- Instructions specific to the Genotyping example experiment data file provided in the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software
- Tips for running your own experiments

Note: First-time users of the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software, please read Booklet 1, *Getting Started with QuantStudio™ 6 and 7 Flex System Software Experiments* in the *QuantStudio™ 6 and 7 Flex System Software Getting Started Guide* (Pub. no. 4489822).

User attention words

Five user attention words may appear in this document. Each word implies a particular level of observation or action as described below:

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

IMPORTANT! Provides information that is necessary for proper instrument operation or accurate chemistry kit use.

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.

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

DANGER! Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury.

Except for IMPORTANTs, the safety alert words in user documentation appear with an open triangle figure that contains a hazard symbol. These hazard symbols are identical to the hazard symbols that are affixed to the instrument. See the "Safety" appendix for descriptions of the symbols.

1 HRM Experiment Overview

About HRM experiments

High Resolution Melt (HRM) analysis is a post-PCR analysis method used for identifying genetic variation in nucleic acid sequences. Simple and fast, this method is based on PCR melt (dissociation) curve techniques and is enabled by the recent availability of improved double-stranded DNA (dsDNA)–binding dyes along with next-generation real-time PCR instrumentation and analysis software. HRM analysis can discriminate DNA sequences based on their composition, length, GC content, or strand complementarity.

The QuantStudio™ 6 and 7 Flex Real-Time PCR System Software can perform:

- **Mutation scanning experiments** – Screen DNA samples for new single-base changes, insertions/deletions, or other unknown mutations.

  The mutation scanning experiment product can be used for subsequent sequencing reactions.
- **Methylation studies** – Determine the percentage of methylated DNA in unknown samples.
- **Genotyping experiments** – Determine the genotype of a DNA sample.

For all types of experiments, the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software compares the melt curves of unknown samples against the melt curves of positive controls to identify groups of variants.

The type of sample used as the positive controls depends on the type of experiment:

- **Mutation scanning experiments** – One or more samples with the wild type sequence are used as the control. For unknowns, the call is either "wild type" (if it matches the control) or "variant X".
- **Methylation studies** – Methylated DNA standards that contain from 0% to 100% methylated DNA are used as the positive controls. The software identifies the % methylation of the variants based on their comparison to the standards.
- **Genotyping experiments** – Three samples are used as controls: one homozygous for Allele 1, one homozygous for Allele 2, and one heterozygous for both alleles (Allele 1 and Allele 2). The software identifies the genotypes of the unknown variants.

  Note: The example experiment shows a basic genotyping experiment. For information on running mutation and methylation experiments, see Chapter 8, "Perform an HRM Mutation Scanning Experiment" on page 63 and Chapter 9, "Perform an HRM Methylation Study" on page 73 respectively.

HRM experiment workflow

The HRM experiment workflow is straightforward; most of the work lies in the design of the PCR primers, reagents, and reaction conditions. For more information see, "Design an HRM Genotyping Experiment" on page 23.

Calibrate the QuantStudio™ 6 or 7 Instrument

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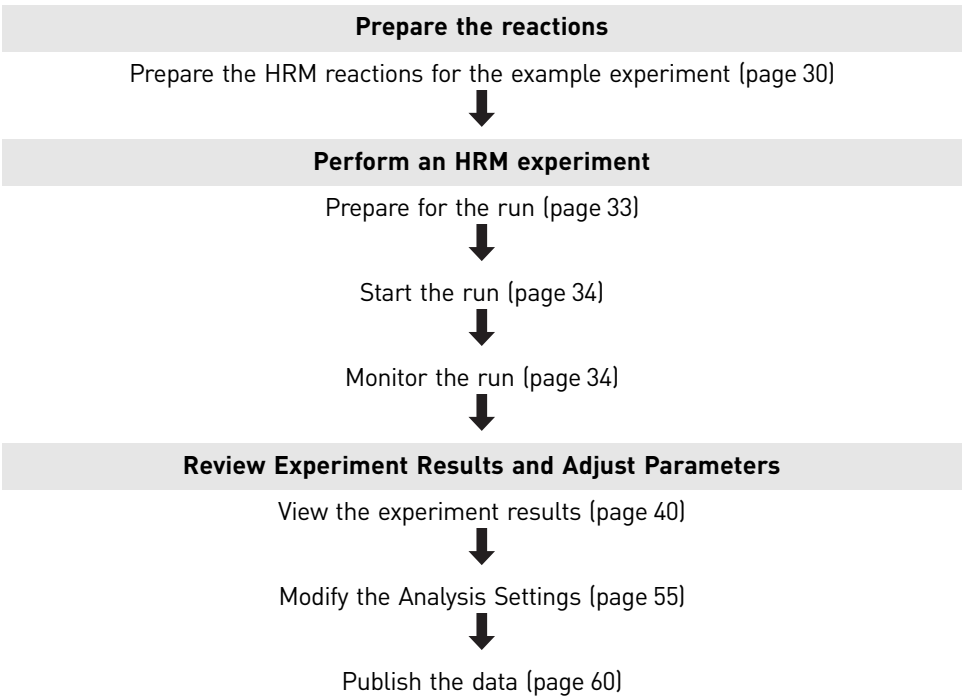


About the HRM example experiment

To illustrate how to perform HRM experiments, the software installs an example High Resolution Melt experiment file to lead you through the process of designing, preparing, running, and analyzing an HRM experiment. The example experiment allows you to quickly familiarize yourself with the process of performing a High Resolution Melt experiment on the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software.

To view the example experiment in the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software: **Open ▸ Program Files ▸ Applied Biosystems ▸ QuantStudio 6 and 7 Flex Software ▸ examples ▸ QS6Flex ▸ QS6_384-Well_High_Resolution_Melt_Example.eds**

Note: To install and activate the HRM license in the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software, see "Activate the HRM license" on page 14.

Note: You can also perform the High Resolution Melt experiment using another example experiment located at C:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\QS7Flex\QS7_384-Well_High_Resolution_Melt_Example.eds

The example experiment is a very basic genotyping experiment and it is intended for instructional purposes only. The experiment has these characteristics:

- It is comprised of 3 samples, Homo 1 (Allele A), Homo 2 (Allele G), and Hetero (Allele A/G).
- The 384-well plate contains 96 wells of each of the above samples. For each sample, 95 wells are designated as "unknowns" and 1 well is designated as the positive control.

- The remaining 96 wells in the example experiment plate are empty.
- There are no negative controls.

Perform the example experiment with the MeltDoctor™ HRM Positive Control Kit. All the materials for the experiment are contained in the MeltDoctor™ HRM Positive Control Kit.

Note: If you are using the MeltDoctor™ HRM Positive Control Kit to run the example experiment, you do not need to design primers because the kit contains primers designed to amplify the alleles in the positive control DNA.

Tips for running your own HRM experiment

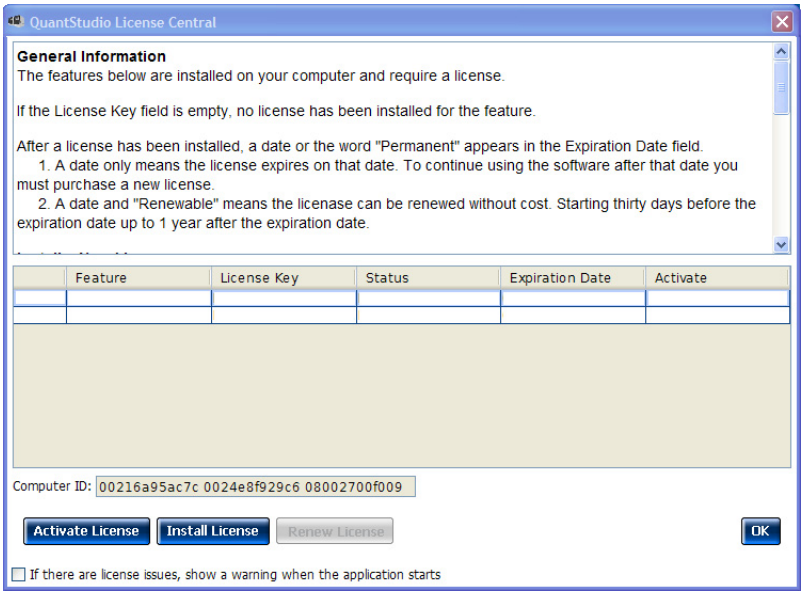
This guide contains instructions specific to the HRM example experiment. It also functions as a guide for your own experiments; tips for running your own experiments are provided at various points.

Note: When you create your own HRM experiments, you may wish to keep the example file open on another tab in the software and use it as a reference.

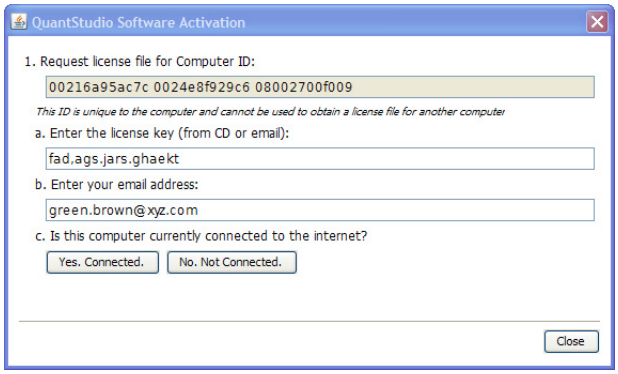
Activate the HRM license

To install and activate the license for the HRM module in the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software:

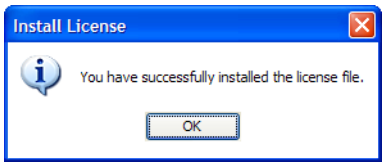
1. On the Home screen, go to **Tools** ▸ **License Central...**
2. In the QuantStudio License Central dialog box, click **Activate License**.



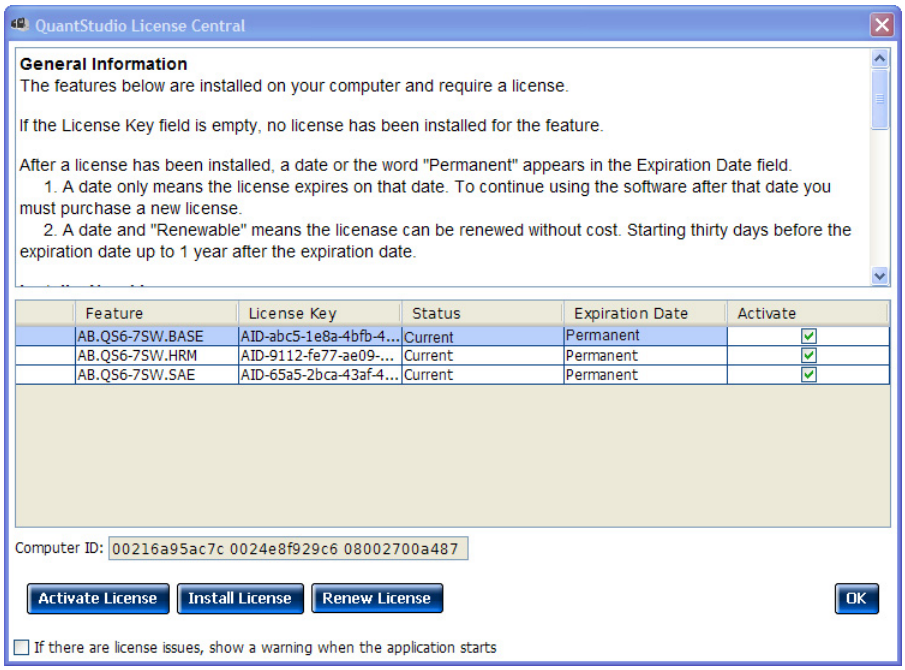
The QuantStudio Software Activation dialog box opens.



3. In the license key field, enter the license key that you have on the software CD or have received on email.
4. Enter your email address to receive the license activation file.
5. Close the QuantStudio Software Activation dialog box after you have clicked the appropriate selection for the computer connectivity.
6. Click **Install License** to navigate to the location of the license file on your computer. Select the appropriate file and click **Open**.
7. Click **OK** to close the Install License dialog box.



8. The HRM license should appear in the licenses list in the QuantStudio License Central dialog box.



2 Calibrate the QuantStudio™ 6 and 7 Flex Instruments

IMPORTANT! Refer to the instrument user guide for safety information and guidelines on using the QuantStudio™ 6 and 7 Instruments.

About HRM calibration

This chapter describes how to calibrate the QuantStudio™ 6 and 7 Flex Instruments using the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software for either the MeltDoctor™ HRM Dye or for an alternative HRM dye.

During HRM calibration, the QuantStudio™ 6 or 7 Instrument performs a PCR amplification of the template in the HRM calibration consumable and a melt curve analysis of the amplified PCR product.

The procedures in this chapter use MeltDoctor™ HRM Dye to perform the calibration. If you use a different HRM dye, you must first perform a custom dye calibration as per the procedure provided in the *QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide* (Pub. no. 4489822). After performing the pure dye calibration with the custom dye, follow the calibration workflow in this chapter, but substitute your HRM dye of choice for the MeltDoctor™ HRM Dye. For component volumes for non-MeltDoctor™ HRM Dye, refer to the manufacturer's instructions.

You should optimize your reactions for any non-MeltDoctor™ HRM Dye that you choose, because each dye interacts uniquely with all other reaction components, affecting the HRM sensitivity of the analysis.

Prepare a MeltDoctor™ HRM calibration plate

IMPORTANT! Before you can perform an HRM calibration on your QuantStudio™ 6 or 7 Flex Instrument using the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software, the region of interest, background, and uniformity calibrations must be current. For more information on instrument calibration, see the instrument user guide.

Note: Immediately prior to the HRM calibration, the background calibration must be run again.

Note: The 96-well standard plate is not available as a ready-to-use calibration plate. If you are using a 96-well 0.2-mL block, you will need to prepare your own HRM calibration plate. See "Prepare a 96-well 0.2-mL HRM calibration plate" on page 90.

Required materials for HRM calibration

- Centrifuge with plate adaptor
- Powder-free gloves
- Safety goggles
- MeltDoctor™ HRM Calibration Plate, 384-Well

  or
- MeltDoctor™ HRM Calibration Plate, 96-Well Fast

Calibration guidelines

- Wear appropriate protective eyewear, clothing, and gloves.
- Prepare and run calibration plates within 120 minutes after thawing them.

  Note: Calibration consumables cannot be frozen again and reused.
- Store calibration plates in a dark place until you are ready to use them.
- Do not allow the bottoms of the plates to become dirty.
- Confirm that your centrifuge is clean. Before centrifugation, wipe down the bucket(s) using a tissue.
- Vortex and centrifuge all calibration plates to ensure complete mixing and that all reagents are contained at the bottom of the wells. The calibration plates must be well mixed and centrifuged before use.

Prepare the MeltDoctor™ HRM calibration plate

IMPORTANT! Wear powder-free gloves and safety glasses when you prepare the plate.

This procedure is for preparing a MeltDoctor™ HRM Calibration Plate. These plates are ready-to-use, and they contain all the components required for pure dye and HRM calibration.

1. Remove the MeltDoctor™ HRM Calibration Plate from the freezer, then thaw it at room temperature for approximately 30 minutes.

   IMPORTANT! Use the MeltDoctor™ HRM Calibration Plate within 2 hours of defrosting it. Until you are ready to run the MeltDoctor™ HRM Calibration Plate, store it in the dark and at ambient temperature (15°C to 30°C). Do not remove the plate from its packaging until you are ready to run it.

2. Remove the MeltDoctor™ HRM Calibration Plate from its packaging. Do not remove the optical film.
3. Vortex and centrifuge the MeltDoctor™ HRM Calibration Plate:
   a. Vortex the MeltDoctor™ HRM Calibration Plate for 5 seconds.
   b. Centrifuge the MeltDoctor™ HRM Calibration Plate for 2 minutes at < 1500 rpm.

      IMPORTANT! The MeltDoctor™ HRM Calibration Plate must be well mixed and centrifuged.

c. Confirm that the liquid in each well of the MeltDoctor™ HRM Calibration Plate is at the bottom of the well. If not, centrifuge the plate again at a higher rpm and for a longer period of time.

Perform the HRM calibration

Perform an HRM calibration to collect information that the software uses when analyzing data from a High Resolution Melt experiment.

IMPORTANT! To perform the HRM calibration, the instrument must be monitored in the Instrument Console.

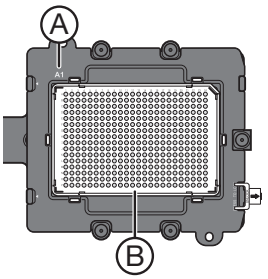
IMPORTANT! Calibrate the instrument at the same ambient temperature at which you will run experiments. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and, in extreme cases, influence experimental results.

1. On the Home page of the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software, click **Instrument Console**.
2. In the Instrument Console, select your instrument from the list of instruments on the network, then click **Manage Instrument** to open the Instrument Manager.

   Note: You must add an instrument to your list before you can manage it. Click **Add to My Instruments** to add an instrument to your list.
3. You can also double-click the instrument icon to open the **Instrument Manager**.
4. In the navigation pane, select **Maintenance** and then **HRM**.
5. Select the type of calibration:
   - For MeltDoctor™ HRM Calibration Plate:
     Maintenance ▸ HRM ▸ MeltDoctor™ HRM Calibration
   - For a custom HRM calibration plate:
     Maintenance ▸ HRM ▸ Non-MeltDoctor™ HRM Calibration

   Note: If you select Non-MeltDoctor™ HRM Calibration, you must have a valid dye calibration for that dye before you can perform the HRM calibration
6. Click **Start Calibration**.

7. Follow the instructions of the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software. When the instrument drawer opens, load the HRM Calibration Plate. Ensure that the plate is properly aligned in the holder.



   - (**A**) Load 96/384-well plates with A1 position at the top-left corner of the plate adapter.
   - (**B**) Load plates with the barcode facing the front of the instrument.

   IMPORTANT! Loading/unloading of plates should be carried out by operators who have been warned of the moving parts hazard and have been adequately trained.

8. Start the calibration:
   a. Select **Check the box when the HRM calibration plate has been loaded** below, then click **Next**.
   b. In the Run tab, click **START RUN** to start the calibration.

   IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the QuantStudio™ 6 or 7 Instrument is in operation.

   Note: Before starting the calibration, the instrument may pause (≤ 10 minutes) to allow the heated cover to reach temperature.

9. When the run is complete, click **Next**, then verify the status of the calibration.
   a. Review the Derivative Melt Curve plot for a single sharp peak.
   b. Review the Plate Layout tab to see that all wells are free of flags.
   c. After you inspect all HRM images, click **Next**, then remove and discard the plate when the instrument ejects the instrument tray.

   IMPORTANT! If the QuantStudio™ 6 or 7 Instrument does not eject the plate, remove the plate as explained in the instrument user guide.

   WARNING! PHYSICAL INJURY HAZARD. During instrument operation, the plate can be heated to 100°C. Before removing the plate, wait until it reaches room temperature.

10. In the HRM Calibration screen, click **Finish** to complete the calibration, then click **Yes** when prompted to save the results.

    Note: If the calibration fails, use the information in the QC tab to troubleshoot the problem, then re-run the calibration.

11. Unload the plate.

Review the HRM Calibration Results

- Review the Pass/Fail status of the calibration.
- Review the calibration data in the analysis plot:

1. Select the **Well Table** tab to:
    a. Review the results for each well in tabular format.
    b. Sort the wells according to well or aligned fluorescence with each filter.
    c. Select wells to review data in the analysis plot.
2. Select the **QC** tab to review the quality of the calibration data.

Troubleshoot the HRM Calibration

If the instrument did not complete the HRM calibration run:

1. Repeat the calibration.
2. If the problem persists, contact Life Technologies.

If the instrument malfunctions:

Symptom	Possible cause	Action
Instrument does not eject the plate	The adhesive cover may have adhered the plate to the heated cover within the instrument	Unload the Instrument.
Instrument malfunction	Multiple possible causes	Contact a local Life Technologies Field Services office.

Note: For troubleshooting failed HRM calibrations, contact Life Technologies.

3 Design an HRM Genotyping Experiment

Design and order the primers

Note: If you are using the MeltDoctor™ HRM Positive Control Kit to run the example experiment, you do not need to design primers because the kit contains primers designed to amplify the alleles in the positive control DNA.

Using Primer Express® Software v3.0 or later, design the primers to amplify the sequence of interest. HPLC-purified primers are recommended for best performance, although desalted primers are usable in some cases.

We recommend using these guidelines when designing primers:

Design attribute	Design guidelines
Amplicon	Length is 60 to 250 basepairs (longer amplicons may require optimization)
Primer length	~20 bases each
Tm	58°C to 62°C (Optimal Tm is 60°C)
% GC content	30–80% GC content in each primer
3′ end	No more than 2 G+C residues in the last 5 nucleotides at the 3′ end
Repeating oligonucleotides	Avoid consecutive identical nucleotides. If you are unable to avoid consecutive identical nucleotides, make sure that each primer contains fewer than 4 consecutive Gs.

To order primers, go to **www.lifetechnologies.com**, then log into the Life Technologies Store if you have an account; register if you are a new user. For more information, see "How to order custom primers" on page 85. For a list of HRM reagents, see Appendix A, "Ordering Information" on page 85.

Plan to use controls

Include controls for each target sequence in your HRM experiment.

- At least one negative control
- At least one positive control to represent each expected variant (for genotyping experiments)

  Run 3-5 replicates of each control to improve your results. Running multiple positive controls allows you to more effectively define the natural spread or variation within different samples of the same sequence, or within replicates of the same genotype

Define the experiment properties

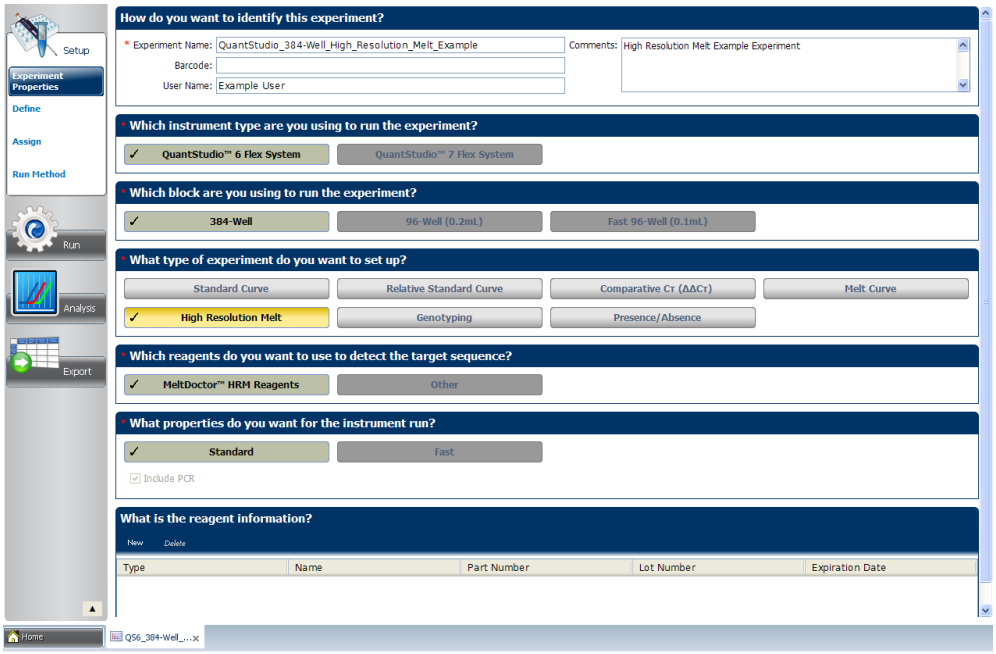
To create a new experiment, access the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software: **New Experiment** ▸ **Experiment Setup**. Enter the experiment properties.

IMPORTANT! Ensure that you select **High Resolution Melt** as the Experiment Type.

Note: For detailed instructions on designing an experiment, refer to Booklet 1, *Getting Started with QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Experiments* in the *QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide* (Pub. no. 4489822).

Save the experiment.

The following is an image of the Experiment Properties screen for the example experiment:



Define targets, samples, and controls

These are the definitions used in the HRM example experiment. For your own HRM experiment, define targets, samples and controls as appropriate.

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

1. Targets

Target Name	Reporter	Quencher	Color
HRM	MELTDOCTOR	None	

2. Samples

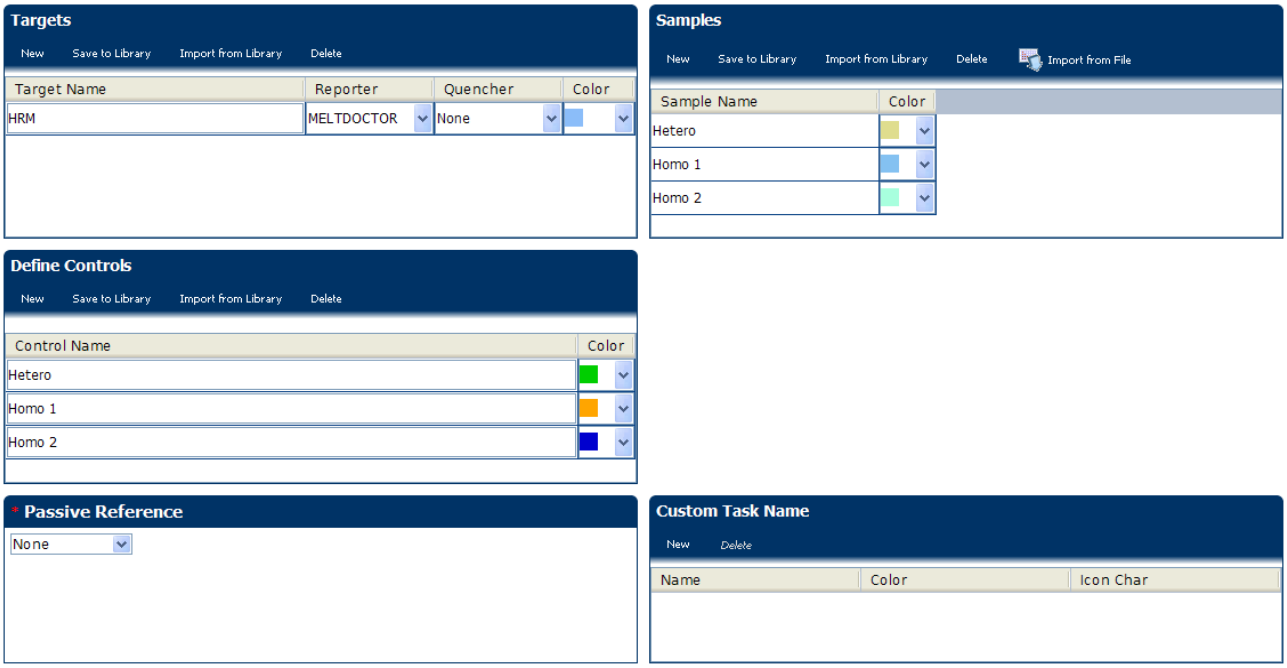
Sample Name	Color
Homo 1 (Allele A/A)	
Homo 2 (Allele G/G)	
Hetero (Allele A/G)	

3. Define Controls

Sample Name	Color
Hetero	
Homo 1	
Homo 2	

4. Dye to be used as a Passive Reference:
   None
5. Custom Task Name:
   Not applicable

The following is an image of the Define screen for the example experiment:



Assign samples and controls

These are the samples and controls as they are assigned in the HRM example experiment. For your own HRM experiment, assign samples and controls as appropriate.

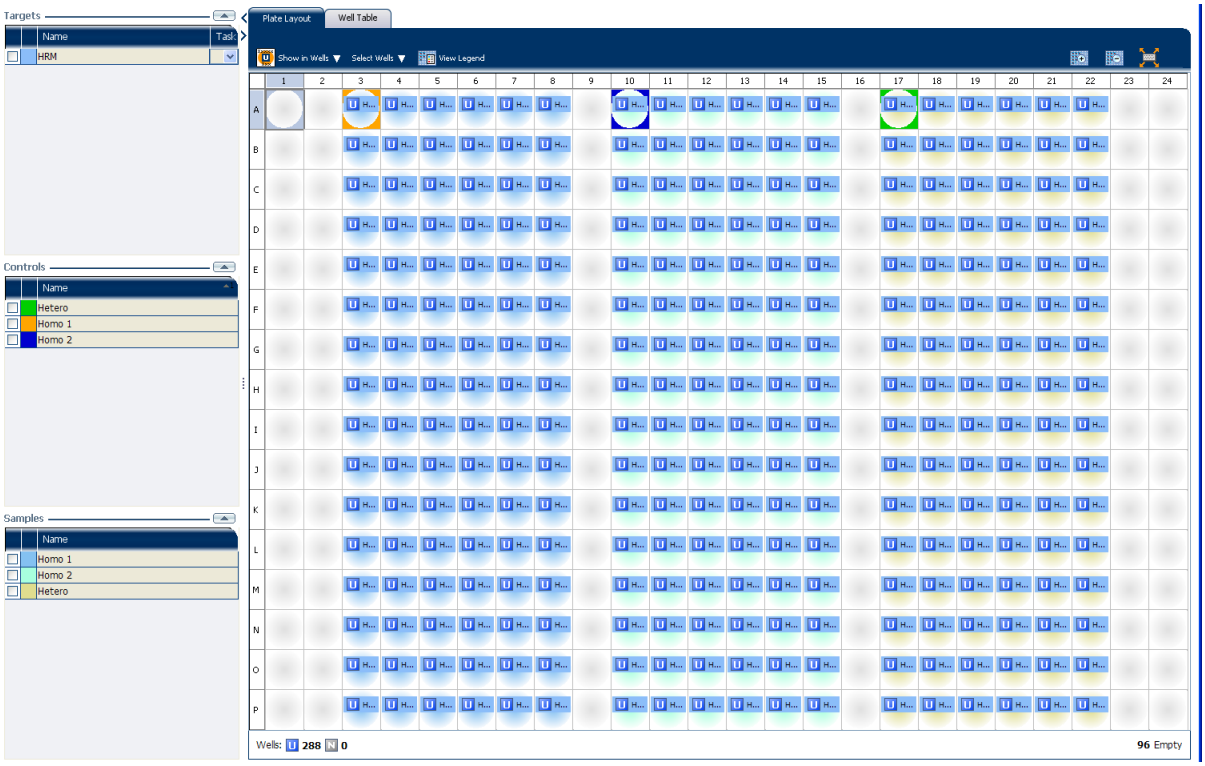
Assign samples

1. Select columns 3-8 in the Plate Layout and click in the check box for Sample Homo 1.
2. Repeat for columns 10-15 with Sample Homo 2 and for columns 17-22 with sample Hetero.

Assign controls

1. Select the top-left corner well of the 96 wells selected for sample Homo 1 and click the check box for **Control Homo 1** to assign it as a control.
2. Repeat for Homo 2 sample wells with **Control Homo 2** and Hetero sample wells with **Control Hetero.**

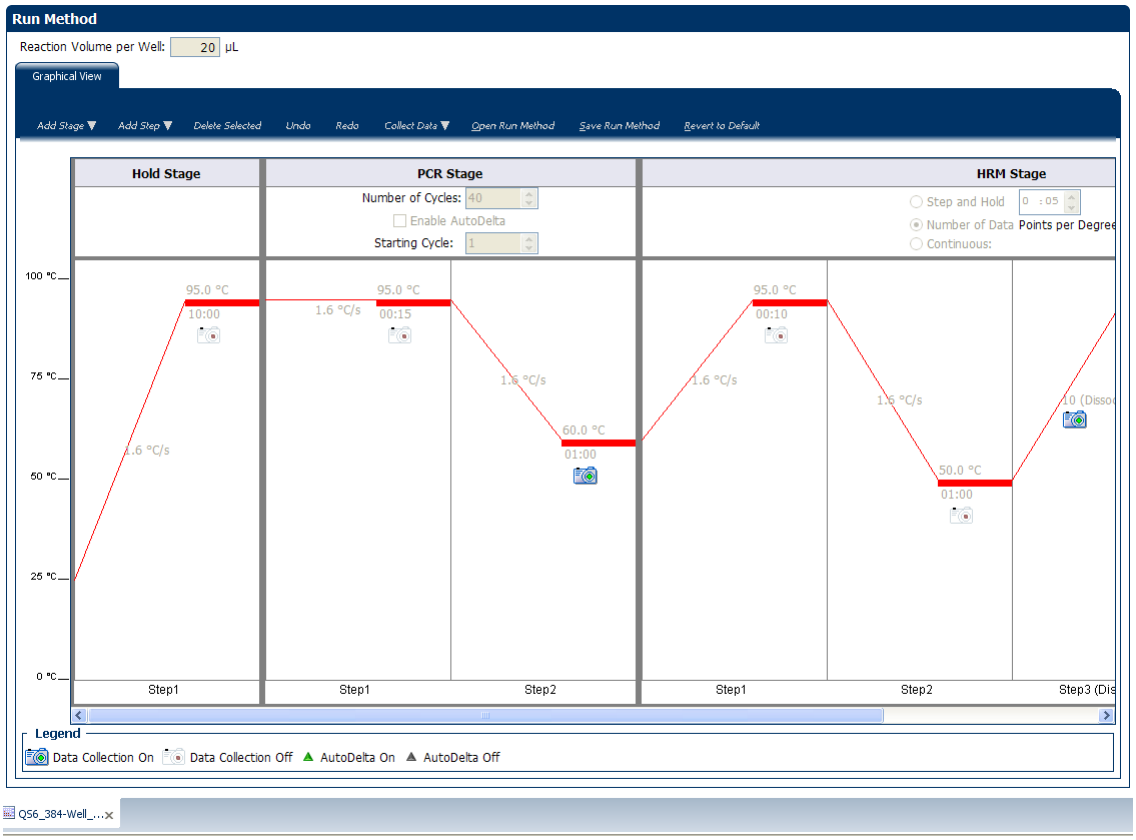
The following is an image of the Assign screen for the example experiment:



Set up the run method

This is the default run method. It has been optimized for use with the MeltDoctor™ HRM Reagents.

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



The default settings are:

Stage	Step	Temp	Time
Holding	Enzyme activation	95°C	10 min
Cycling (40 cycles)	Denature	95°C	15 sec
	Anneal/extend	60°C	1 min
Melt curve/dissociation	Denature	95°C	10 sec
	Anneal	60°C	1 min
	High resolution melting	95°C	15 sec
	Anneal	60°C	15 sec

Note that the Reaction Volume per Well is 20-µL.

If you wish to change the settings for your own experiment, see **QuantStudio™ 6 and 7 Flex System Software Help** (click ? or press **F1**).

2. If you have modified the settings, **Save** the file.

4 Prepare HRM Genotyping Reactions

Required materials

Basic materials for all HRM experiments

You need the following basic materials, plus additional materials for either the example experiment or for your own experiment.

- Microcentrifuge tubes
- MicroAmp® Optical 96/Fast 96/384-Well Reaction Plate
- MicroAmp® Optical Adhesive Film
- Deionized water
- Pipettors and pipette tips
- Vortexer
- Centrifuge

Additional materials for example experiment

In addition to the basic materials you need:

- MeltDoctor™ HRM Positive Control Kit, components from the kit:
  - MeltDoctor™ HRM Primer Mix (20X)
  - MeltDoctor™ HRM Allele A DNA (20X)
  - MeltDoctor™ HRM Allele G DNA (20X)
  - MeltDoctor™ HRM Allele A/G DNA (20X)
- MeltDoctor™ HRM Master Mix

Additional materials for your own HRM experiments

In addition to the basic materials you need:

- Forward and reverse primers (5 µM each)
- DNA samples
- MeltDoctor™ HRM Master Mix

Note: If you use HRM reagents from another manufacturer you must first perform calibration using those dyes. See "Prepare a custom HRM calibration plate" on page 91.

Prepare the HRM reactions for the example experiment

This procedure describes how to prepare reactions for the HRM example genotyping experiment. For your own HRM experiment, see "Tips for preparing the reactions for your own HRM experiment".

Prepare the reactions for each replicate group separately, then transfer the reactions to a reaction plate appropriate for your instrument.

1. Prepare the reactions using the Positive Control Kit in separate appropriately sized, labeled tubes:

Components	384-well reaction plate	
	Volume for one 20-µL reaction	**Volume for three 20-µL replicates plus 10% excess**
MeltDoctor™ HRM Master Mix	10 µL	33.0 µL
One type of allele DNA: • MeltDoctor™ HRM Allele A DNA (20X) • MeltDoctor™ HRM Allele G DNA (20X) • MeltDoctor™ HRM Allele A/G DNA (20X)	1 µL	3.3 µL
MeltDoctor™ HRM Primer Mix (20X)	1 µL	3.3 µL
Deionized water	8 µL	26.4 µL
Total volume	**20 µL**	**66 µL**

IMPORTANT! Include excess volume in your calculations to compensate for the loss that occurs during reagent transfers. Life Technologies recommends an excess volume of at least 10%.

2. Vortex the reactions to mix, then spin the tubes briefly.
3. Prepare a reaction plate appropriate for your instrument:
   a. Pipet each reaction replicate into the appropriate wells of the optical reaction plate.
   b. Seal the reaction plate with optical adhesive film, then spin the reaction plate.
   c. Confirm that the liquid is at the bottom of the wells in the reaction plate.

Note: If you plan to wait more than 24 hours before running the plate, store the plate at 4°C. Allow the plate to warm to room temperature, then spin the plate briefly before running it.

Note: For detailed information on preparing a reaction plate, see *Prepare Reactions*, in *Getting Started with QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Experiments*. It is the first booklet of the *QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide* (Pub. no. 4489822).

Tips for preparing the reactions for your own HRM experiment

When you perform your own HRM genotyping experiment, you may wish to include negative controls. If you are performing PCR on the QuantStudio™ 6 or 7 Flex Instrument, negative controls will tell you if you have contamination in your samples.

1. Follow the same procedure for creating positive controls as in "Prepare the HRM reactions for the example experiment", but replace Primer mix with your forward and reverse primers and the HRM Alleles with your DNA samples.
2. In addition to the positive control reactions, also prepare negative control reactions in an appropriately sized, labeled tube.

Components	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10 µL	33.0 µL
MeltDoctor™ HRM Primer Mix (20X)	1 µL	3.3 µL
Deionized water	9 µL	29.7 µL
Total volume	**20 µL**	**66.00 µL**

IMPORTANT! Include excess volume in your calculations to compensate for the loss that occurs during reagent transfers. We recommend an excess volume of at least 10%.

3. Vortex the reactions to mix, then spin the tubes briefly.
4. Prepare a reaction plate appropriate for your instrument:
   a. Pipet the negative controls, positive controls, and your samples into the appropriate wells of the optical reaction plate.
   b. Seal the reaction plate with optical adhesive film, then spin the reaction plate.
   c. Confirm that the liquid is at the bottom of the wells in the reaction plate.

Note: If you plan to wait more than 24 hours before running the plate, store the plate at 4°C. Allow the plate to warm to room temperature, then spin the plate briefly before running it.

Note: For detailed information on preparing a reaction plate, see *Prepare Reactions*, in *Getting Started with QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Experiments*. It is the first booklet of the *QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide* (Pub. no. 4489822).

Note: For information about using the MeltDoctor™ HRM Reagent Kit to optimize your reactions, see "Optimizing the reaction conditions" on page 93.

5 Run an HRM Genotyping Experiment

Prepare for the run

Open the HRM experiment

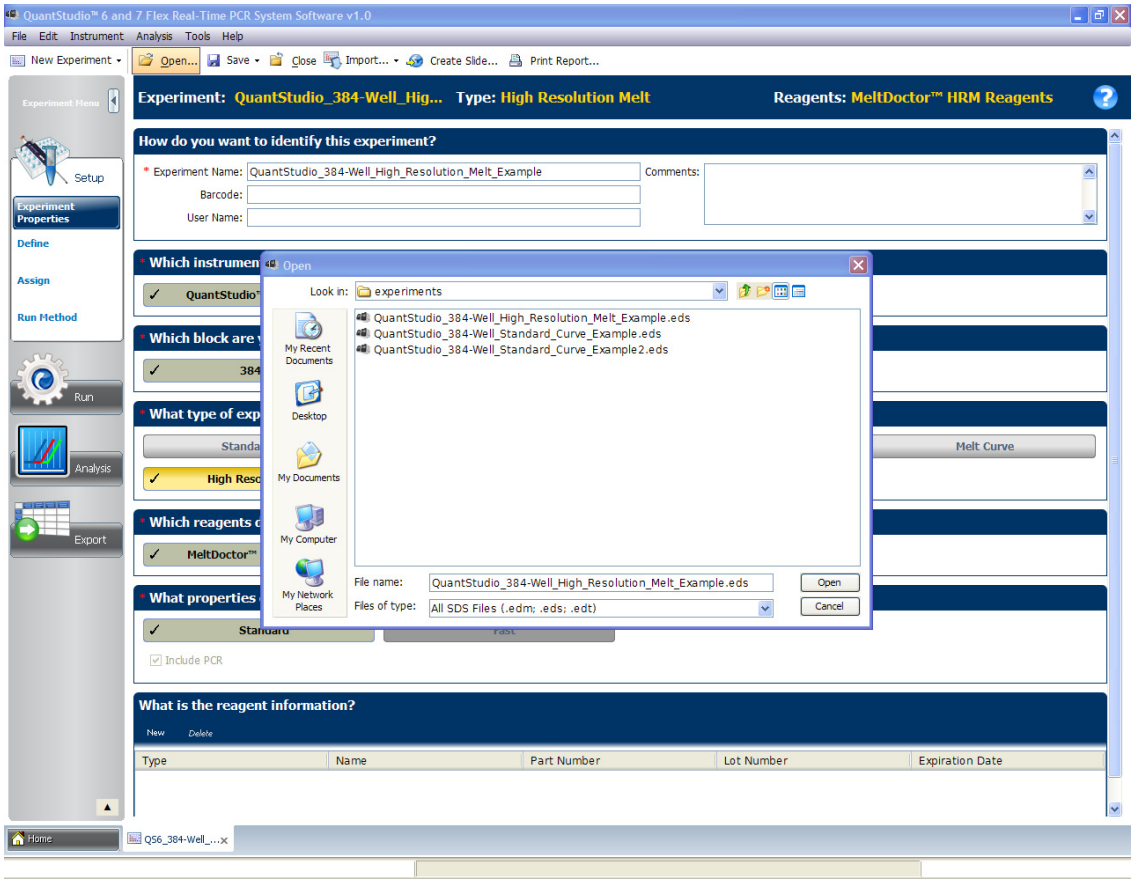
From the Home screen, open one of the following:

- The example experiment:

  Open ▸ Program Files ▸ Applied Biosystems ▸ QuantStudio 6 and 7 Flex Software ▸ examples ▸ QS6Flex ▸ QS6_384-Well_High_Resolution_Melt_Example.eds

- Your experiment:

  Open ▸ Applied Biosystems ▸ QuantStudio 6 and 7 Flex Software ▸ experiments ▸ <your HRM experiment.eds>



Load the reaction plate Into the instrument

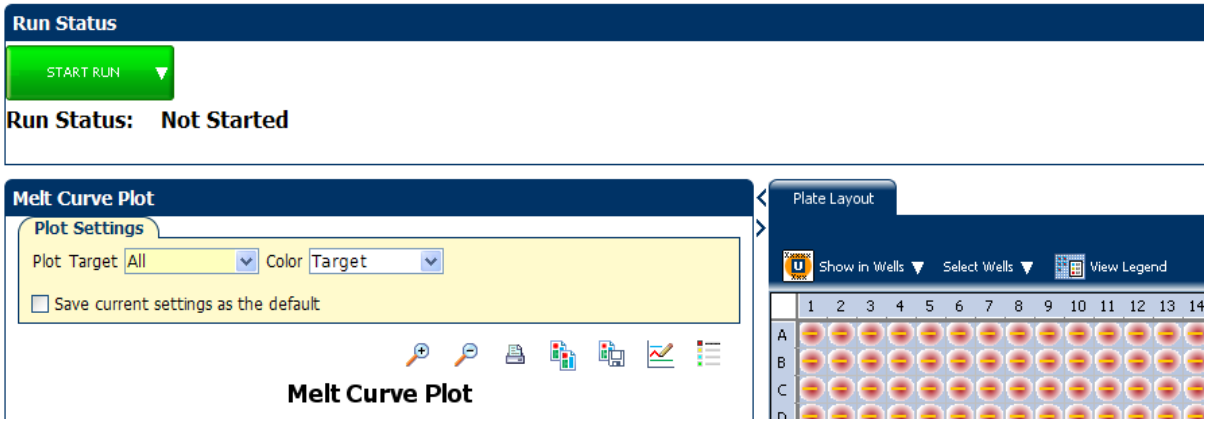
IMPORTANT! Loading/unloading of plates should be carried out by operators that have been warned of the moving parts hazard and have been adequately trained.

1. In the Home screen of the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software, click **Instrument Console**.
2. Select your instrument from the list of instruments.
3. If your instrument is in the "On the Network" group, add it to **My Instrument**.
4. Click **Open Door** in the Instrument Console tool bar.
5. Load your prepared reaction plate.
6. Close the door, either from the touchscreen or the software.

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

Start the run

Once the reaction plate is loaded, start the run by clicking **Start Run** in the Melt Curve window.



Monitor the run

You can view the progress of the run in real-time as described in the following table. During the run, periodically view all three plots available from the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software for potential problems.

To...	Action
View temperature data for the run in real-time.	Select **Temperature**.

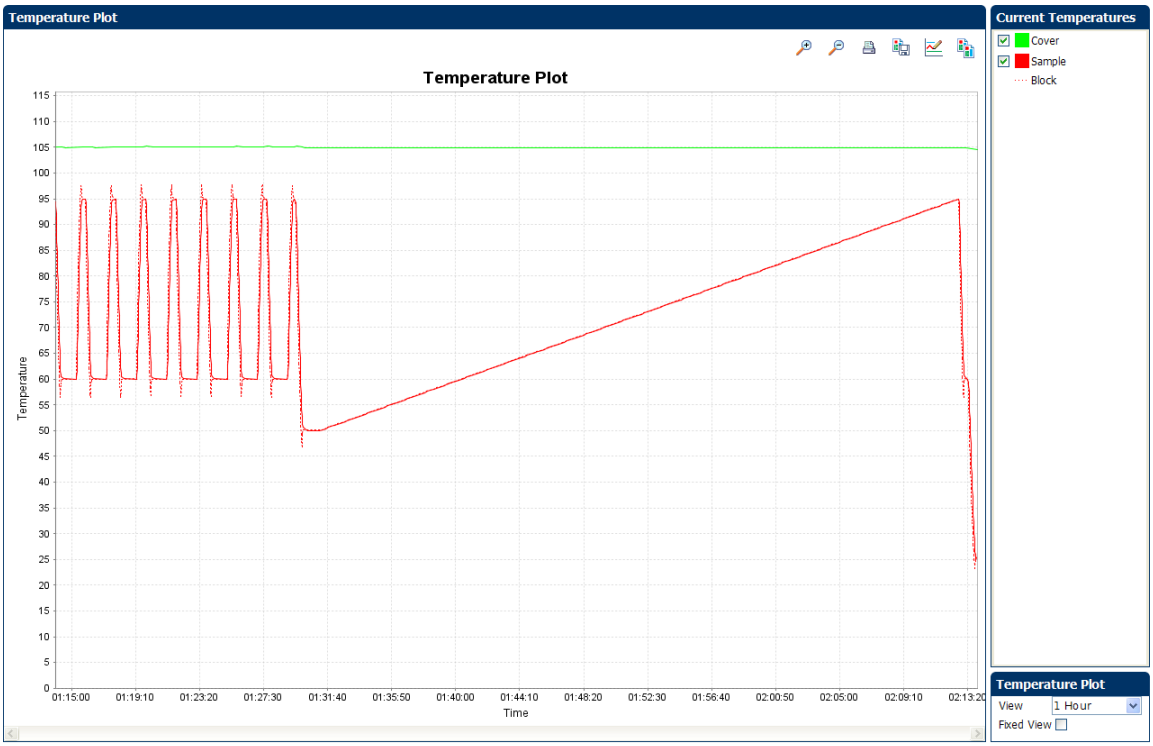
To...	Action
View progress of the run in the Run Method screen.	Select **Run Method**.
Enable/disable the Notification Settings.	Select or deselect **Notification Settings**.
Stop the run.	1. In the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software, click **STOP RUN**. 2. In the Stop Run dialog, click one of the following: • **Stop Immediately** to stop the run immediately. • **Cancel** to continue the run.

About the Temperature Plot screen

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/remove temperature plots.	Select **Cover** or **Sample Block** to view the presence of the associated data in the plot.
Change the time displayed by plot.	Click the **Plot Properties** icon. Select the amount of time to display in the plot.
Display a fixed time window during the instrument run.	Select **Fixed View**. 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 run lasts more than 10 minutes. • The plot updates as the run progresses with Fixed View deselected. • The plot updates as the run progresses with Fixed View deselected.

The following is an image of the Temperature Plot screen as it appears during the example experiment.



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.

- In general, 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.

If you notice an abnormal temperature plot, refer to the instrument user guide to troubleshoot the error.

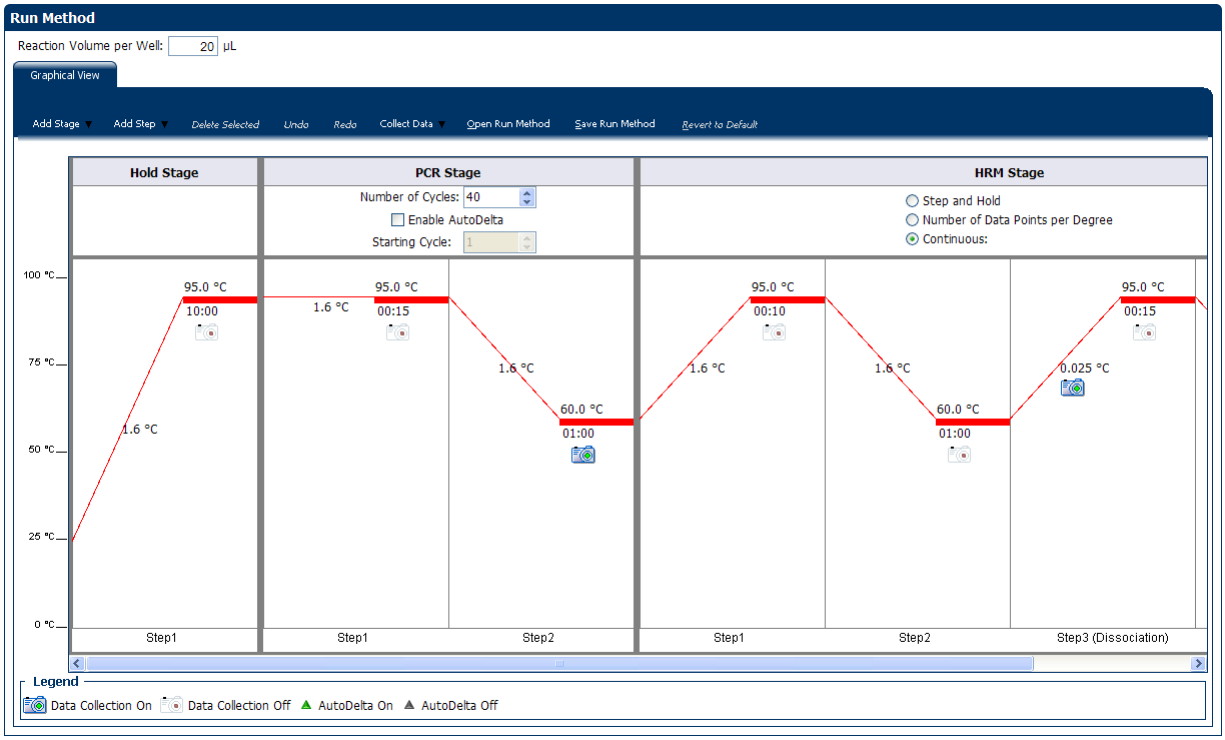
Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

About the Run Method screen

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

The following is an image of the Run Method screen as it appears in the example experiment.



If an alert appears, click the error for more information and troubleshoot the problem as explained in the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Help (click or press F1).

6 Review HRM Genotyping Experiment Results and Adjust Parameters

How to evaluate the results

Review of the results occurs in three steps:

1. Perform an initial review of the High Resolution Melt Plots (see page 40), the Plate Layout (see page 43), and the Well Table (see page 45) to evaluate the genotype calls made by the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software.
2. Perform a thorough review of the QC Summary (see page 48) to evaluate the samples that triggered QC flags. Review the raw data (see page 49) and amplification data (see page 52) for the samples that exhibit abnormal amplification.
3. If necessary, define the analysis settings (see page 55) or modify the calls manually (see page 58).

After evaluating the results, you can publish the results as explained in "Publish the data" on page 60.

View the experiment results

If this is an active experiment, select **Analyze** from the Experiment menu. If you are viewing a saved experiment, open the experiment file.

- To view the HRM example experiment:
  Open ▸ Applied Biosystems ▸ QuantStudio 6 and 7 Flex Software ▸ User Files ▸ experiments ▸ examples ▸ QuantStudioTD 384 Well High Resolution Melt Example.eds.
- To view your own experiment:
  Open ▸ experiments ▸ <your experiment.eds>

Review the High Resolution Melt Plots

Perform an initial review of the experiment results in the High Resolution Melt Plots.

The High Resolution Melt Plots are:

- Raw Melt Curves
- Derivative Melt Curves
- Aligned Melt Curves
- Difference Plot

View the plots

1. From the Experiment Menu pane, select **Analysis ▸ High Resolution Melt Plots**.

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

2. If you wish to examine a certain well or set of wells, click the **Plate Layout** tab, then select a well or set of wells.

You can view up to four plots simultaneously. From the Experiment Menu, select **Analysis ▸ Multiple Plots View**.

- To display four plots, select Show plots in a 2x2 matrix.
- To display two plots in rows, select Show plots in two rows. To display two plots in columns, select **Show plots in two columns**.
- To display a specific plot, select the plot from the drop-down menu above each plot display.

Analysis guidelines for High Resolution Melt plots

Confirm that all controls have the correct genotype.

1. If using positive controls, confirm the calls for the positive controls:
   a. From the well table, select the wells containing a positive control to highlight the corresponding melt curve in the analysis plots.
   b. Confirm that the color of the line corresponds to the correct genotype.
   c. Repeat steps a and b for the wells containing the other positive controls.
2. Screen the negative controls to ensure that samples failed to amplify:
   a. From the well table, select the wells containing a negative control to highlight the corresponding melt curve in the analysis plots.

b. Check that the selected wells in the well table are negative controls, and not unknown samples.

   Samples that grouped with the negative controls may:

   - Contain no DNA
   - Contain PCR inhibitors
   - Be homozygous for a sequence deletion

3. Confirm the results of the samples that did not group tightly or are grouped with negative controls by retesting them.
4. If you select to run replicate reactions, carefully review your data set for curves that do not align tightly with the other samples in the group (outliers) to ensure the accuracy of the genotype calls. If outliers are present, confirm the results of the associated samples by retesting them.
5. Look for how many different variant groups (different colors) are displayed. If you see more than you were expecting, you may have sample contamination or may need to modify the analysis settings.

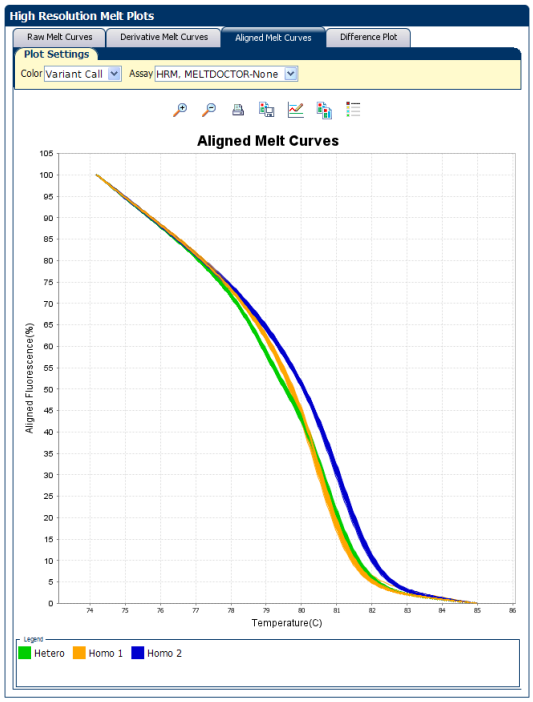
About melt curves

The melt profile of a PCR product depends on its GC content, length, sequence, and heterozygosity. High resolution melt analysis calls variants or genotypes based on the differences in the shape of the melt curves and the differences in the Tm values.

The **Aligned Melt Curves** plot displays the melt curves as % melt (0-100%) over temperature. The melt curves are aligned to the same fluorescence level using the Pre- and Post-melt regions.

Note the following in the screen shot of the example experiment results:

- Hetero has a different curve shape compared to Homo 1 and Homo 2. The shape of the melt curve is an indicator of heteroduplex formation.
- Homo 1 and Homo 2 are distinguished from each other based on the difference in Tm values.



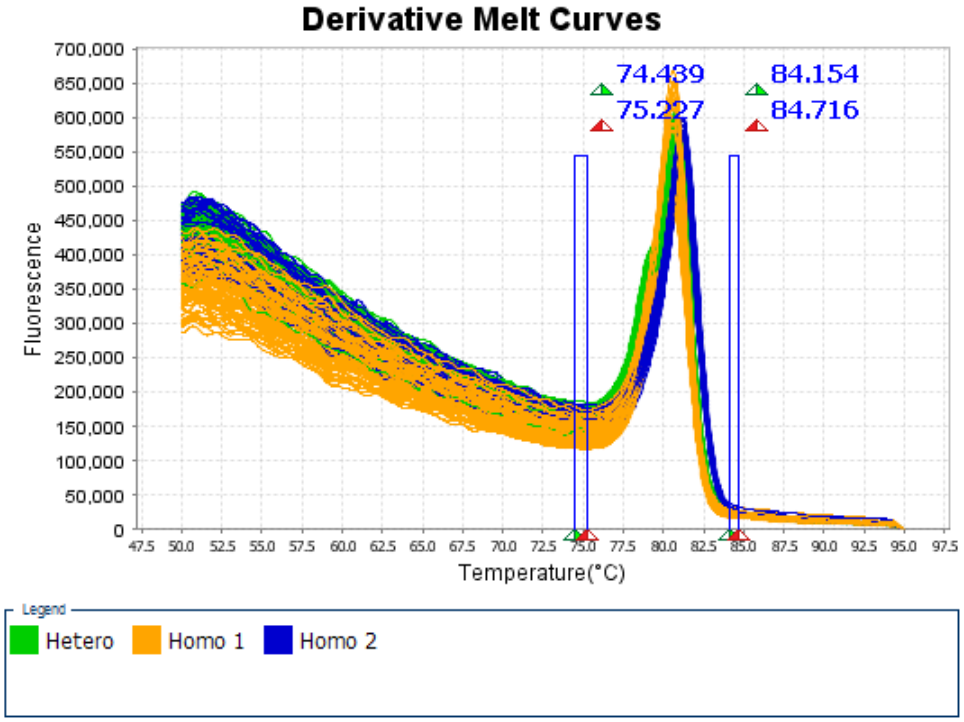
About the Pre- and Post-melt regions

In the **Derivative Melt Curves** plot and the **Raw Melt Curves** plot, there are two pairs of vertical lines before and after the data peak. These lines define the Pre- and Post-melt regions used to scale the data in the Aligned Melt Curves and Difference Plot.

- Pre-melt region: The pair of lines to the left of the peak indicate the Pre-melt Start and Stop temperatures when every amplicon is double-stranded.

  Fluorescence data from the Pre-melt region corresponds to 100% fluorescence in the Aligned Melt Curves Plot.
- Active melt region: The data peak indicates the active melt region of the plot. Data from the active melt region are used to plot the Aligned Melt Curves Plot.
- Post-melt region: The set of lines to the right of the peak indicate the Post-melt Start and Stop temperatures when every amplicon is single-stranded.

  Fluorescence data from the Post-melt region correspond to 0% fluorescence in the Aligned Melt Curves Plot.

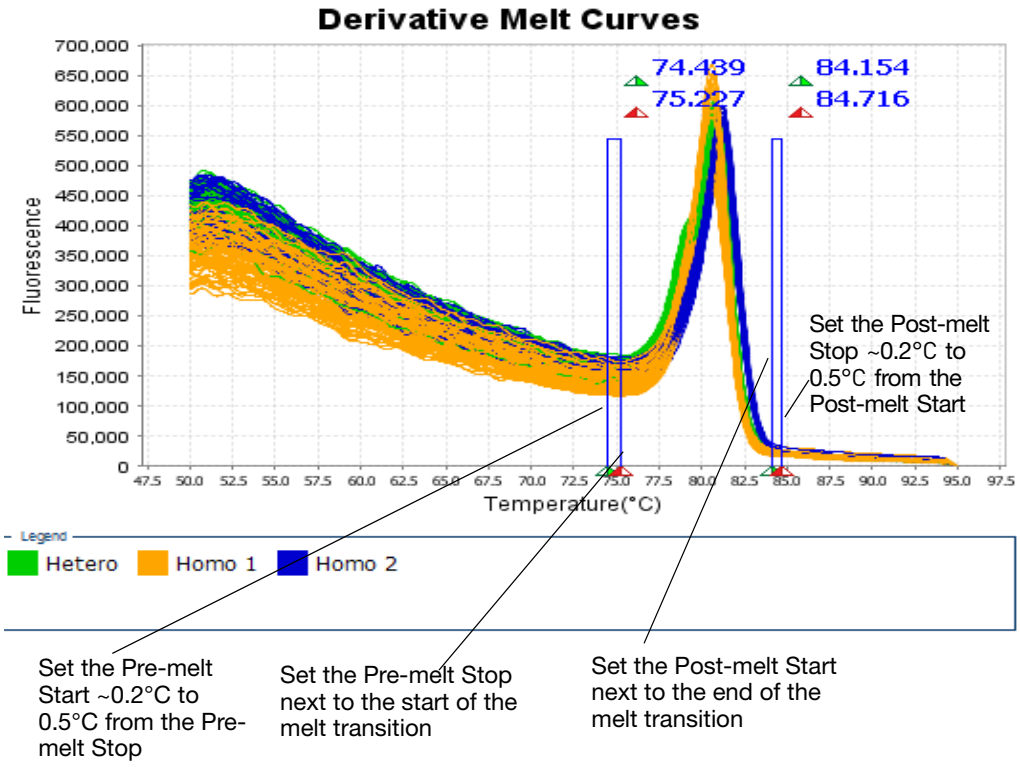


Review and adjust the Pre- and Post-melt regions

When you analyze an HRM experiment, the software calculates the Pre- and Post-melt regions using default settings. You can review and adjust the Pre- and Post-melt regions to optimize your separation and variant calls. For most experiments, set the Pre- and Post-melt regions as close as possible to the melt transition region.

1. In the Data pane, select the **Derivative Melt Curves** tab.
2. Click in the Plate Layout and press **Ctrl+A** to select all wells.
3. Set the Pre-melt region:
   a. Click and drag the Pre-melt Stop temperature line (red arrow on the left) adjacent to the start of the melt transition region.
   b. Click and drag the Pre-melt Start temperature line (green arrow on the left) approximately 0.2°C to 0.5°C from the Pre-melt Stop temperature line.

Note: The Pre-melt region should be within a flat area where there are no large spikes or slopes in the fluorescence levels.



4. Set the Post-melt region:
   a. Click and drag the Post-melt Start temperature line (green arrow on the right) adjacent to the end of the melt transition region.
   b. Click and drag the Post-melt Stop temperature line (red arrow on the right) approximately 0.2°C to 0.5°C from the Post-melt Start temperature line.

      Note: The Post-melt region should be within a flat area where there are no large spikes or slopes in the fluorescent levels.

5. Click Analyze **Analyze**.

   The software reanalyzes the data using the new Pre- and Post-melt regions. The colors of the melt curves change to reflect the new results.

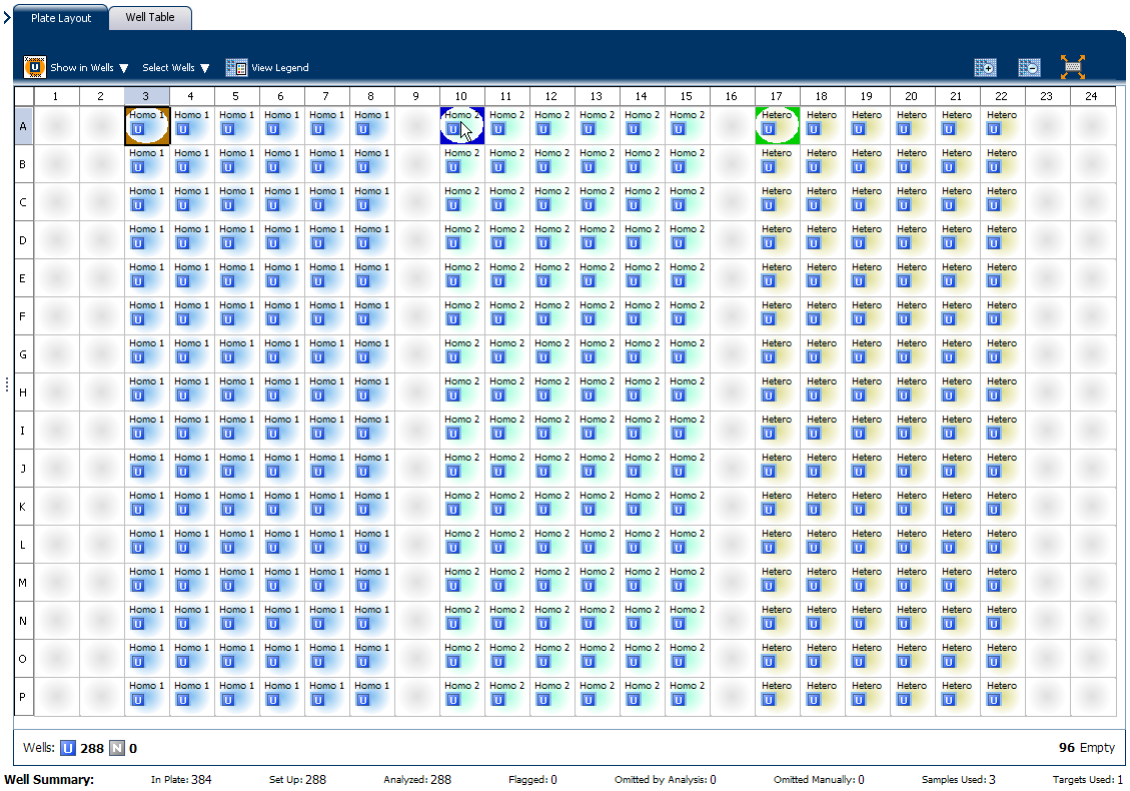
   For information on saving these settings, see "Apply custom Pre- and Post-melt settings to an assay" on page 57.

View the 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.

1. Click ‹ to maximize the plate layout and hide the plots.

2. Click **Show in Wells**, then select or deselect a parameter that you want the wells to display.
3. Repeat step 2 until the plate layout contains all of the desired parameters.



Parameter	Description
Sample Color	The color of the sample applied to the well.
Target	The nucleic acid sequence in the plate layout that you want to amplify and detect.
Control Color	The color assigned to the control samples in the plate layout.
TM	The temperature at which 50% of the DNA is double-stranded and 50% of the DNA is dissociated into single-stranded DNA.
Control Value	The name of the variant in the well.
Flags	The number of QC flags the well triggered.

Example experiment plate layout results

For the example experiment, confirm that the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software called:

- 95 samples as Homo 1 (homozygous), plus one control (Variant: Control-Homo 1)
- 95 samples as Homo 2 (homozygous), plus one control (Variant: Control-Homo 2)
- 95 samples as Hetero (heterozygous), plus one control (Variant: Control-Hetero)

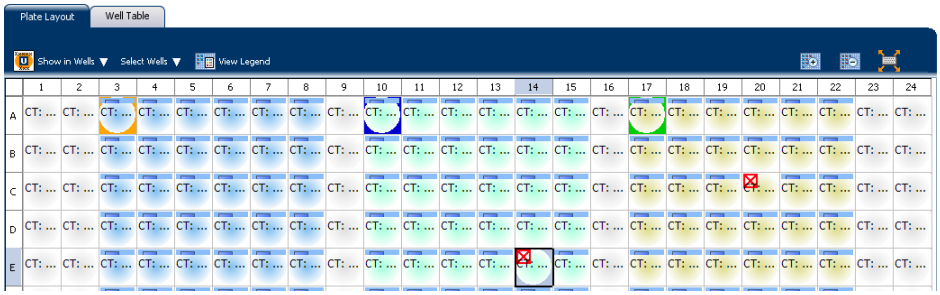
If necessary, click  to zoom in and read the contents of a well.

Confirm that no wells of the reaction plate triggered QC flag(s).

Analysis guidelines for the plate layout view

When you analyze your own experiment:

- You may wish to omit wells with outliers.
  The plate layout displays  in the top-left corner of wells omitted by the user; and it displays  in the corner of wells omitted by the QC flag settings.
  To omit wells, select wells with the mouse and right-click. Select **Omit**



- Note the location of any samples that triggers 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 wells.
  - Left-click the mouse and drag across an area to select it.
  - Select Sample, Target, or Task from the Select Items menu in the View Plate tab. Then select the sample, target, or task name from the second Select Items menu to select wells of a specific type using the well-selection tool.
- You can adjust the plate layout:
  - Use the  (Zoom In),  (Zoom Out), and  (Fit All) buttons to increase or decrease the wells shown.
  - Use the arrow tabs to expand the plate layout to cover the entire screen.

View the well table

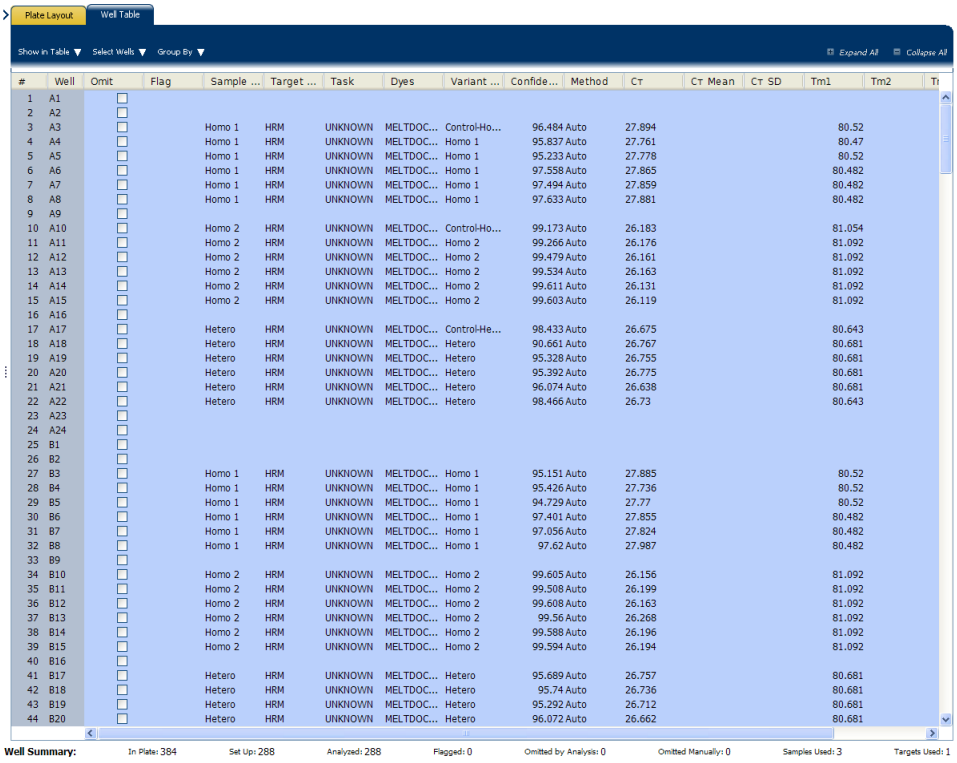
Review the details of the experiment results in the well table and identify flagged wells. The well table displays the assay-specific setup and analysis properties for the experiment in a table format.

Example experiment well data

If you are running the example experiment, confirm that no wells of the reaction plate triggered QC flags ().

Review the well table

1. Select the Well Table tab.



#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	Variant ...	Confide...	Method	CT	CT Mean	CT SD	Tm1	Tm2
1	A1														
2	A2														
3	A3			Homo 1	HRM	UNKNOWN	MELTDOC...	Control-Ho...	96.484	Auto	27.894			80.52	
4	A4			Homo 1	HRM	UNKNOWN	MELTDOC...	Homo 1	95.837	Auto	27.761			80.47	
5	A5			Homo 1	HRM	UNKNOWN	MELTDOC...	Homo 1	95.233	Auto	27.778			80.52	
6	A6			Homo 1	HRM	UNKNOWN	MELTDOC...	Homo 1	97.558	Auto	27.865			80.482	
7	A7			Homo 1	HRM	UNKNOWN	MELTDOC...	Homo 1	97.494	Auto	27.859			80.482	
8	A8			Homo 1	HRM	UNKNOWN	MELTDOC...	Homo 1	97.633	Auto	27.881			80.482	
9	A9														
10	A10			Homo 2	HRM	UNKNOWN	MELTDOC...	Control-Ho...	99.173	Auto	26.183			81.054	
11	A11			Homo 2	HRM	UNKNOWN	MELTDOC...	Homo 2	99.266	Auto	26.176			81.092	
12	A12			Homo 2	HRM	UNKNOWN	MELTDOC...	Homo 2	99.479	Auto	26.161			81.092	
13	A13			Homo 2	HRM	UNKNOWN	MELTDOC...	Homo 2	99.534	Auto	26.163			81.092	
14	A14			Homo 2	HRM	UNKNOWN	MELTDOC...	Homo 2	99.611	Auto	26.131			81.092	
15	A15			Homo 2	HRM	UNKNOWN	MELTDOC...	Homo 2	99.603	Auto	26.119			81.092	
16	A16														
17	A17			Hetero	HRM	UNKNOWN	MELTDOC...	Control-He...	98.433	Auto	26.675			80.643	
18	A18			Hetero	HRM	UNKNOWN	MELTDOC...	Hetero	90.661	Auto	26.767			80.681	
19	A19			Hetero	HRM	UNKNOWN	MELTDOC...	Hetero	95.328	Auto	26.755			80.681	
20	A20			Hetero	HRM	UNKNOWN	MELTDOC...	Hetero	95.392	Auto	26.775			80.681	
21	A21			Hetero	HRM	UNKNOWN	MELTDOC...	Hetero	96.074	Auto	26.638			80.681	
22	A22			Hetero	HRM	UNKNOWN	MELTDOC...	Hetero	98.466	Auto	26.73			80.643	
23	A23														
24	A24														
25	B1														
26	B2														
27	B3			Homo 1	HRM	UNKNOWN	MELTDOC...	Homo 1	95.151	Auto	27.885			80.52	
28	B4			Homo 1	HRM	UNKNOWN	MELTDOC...	Homo 1	95.426	Auto	27.736			80.52	
29	B5			Homo 1	HRM	UNKNOWN	MELTDOC...	Homo 1	94.729	Auto	27.77			80.52	
30	B6			Homo 1	HRM	UNKNOWN	MELTDOC...	Homo 1	97.401	Auto	27.855			80.482	
31	B7			Homo 1	HRM	UNKNOWN	MELTDOC...	Homo 1	97.056	Auto	27.824			80.482	
32	B8			Homo 1	HRM	UNKNOWN	MELTDOC...	Homo 1	97.62	Auto	27.987			80.482	
33	B9														
34	B10			Homo 2	HRM	UNKNOWN	MELTDOC...	Homo 2	99.605	Auto	26.156			81.092	
35	B11			Homo 2	HRM	UNKNOWN	MELTDOC...	Homo 2	99.508	Auto	26.199			81.092	
36	B12			Homo 2	HRM	UNKNOWN	MELTDOC...	Homo 2	99.608	Auto	26.163			81.092	
37	B13			Homo 2	HRM	UNKNOWN	MELTDOC...	Homo 2	99.56	Auto	26.268			81.092	
38	B14			Homo 2	HRM	UNKNOWN	MELTDOC...	Homo 2	99.588	Auto	26.196			81.092	
39	B15			Homo 2	HRM	UNKNOWN	MELTDOC...	Homo 2	99.594	Auto	26.194			81.092	
40	B16														
41	B17			Hetero	HRM	UNKNOWN	MELTDOC...	Hetero	95.689	Auto	26.757			80.681	
42	B18			Hetero	HRM	UNKNOWN	MELTDOC...	Hetero	95.74	Auto	26.736			80.681	
43	B19			Hetero	HRM	UNKNOWN	MELTDOC...	Hetero	95.292	Auto	26.712			80.681	
44	B20			Hetero	HRM	UNKNOWN	MELTDOC...	Hetero	96.072	Auto	26.662			80.681	

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 QC flags ( ).
   c. Click the – icons to collapse the negative and positive controls.
4. Click > beside the Plate Layout tab to display the Well Table and the plots simultaneously.

The following table shows the information in the Well Table view.

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 test assay evaluated in the well.
Task	The task assigned to the well (Unknown, Negative Control, or Positive Control).
Dyes	Reporter dyes in wells.

Column	Description
Variant Call	Call for the sample in well. Can be assigned by software (Auto) or manually. See "Manually set the number of variants" on page 57.
Confidence (%)	The quality value calculated for the genotype call.
Method	The method used to assign the call to the sample (Auto if assigned by the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software, or Manual if applied by a user).
C_T (Cycle Threshold)	The PCR cycle number at which the fluorescence crosses the threshold in the amplification plot.
C_TMean	The arithmetic average of the PCR cycle numbers at which the fluorescence crosses the threshold in the amplification plot for all selected samples.
C_T SD	The standard deviation of the C_T Mean.
TM1	Melting temperature at which half of the DNA has dissociated into single strands.
TM2	The secondary melting temperature.
TM3	The tertiary melting temperature.
Comments	Comments that have been added to the sample descriptions.

Analysis guidelines for the well table view

When you analyze your own experiment:

1. If you are using positive controls, confirm the integrity of the positive controls:
   a. From the Group By menu, select Variant Call to organize the table rows by their function on the reaction plate. Then select the positive control rows.
   b. Confirm that the positive controls do not display flag(s).
   c. Repeat steps a and b for each positive control.
2. Review the data for the Unknown samples. For each row that displays a flag, note the data and the flag(s) triggered by the associated well.
3. Select areas of the table or wells of a specified type by:
   - Clicking and dragging across the rows you want to select.
   - Selecting Sample, Target, or Task from the Select Wells menu, then selecting the sample, target, or task name from the submenu to select specific wells.
4. 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.
5. 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.

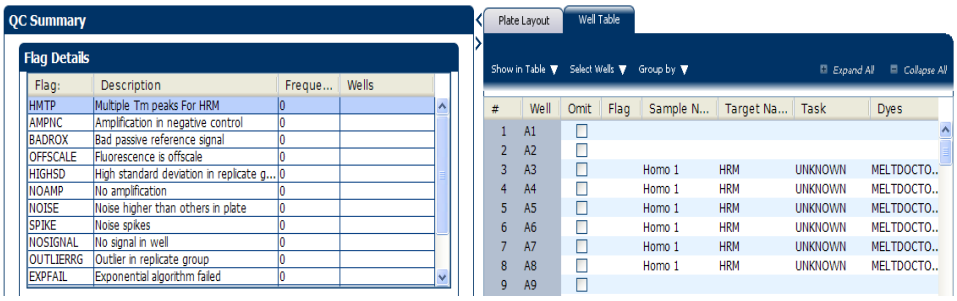
   Note: You must reanalyze the experiment each time you omit or include a well.

View the QC Summary

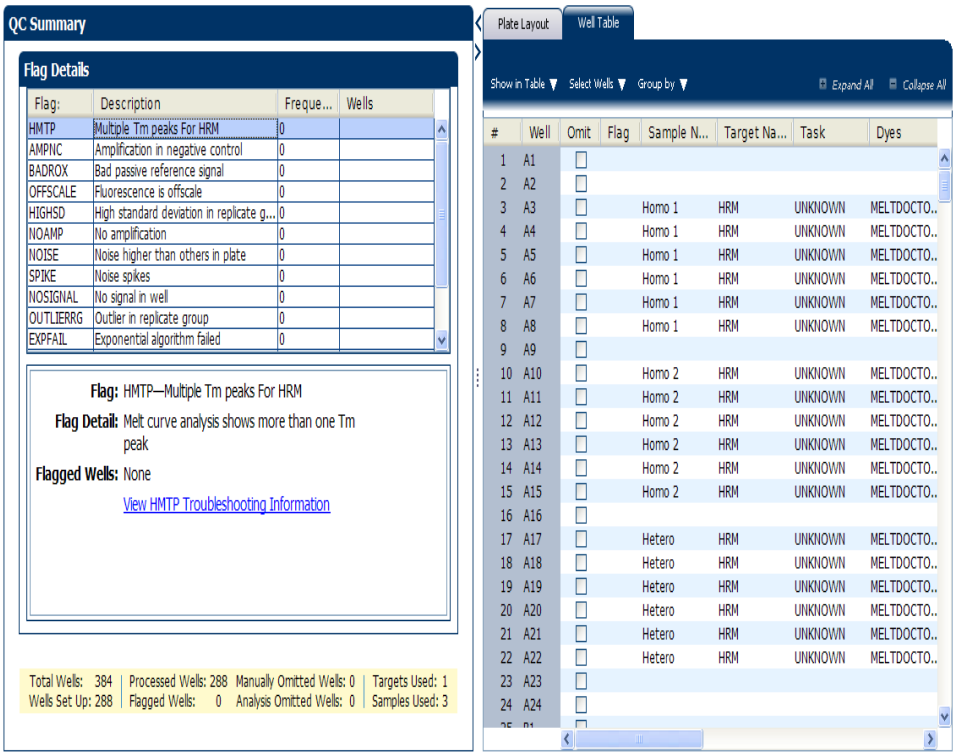
Review the summary of the QC flags triggered by the experiment data and troubleshoot the flags. The QC summary displays a frequency and location of all QC flags. If a flag does not appear in the experiment, its frequency is 0. If the frequency is not 0, that flag appears at the well position listed in the location column. Clicking a flag displays the flag details, including a list of all flagged wells.

Review the QC Summary

1. In the Experiment Menu, select **QC Summary**.



2. In the Flag Details table, look in the Frequency and Wells columns to determine which flags appear in the experiment.



3. In the Flag Details table, check each flag with a frequency >0 to display detailed information about the flag.

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

Possible flags

The flags listed in the following table may be triggered by the experiment data.

Note: To change the flag settings, refer to "Adjust the flag settings" on page 56.

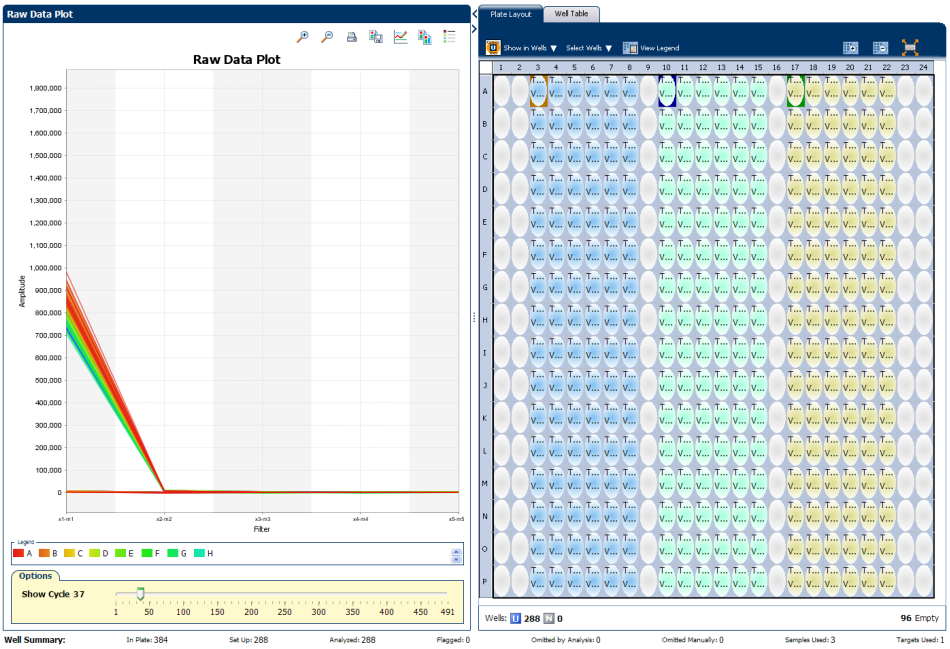
Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
HMTP	Multiple T_m peaks for HRM
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
Secondary analysis flags	
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group

View the Raw Data Plot

The Raw Data Plot displays the amplitude of the raw fluorescence collected in each of the filters during the run cycle indicated by the Show Cycle slider. The plot displays the raw spectra for the wells selected in the plate layout or the well table.

Review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

1. In the navigation pane, select **Raw Data Plot**.



2. In the well table, select the wells that you want to inspect.

   Note: The legend displays the color code for each row of the reaction plate.

3. Click and drag the Show Cycle pointer from cycle 1 to cycle 491 (There are 40 PCR amplification cycles in the default Run Method. After 40 cycles there is only one filter being used in the default protocol). In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.

View the Multicomponent Plot

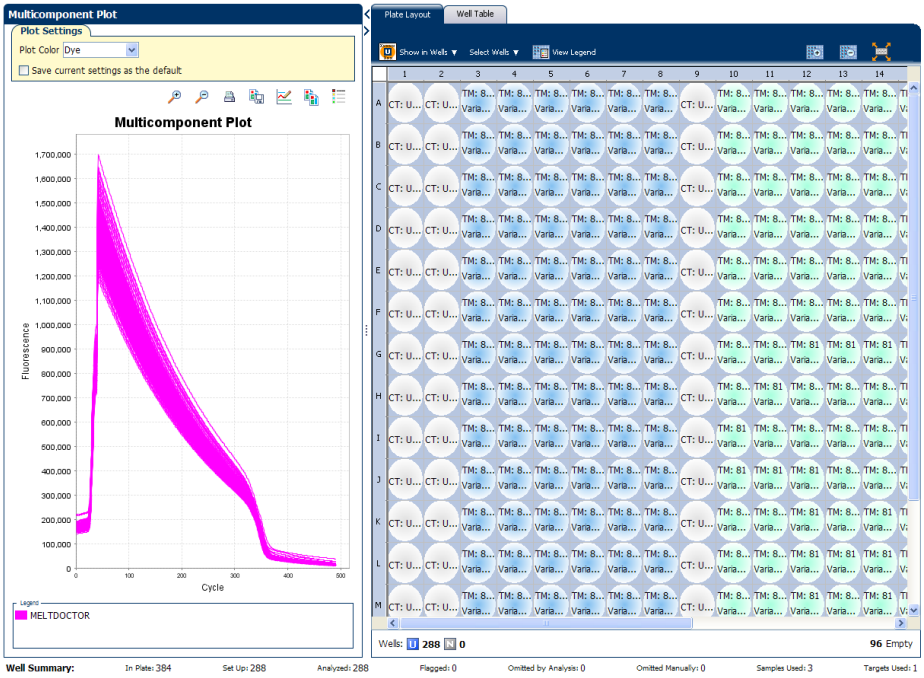
The Multicomponent Plot displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Review the Multicomponent Plot for:

- Any dye you have included as a passive reference
- MeltDoctor™ HRM dye or your custom reporter dye
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

Review the Multicomponent plot

1. In the navigation column, select Multicomponent Plot.



2. Select one unknown well in the plate layout to display the corresponding data in the Multicomponent Plot.

   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. If the Legend is not displayed, click **Show a legend for the plot**.
5. Check the dye signals in the Amplification Plot. The signals should increase throughout the PCR, indicating normal amplification.
6. If you have included one, check the Passive Reference dye signal. It should remain constant throughout the PCR process.

   In the HRM example experiment, there is no Passive Reference dye.
7. Select the negative control wells one at time and check for amplification. If amplification has taken place, there may be contamination in your plate wells.

   In the HRM example experiment, the negative control wells are empty.

Analysis guidelines for Multicomponent Plot

When reviewing the Multicomponent Plot 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.

- **Any 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.

View the Amplification Plot

If you collected real-time data for your experiment, review the amplification data to further understand the flags triggered by the experiment data.

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** – This plot displays ΔR_n 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** – 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** – This plot displays C_T as a function of well position. You can use this plot to locate outlying amplification (outliers)

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

Review the results

1. In the Experiment Menu, select Amplification Plot.
2. In the Amplification Plot:
   a. From the Plot Type drop-down menu, select ΔRn vs Cycle.
   b. From the Plot Color drop-down menu, select Sample.
   c. If the Legend is not displayed, click **Show a legend for the plot**.
3. View the baseline values:
   a. From the Graph Type drop-down menu, select **Linear**.

b. Select **Baseline Start:** to show the start cycle and end cycle.

4. 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:

   a. From the Graph Type drop-down menu, select **Log**.

   b. Select **Threshold** to show the threshold.

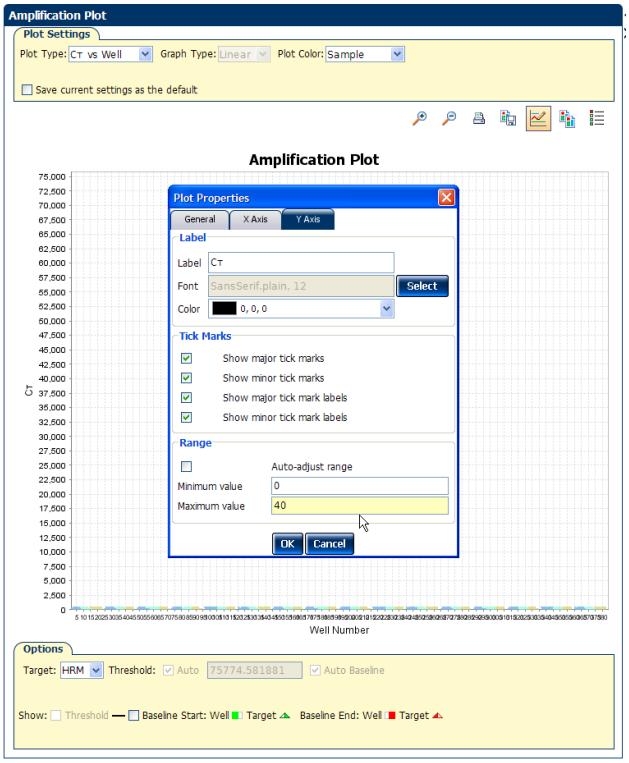
   c. Verify that the threshold is set correctly.

      In the example experiment, the threshold is in the exponential phase.

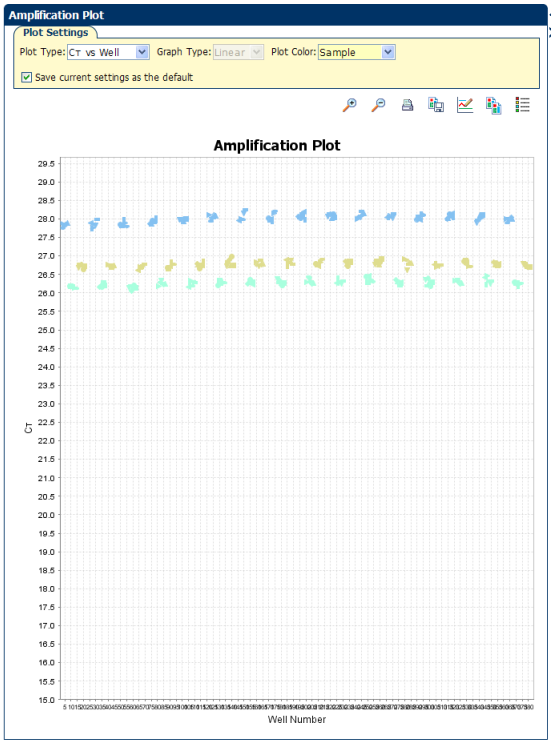
6. Repeat above steps for all targets.

7. From the Plot Type drop-down menu, select **CT vs Well** and from the Plot Color drop-down menu, select **Sample**.

8. If the Y axis scale is set too high, you will need to change the plot properties, so that the Y axis goes to 40.
   Click **Plot Properties** , select **Y axis** tab, change Y axis to **40**, and **Save**.



9. Confirm that the replicas have achieved similar amplification.



Analysis guidelines for Amplification Plot

When you analyze the Amplification Plot, look for:

- **Outliers**
- **A typical amplification plot** – The QuantStudio™ 6 and 7 Flex Real-Time PCR System 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

For information on troubleshooting issues with amplification, see Appendix C, "Troubleshooting HRM Experiments" on page 95.

Modify the Analysis Settings

If you are dissatisfied with how the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software is calling genotypes or the wells which are flagged, review and adjust the analysis settings and/or calls as needed.

Use the example experiment data to review and adjust the analysis settings to learn how the C_T, flag, and call settings contribute to the analysis of the genotyping data.

Adjust the C_T settings

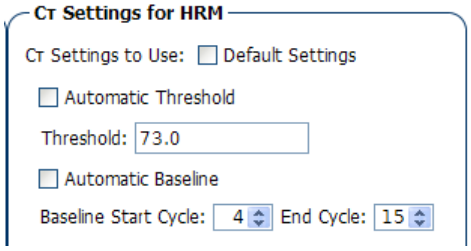
The C_T Settings are available only for experiments that include amplification data.

HRM analysis does not use C_T data. The main use of C_T data is to confirm that amplification proceeded correctly in all samples.

1. Select **Analysis** ▸ **Analysis Settings** ▸ **C_T Settings** tab.

   Note: Use Baseline Threshold if you wish to adjust parameters.

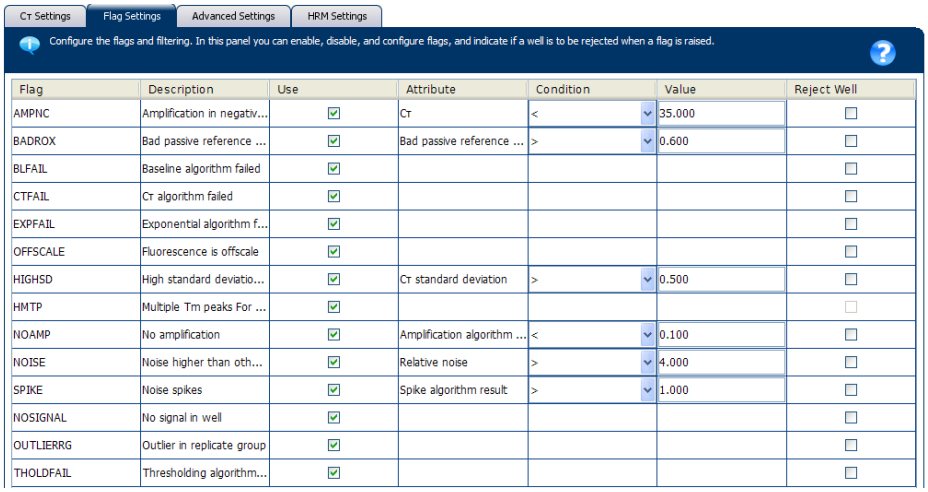
2. Select a target in the **Select a Target** list. (HRM is the only target in the example experiment.)
3. In the **C_T Settings for HRM** section, uncheck **Default Settings.**
4. Uncheck **Automatic Threshold**, then enter a new threshold value.
5. Uncheck **Automatic Baseline**, then enter new baseline values.



6. Repeat steps 1-5 for any other targets for which you wish to modify C_T settings.
7. Click **Apply Analysis Settings** to analyze the data using the new settings.

Adjust the flag settings

1. Select the **Flag Settings** tab.
2. In the Use column, select the check box of each flag that you want to enable
3. Adjust the value(s) for the enabled flags as needed.
4. If you want an enabled QC flag to automatically omit wells that test positive for the condition it defines, select the **Reject Well** check box for the flag.



Flag	Description	Use	Attribute	Condition	Value	Reject Well
AMPNC	Amplification in negativ...	☑	CT	<	35.000	☐
BADROX	Bad passive reference ...	☑	Bad passive reference ...	>	0.600	☐
BLFAIL	Baseline algorithm failed	☑				☐
CTFAIL	CT algorithm failed	☑				☐
EXPFAIL	Exponential algorithm f...	☑				☐
OFFSCALE	Fluorescence is offscale	☑				☐
HIGHSD	High standard deviatio...	☑	CT standard deviation	>	0.500	☐
HMTP	Multiple Tm peaks For ...	☑				☐
NOAMP	No amplification	☑	Amplification algorithm ...	<	0.100	☐
NOISE	Noise higher than oth...	☑	Relative noise	>	4.000	☐
SPIKE	Noise spikes	☑	Spike algorithm result	>	1.000	☐
NOSIGNAL	No signal in well	☑				☐
OUTLIERRG	Outlier in replicate group	☑				☐
THOLDFAIL	Thresholding algorithm...	☑				☐

5. Click **Apply Analysis Settings** to analyze the data using the new settings.

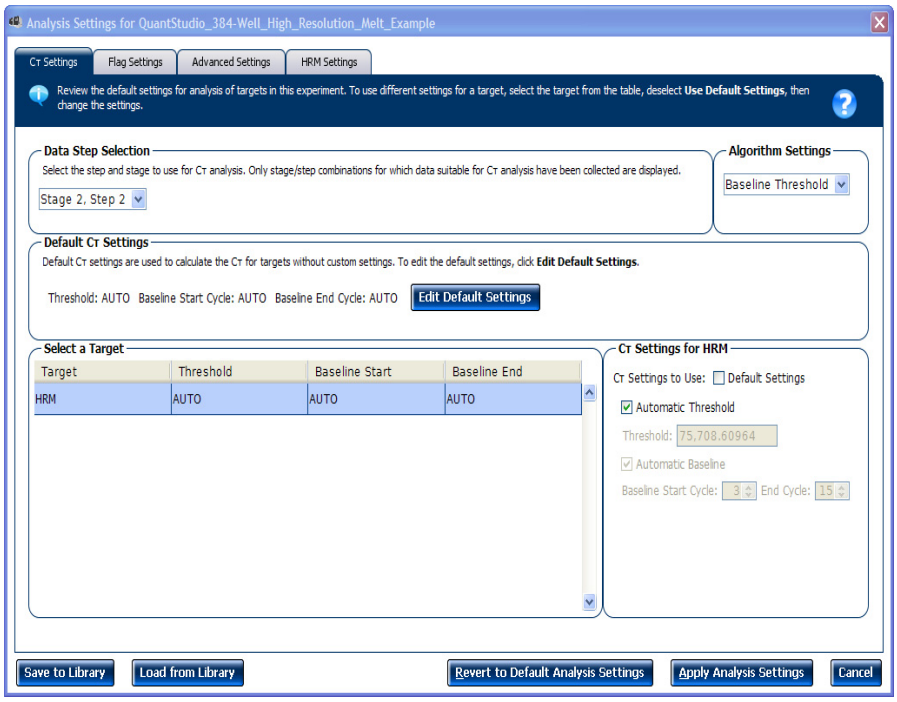
Adjust Pre- and Post-melt call settings to automatic or manual

Assays can use either the default Pre- and Post-melt settings or settings that are manually assigned.

You can individually assign manual or automatic settings to different assays.

1. Select **Analysis** ▸ **Analysis Settings** ▸ **HRM Setting**s tab.
2. Adjust the call assay settings:
   a. If you want to set the Pre- and Post-melt regions to specific values, uncheck **Automatically set the Pre-melt and Post-melt regions**.

b. Enter new settings for Pre-melt Start and Stop temperatures and for Post-melt Start and Stop temperatures.



c. Click **Apply Analysis Settings** to analyze the data using the new settings.

Apply custom Pre- and Post-melt settings to an assay

If you have manually adjusted the Pre- and Post-melt settings, you can uncheck Automatic Calling to apply these settings to any future analysis of a selected assay.

1. Select **Analysis** ▸ **Analysis Settings** ▸ **HRM Settings** tab.
2. Uncheck **Automatically set the Pre-melt and Post-melt regions** in the **HRM Settings** pane.
3. Click **Apply Analysis Settings**.
4. Save the experiment

   The software will use the Pre-and Post-melt settings you entered for any future analysis performed on this assay.

Manually set the number of variants

You can adjust the sensitivity of the software algorithm by manually setting the number of expected variants.

1. Select **Analysis** ▸ **Analysis Settings** ▸ **HRM Settings** tab.
2. Uncheck **Automatically determine the number of variant groups** in the **HRM Settings** pane.
3. Enter the number of variant groups you wish the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software to use when analyzing.
4. Click **Apply Analysis Settings** to have the software reanalyze your file. Click **Save to Assay Settings Library...** to save your settings.

Perform manual calls

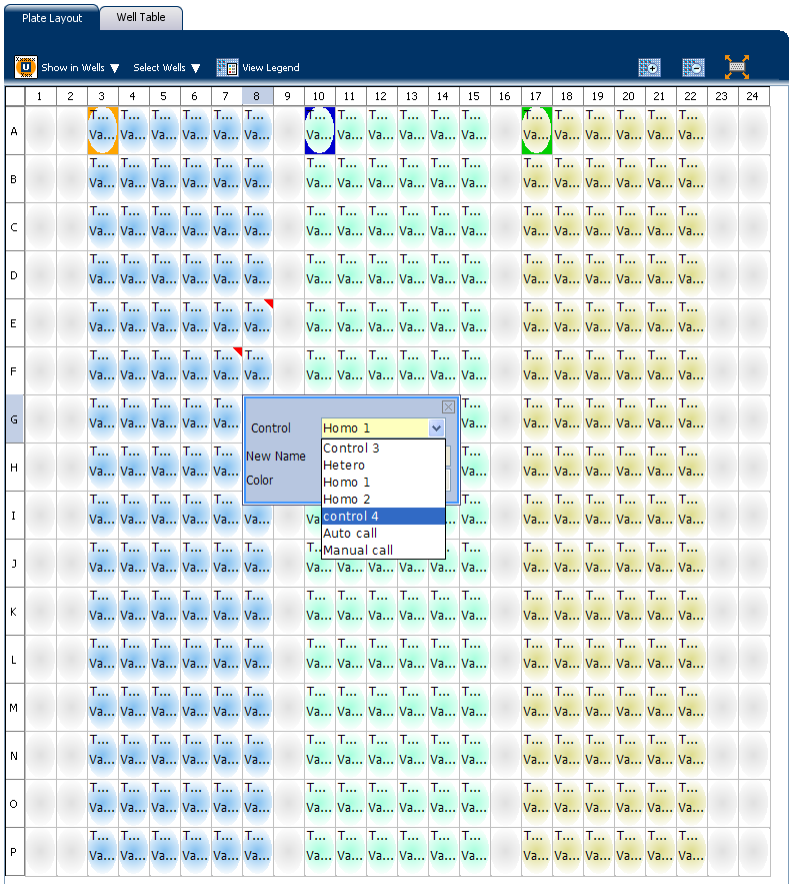
Perform manual calls when you want to manually assign a sample to a variant group.

Perform manual calls in the plate layout

1. In the **Plate Layout** tab, double-click on a well in the plate view.
2. From the **Manual Call** dialog box, you can assign the sample to:
   - **An existing variant call** - Click **Select**, select the appropriate call from the drop-down menu, then click **OK**.
   - **A new variant call** - Click **New**, enter a name for the new call, select a color, then click **OK**.

     In the **Plate Layout** tab, the upper right corner of the sample well is marked with a red triangle.

     In the **Well Table** tab, in the Method column, *Manual* appears next to the selected sample.



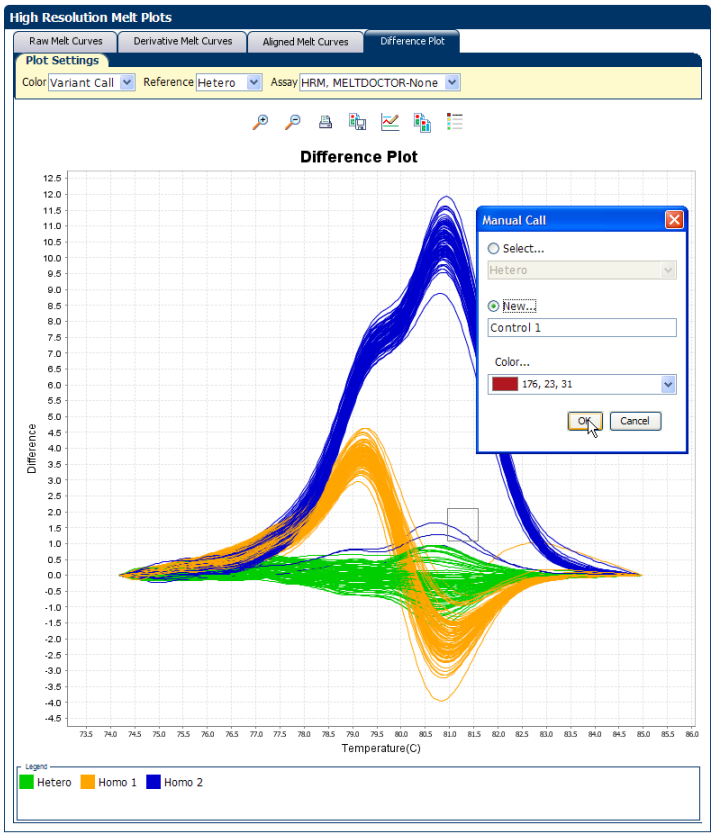
3. Repeat steps 1 and 2 to assign more manual calls.
4. Click **Analyze**. The software reanalyzes the data using the manual calls.

Change selected manual calls to auto calls in the plate layout

1. Double-click on the well to be assigned as an auto call.
2. Right-click in the selection box and select **Auto Call**.
3. Repeat steps 1 and 2 to change other manual calls to auto calls.
4. Click **Analyze**. The software reanalyzes the data.

Perform manual calls in the HRM plots

1. In the **Difference Plot** or **Aligned Melt Curves** plot, click and drag with the mouse to select samples to be called manually. Right-click on the selection box and select **Manual Call** from the menu.



2. From the **Manual Call** dialog box, you can assign the sample to:
   - **An existing variant call** - Click **Select**, select the appropriate call from the drop-down menu, then click **OK**.
   - **A new variant call** - Click **New**, enter a name for the new call, select a color, then click **OK**.

     In the **Plate Layout** tab, the upper right corner of each sample well selected is marked with a red triangle.

     In the **Well Layout** tab, in the Method column, *Manual* appears next to the selected sample.
3. Click **Analyze**. The software reanalyzes the data

Change selected manual calls to auto calls in the HRM plots

1. In the **Difference Plot** or **Aligned Melt Curves** plot, click and drag to select the wells you wish to Auto call.
2. Right-click in the selection box and select **Auto Call**.
3. Repeat steps 1 and 2 to change other manual calls to auto calls.
4. Click **Analyze**. The software reanalyzes the data using the auto call.

Change all manual calls to auto calls

You can quickly change all manual calls to auto calls:

1. Click **Analysis** ▸ **Analysis Settings** ▸ **HRM Settings** tab.
2. Check **Remove all manual variant calls upon reanalysis**.
3. Click **Apply Analysis Settings**.
   The software removes all manual calls and reanalyzes the data using auto calls.

Publish the data

You can publish the experiment data in several ways:

- Save the plot as an image file
- Print the plot
- Print the plate layout
- Create slides
- Print a report
- Export data (see "How to export analysis results" on page 61)

7 Export HRM Genotyping Analysis Results

How to export analysis results

This procedure shows how to export the HRM example experiment to a .txt file. For information on advanced export, refer to the *QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Programming Supplement* (Pub. no. 4489825).

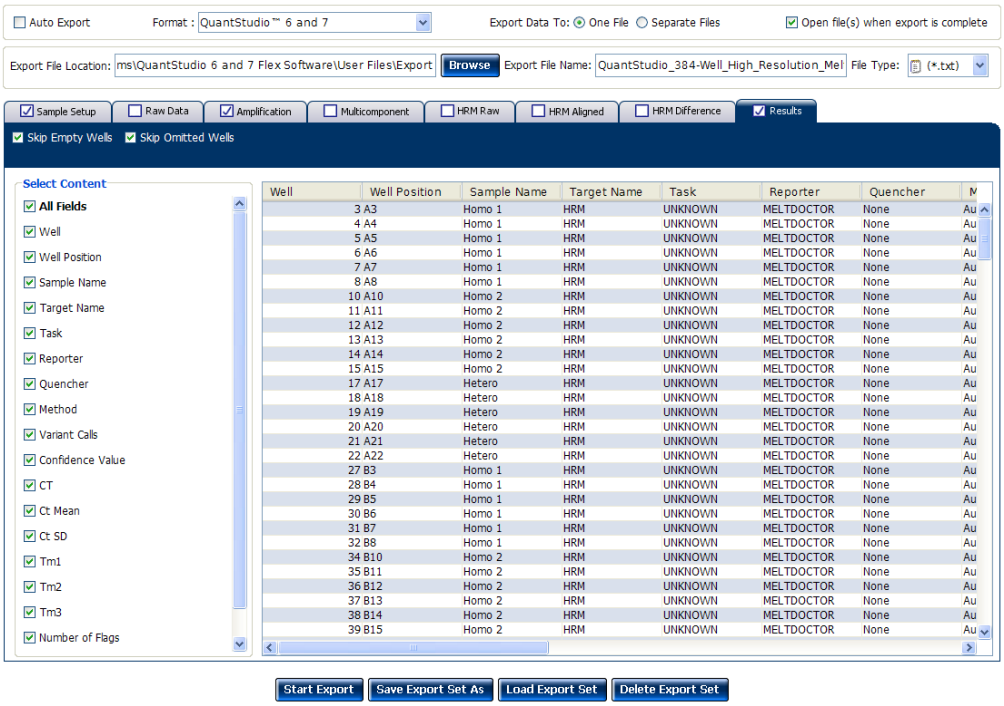
1. Open the HRM example experiment file. **Open ▸ Program Files ▸ Applied Biosystems ▸ QuantStudio 6 and 7 Flex Software ▸ examples ▸ QS6Flex ▸ QuantStudioTD 384 Well High Resolution Melt Example.eds**.
2. In the Experiment Menu, click  **Export.**

Note: If you want the data to be exported 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 **QuantStudio™ 6 and 7** format.
4. Complete the Export dialog box and save the export file to the default location (*<drive>*:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\Export) or to the location of your choice.

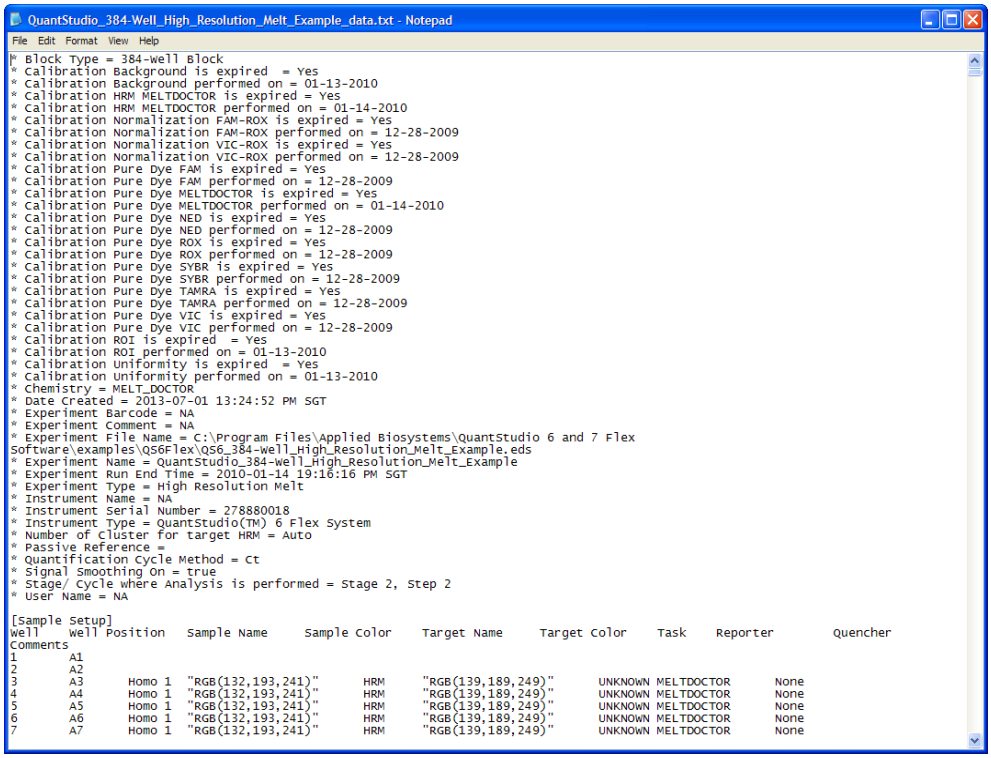
Note: By default, all of the experiment data will be exported. This file can be quite large. If you wish, you can uncheck tabs that contain data that you wish to exclude from the file.

The following is an image of the export screen for the example experiment:



Well	Well Position	Sample Name	Target Name	Task	Reporter	Quencher
3	A3	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
4	A4	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
5	A5	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
6	A6	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
7	A7	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
8	A8	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
10	A10	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None
11	A11	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None
12	A12	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None
13	A13	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None
14	A14	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None
15	A15	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None
17	A17	Hetero	HRM	UNKNOWN	MELTDOCTOR	None
18	A18	Hetero	HRM	UNKNOWN	MELTDOCTOR	None
19	A19	Hetero	HRM	UNKNOWN	MELTDOCTOR	None
20	A20	Hetero	HRM	UNKNOWN	MELTDOCTOR	None
21	A21	Hetero	HRM	UNKNOWN	MELTDOCTOR	None
22	A22	Hetero	HRM	UNKNOWN	MELTDOCTOR	None
27	B3	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
28	B4	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
29	B5	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
30	B6	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
31	B7	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
32	B8	Homo 1	HRM	UNKNOWN	MELTDOCTOR	None
34	B10	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None
35	B11	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None
36	B12	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None
37	B13	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None
38	B14	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None
39	B15	Homo 2	HRM	UNKNOWN	MELTDOCTOR	None

The following is an image of the exported file as it should appear when opened in Notepad:



8 Perform an HRM Mutation Scanning Experiment

Perform an HRM mutation scanning experiment to screen DNA samples for new single-base changes, insertions/deletions, or other unknown mutations.

Note: This chapter provides basic instructions for running a Mutation Scanning experiment. For more information on the Mutation Scanning experiments, refer to the applicable Application Note or contact Life Technologies (see "Documentation and Support" on page 99).

Design the HRM experiment

Design and order the primers

Using Primer Express® Software v3.0 or later, design the primers to amplify the genomic DNA that spans the mutations of interest. Order the primers from the Life Technologies Store.

1. Design the primers so that they meet these guidelines:

Design attribute	Design guidelines
Amplicon	Length is less than 250 basepairs
Primer length	~20 bases each
Tm	58°C to 60°C (Optimal Tm is 59°C)
% GC content	30–80% GC content in each primer
3′ end	No more than 2 G+C residues in the last 5 nucleotides at the 3′ end
Repeating oligonucleotides	Avoid consecutive identical nucleotides. If you are unable to avoid consecutive identical nucleotides, make sure that each primer contains fewer than 4 consecutive Gs.

2. If you want to use M13F and M13R primers in the sequencing reaction, add the appropriate M13 tail to the 5′ end of the primers:
   - M13F (add to the 5′ end of the forward primer): TGTAAAACGACGGCCAGT
   - M13R (add to the 5′ end of the reverse primer): CAGGAAACAGCTATGACC

3. Go to **www.lifetechnologies.com**, then log into the Life Technologies Store if you have an account; register if you are a new user. For more instructions, see "How to order custom primers" on page 85.

Select controls

Include controls for each target sequence in your HRM mutation scanning experiment:

- At least one negative control
- At least one wild type control

  Run up to 5 replicates for each wild type control to improve your results. Running multiple wild type controls allows you to more effectively define the natural spread or variation within the normal population.

Create and set up the HRM run file

Create and set up an HRM mutation scanning experiment in the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software.

The following table mentions the recommended settings for all Mutation Scanning experiments in the order that they should be performed:

Step no.	Setup	Recommended setting
1	Experiment properties	• Experiment type: **High Resolution Melt** • Reagents: **MeltDoctor™ HRM Reagents**, then select the **Include PCR** check box • Ramp speed: **Standard (~ 2 hours to complete a run)**
2	Define Target properties	• Reporter: **MELTDOCTOR** • Quencher: **None**
	Define Samples	Set up samples For each control sample, enter information about that control in the HRM Software and assign the control to the appropriate wells. **Note:** For control names, do not use the convention *variantN*, where *N* is any number (for example, *variant1*, *variant2*, and so on). The QuantStudio™ 6 and 7 Flex Real-Time PCR System Software uses the convention *variantN* when automatically assigning the variant calls. **Note:** This step can be done before or after running a plate.
3	Plate layout	Assign samples to appropriate wells
4	Run method	• Reaction Volume Per Well: **20 µL** • Ramp mode and rate: Select **Continuous**, then set the ramp rate to 0.3%

Note: If you use HRM reagents from another manufacturer you must first perform calibration using those dyes. See "Custom HRM dyes" on page 89.

Note: For detailed instructions on how to create and set up an HRM experiment, see Chapter 3, "Design an HRM Genotyping Experiment" on page 23.

Prepare the HRM reactions

Combine purified genomic DNA templates with MeltDoctor™ HRM Master Mix and primers to amplify the target sequence.

Note: If you are using the MeltDoctor™ HRM Reagent Kit instead of the MeltDoctor™ HRM Master Mix, see page 93 for reaction component volumes.

Required materials

- Microcentrifuge tubes
- Optical reaction plate appropriate for your Real-Time PCR instrument
- MicroAmp™ Optical Adhesive Film
- MeltDoctor™ HRM Master Mix
- For each target sequence:
  - Forward and reverse primers (5 µM each)
  - DNA samples
- Deionized water
- Pipettors and pipette tips
- Vortexer
- Centrifuge

Prepare the HRM reactions

Prepare the reactions for each replicate group separately, then transfer the reactions to a reaction plate appropriate for your instrument.

For information about using the MeltDoctor™ HRM Reagent Kit to optimize your reactions, see "Optimizing the reaction conditions" on page 93.

1. Prepare the negative control reactions in an appropriately sized, labeled tube:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess	Volume for one 50-µL reaction	Volume for three 50-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL	25.0 µL	82.5 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Deionized water	7.6 µL	25.08 µL	19.0 µL	62.7 µL
Total reaction volume	**20.0 µL**	**66.00 µL**	**50.0 µL**	**165.0 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Life Technologies recommends an excess volume of at least 10%.

2. Prepare positive control reactions and unknown reactions in separate appropriately sized, labeled tubes:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess	Volume for one 50-µL reaction	Volume for three 50-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL	25.0 µL	82.5 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Genomic DNA (20 ng/µL)	1.0 µL	3.30 µL	2.5 µL	8.25 µL
Deionized water	6.6 µL	21.78 µL	16.5 µL	54.45 µL
Total reaction volume	**20 µL**	**66 µL**	**50 µL**	**165 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Life Technologies recommends an excess volume of at least 10%.

3. Vortex the reactions to mix, then spin the tubes briefly.
4. Prepare a reaction plate appropriate for your instrument:
   a. Pipet each reaction replicate to the appropriate wells of the optical reaction plate:

Reaction plate	Reaction volume
Fast 384-well plate	20 µL
Fast 96-well plate	20 µL
Standard 96-well plate	50 µL

   b. Seal the reaction plate with optical adhesive film, then spin the reaction plate.
   c. Confirm that the liquid is at the bottom of the wells in the reaction plate.

Note: If you plan to wait more than 24 hours before running the plate, store the plate at 4°C. Allow the plate to warm to room temperature, then spin the plate briefly before running it.

Amplify and melt the DNA

After you have prepared the reaction plate, run the plate to amplify and melt the DNA and to generate HRM fluorescence data using the following settings:

Stage	Step	Temp	Time
Holding	Enzyme activation	95°C	10 min
Cycling (40 cycles)	Denature	95°C	15 sec
	Anneal/extend	60°C	1 min
Melt curve/dissociation	Denature	95°C	10 sec
	Anneal	60°C	1 min
	High resolution melting	95°C	15 sec
	Anneal	60°C	15 sec

Note: See Chapter 5, "Run an HRM Genotyping Experiment" on page 33 for information on running an HRM experiment.

Verify that the samples amplified

Review the Amplification Plot

Review the Amplification Plot for normal characteristics:

- Fluorescence levels that exceed the threshold between cycles 8 and 35
- An exponential increase in fluorescence

Note the following:

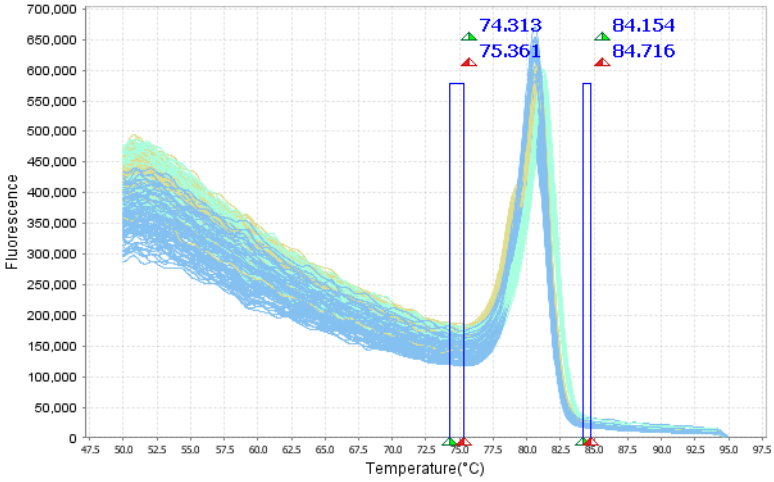
- Outlier wells with C_T values that differ from replicates by more than 2.
- The outliers may produce erroneous HRM results.
- If the Amplification Plot looks abnormal, refer to Appendix C, "Troubleshooting HRM Experiments" on page 95 to identify and resolve the problem.

Review the peaks in the melt curve

1. Verify that the Derivative Melt Curve shows no unexpected T_m peaks:
   If the sequence you amplified contains more than 1 variant or a more complex mutation, you may see more than 1 T_m peak.

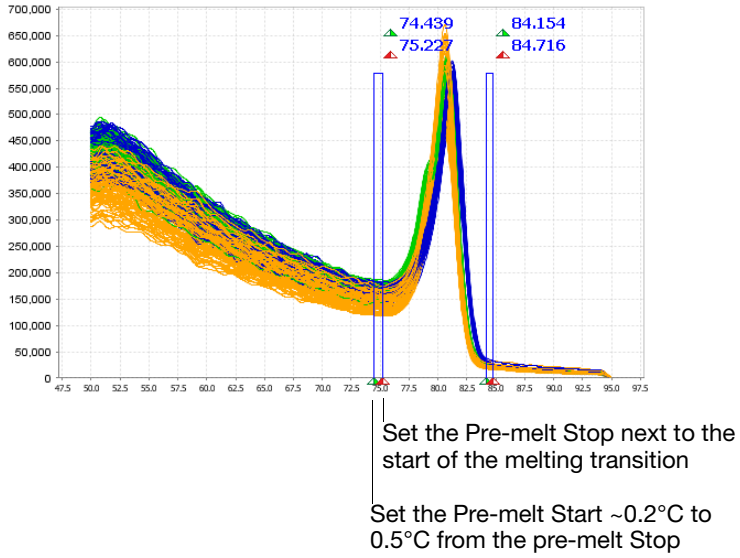
   Note the following:
   Unexpected peaks may indicate:
   - Contamination
   - Primer dimers, or
   - Non-specific amplification

   Note the following:
   The data appear noisy because:
   - More data is collected during a high resolution melt curve than during a standard melt curve
   - The extra data are required for analysis with the High Resolution Melt Module for the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software

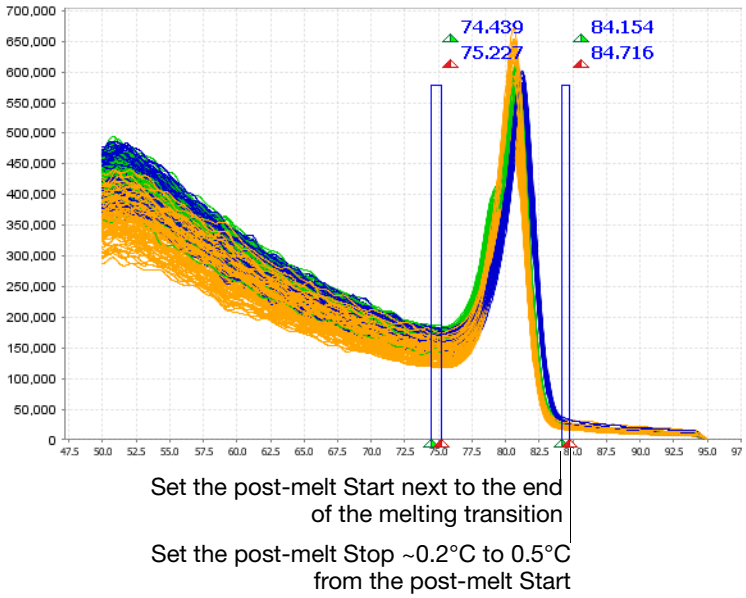


2. In the Derivative Melt Curves plot, review and adjust the Pre- and Post-melt regions to optimize your separation and variant calls. For most experiments, set the Pre- and Post-melt regions as close as possible to the melting transition region:

- The Pre-melt Start and Stop temperature lines (green and red arrows on the left) should be approximately 0.2°C to 0.5°C apart from each other.



- The post-melt Start and Stop temperature lines (green and red arrows on the right) should be approximately 0.2°C to 0.5°C apart from each other.



Review the high-resolution melting data

This section provides tips and guidelines to review the high-resolution data for a mutation scanning experiment.

After you create, run, and analyze the *.eds file using the QuantStudio™ 6 or 7 Instrument with the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software, use the HRM module for the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software to perform high resolution melting analysis of the data and screen the samples for mutations.

About the melting profiles

The melting profile of a PCR product depends on its GC content, length, sequence, and heterozygosity. High-resolution melting analysis calls variants based on the differences in the shape of the melt curves and the differences in the T_m values.

In mutation scanning experiments, the variants have a different curve shape or T_m compared to the wild type.

Review the populations in the Aligned Melt Curves plot

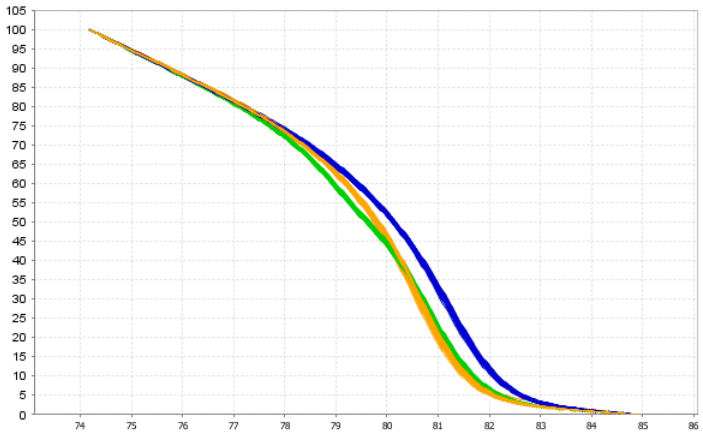
The Aligned Melt Curves plot displays the melt curves as % melt (0-100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions that you set (see page 68).

1. In the Analyzed Data pane, select the **Aligned Melt Curves** tab.
2. Review:
   - Wild type controls – Do the melt curves for the wild type controls cluster well? Are there any outliers?
   - Possible mutations – Are there any samples with melt curves that are different from the wild type melt curves?

Aligned Melt Curves example

In the following example, there is 1 distinct variant group for the wild type samples. There are 2 samples that vary from the wild type samples and may contain mutations.

Note: The colors represent three different variants.



Review the Difference Plot for outliers

The Difference Plot displays the aligned data as the difference in fluorescence between the melt curve for a reference sample and the other melt curves. You can select a control or any well as a reference. After you select the reference, the software subtracts the reference curve from the other curves.

The Difference Plot allows you to more easily see small differences between curves and identify outliers.

1. In the Analyzed Data pane, select the **Aligned Data - Difference Plot** tab.

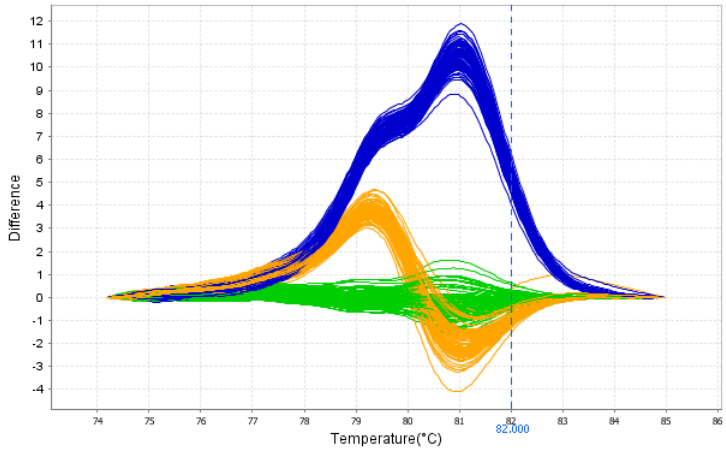
2. From the **Reference** drop-down menu, select a control or any well as the reference, then review:
   - Variant clusters – How many distinct clusters are displayed?
   - Outliers – How tight are the curves within each variant cluster?

   Note: Try selecting different reference samples to find the optimal display of the clusters.

Difference Plot example

In the following example, there is 1 distinct variant group for the wild type samples. There are 2 samples that vary from the wild type samples and may contain mutations.

Note: The colors represent three different variants.



Review the software calls

The QuantStudio™ 6 and 7 Flex Real-Time PCR System Software automatically makes a call for each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. In the Results pane, click the **Well** column header to sort the results according to the well position.
2. For each replicate group, review:
   - Variant Call column – Do all replicates have the same call?
   - Confidence column – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?
3. To view the fluorescence data for certain wells, select the rows in the Results table.
4. In the Results pane, click the **Variant Call** column header to sort the results according to the variant call. Scan the results for samples that were not assigned the same call as the wild type control.

Omit outliers or change calls

After you review the software calls, you can omit outliers or change calls. Remember to click Analyze to reanalyze the data after you omit outliers or change calls.

For more detailed instructions on the following, see page 39:

- Omit outliers from analysis
- Change calls made by the software

- Revert selected manual calls to the software Auto call
- Revert all manual calls to the software Auto call

(Optional) Sequence the variants

After you identify the variants in the HRM software, dilute or purify the PCR product from the HRM reactions, then sequence the variants. For more information on sequencing the variants, contact Life Technologies.

9 Perform an HRM Methylation Study

Perform an HRM methylation study to determine the percentage of methylated DNA in unknown samples. This chapter covers:

Note: This chapter provides basic instructions for running a Methylation Study experiment. For more information on the Methlylation study, refer to the applicable Application Note or contact Life Technologies (see "Documentation and Support" on page 99).

Design the HRM Experiment

Design and order the primers

Using Life Technologies Methyl Primer Express® Software, design the primers to amplify the genomic DNA that spans the methylation sites of interest. With Methyl Primer Express® Software, you can specify the number of CpG dinucleotides to include in the PCR primers and their position. Order the primers from the Life Technologies Store.

1. Design the primers so that they meet these guidelines:

Design attribute	Design guidelines
Amplicon	• Length is less than 250 basepairs • To detect high levels of methylation, primers lie outside of the CpG island • To detect low levels of methylation, primer sequences include CpG dinucleotides
Primer length	~20 bases each
T_m	58°C to 60°C (Optimal Tm is 59°C)
% GC content	30–80% GC content in each primer
3′ end	No more than 2 G+C residues in the last 5 nucleotides at the 3′ end

Design attribute	Design guidelines
Repeating oligonucleotides	Avoid consecutive identical nucleotides. If you are unable to avoid consecutive identical nucleotides, make sure that each primer contains fewer than 4 consecutive Gs.

2. Go to **www.lifetechnologies.com**, then log into the Life Technologies Store if you have an account; register if you are a new user. For more instructions, see "How to order custom primers" on page 85.

Select controls

Including controls for each target sequence in your HRM methylation study:

- At least one negative control
- Methylated DNA standards that contain from 0% to 100% methylated DNA

Create and set up the HRM run file

Create and set up an HRM methylation experiment in the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software.

The following table mentions the recommended settings for all Methylation Study experiments in the order that they should be performed:

Step no.	Setup	Recommended setting
1	Experiment properties	• Experiment type: **High Resolution Melt** • Reagents: **MeltDoctor™ HRM Reagents**, then select the **Include PCR** check box • Ramp speed: **Standard (~ 2 hours to complete a run)**
2	Define Target properties	• Reporter: **MELTDOCTOR** • Quencher: **None**
	Define Samples	Set up samples For each control sample, enter information about that control in the HRM Software and assign the control to the appropriate wells. **Note:** For control names, do not use the convention *variantN*, where *N* is any number (for example, *variant1*, *variant2*, and so on). The QuantStudio™ 6 and 7 Flex Real-Time PCR System Software uses the convention *variantN* when automatically assigning the variant calls. **Note:** This step can be done before or after running a plate.
3	Plate layout	Assign samples to appropriate wells
4	Run method	• Reaction Volume Per Well: **20 µL** • Ramp mode and rate: Select **Continuous**, then set the ramp rate to 0.3%

Note: If you use HRM reagents from another manufacturer you must first perform calibration using those dyes. See "Custom HRM dyes" on page 89.

Note: For detailed instructions on how to create and set up an HRM experiment, see Chapter 3, "Design an HRM Genotyping Experiment" on page 23.

Optimize the HRM reactions

Optimize the HRM reactions to identify the most suitable PCR reaction to study a differentially methylated region.

1. Prepare the HRM reactions: Test different reaction conditions.
2. Amplify and melt the DNA: Review the C_T values to quantify the efficiency of the PCR reaction.
3. Review the HRM data: Review the specificity of the PCR reaction and the melting behavior of the PCR fragments.
4. Perform electrophoresis of the PCR products on high-percentage agarose gels: Verify the size of the amplicon and review the specificity of the PCR reaction.

Prepare the HRM reactions

Combine purified genomic DNA templates with MeltDoctor™ HRM Master Mix and primers to amplify the target sequence.

Note: If you are using the MeltDoctor™ HRM Reagent Kit instead of the MeltDoctor™ HRM Master Mix, see "Optimizing the reaction conditions" on page 93.

Prepare the methylated DNA standards

1. Obtain universally methylated DNA to represent DNA that is 100% methylated.
2. Select DNA that is non-methylated to represent DNA that is 0% methylated.
3. Mix different ratios of 100% methylated and 0% methylated DNA of equal concentration. For example:

DNA	Volume to prepare the methylated DNA standard					
100% methylated DNA (20 ng/µL)	10 µL	7.5 µL	5 µL	2.5 µL	1 µL	0 µL
Non-methylated DNA (20 ng/µL)	0 µL	2.5 µL	5 µL	7.5 µL	9 µL	10 µL
% methylated DNA	100%	75%	50%	25%	10%	0%

Note: To detect low levels of methylation, add more standards between 0% and 2% methylation. For example, prepare standards to represent 0.0%, 0.1%, 0.5%, 1%, 2%, 5%, 10%, and 100% methylation.

Treat the samples and methylated DNA standards with bisulfite

Before you perform the HRM reactions for your methylation study, treat your samples and methylated DNA standards with bisulfite to convert non-methylated cytosines (C) in your DNA to uracil (U). Samples that vary in the number of U residues within the amplified sequence will have distinct melt curve shapes and T_m values.

Required materials

- Microcentrifuge tubes
- Optical reaction plate appropriate for your Real-Time PCR instrument
- MicroAmp™ Optical Adhesive Film
- MeltDoctor™ HRM Master Mix

- For each target sequence:
  - Forward and reverse primers (5 µM each)
  - DNA samples
- Methylated DNA standards
- Deionized water
- Pipettors and pipette tips
- Vortexer
- Centrifuge

Prepare the HRM reactions

Prepare the reactions for each replicate group separately, then transfer the reactions to a reaction plate appropriate for your instrument.

For information about using the MeltDoctor™ HRM Reagents Kit to optimize your reactions, see "Optimizing the reaction conditions" on page 93.

1. Prepare the negative control reactions in an appropriately sized, labeled tube:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess	Volume for one 50-µL reaction	Volume for three 50-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL	25.0 µL	82.5 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Deionized water	7.6 µL	25.08 µL	19.0 µL	62.7 µL
Total reaction volume	**20.0 µL**	**66.00 µL**	**50.0 µL**	**165.0 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Life Technologies recommends an excess volume of at least 10%.

2. Prepare methylated DNA standards and unknown reactions in separate appropriately sized, labeled tubes:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	**Volume for three 20-µL replicates plus 10% excess**	**Volume for one 50-µL reaction**	**Volume for three 50-µL replicates plus 10% excess**
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL	25.0 µL	82.5 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Genomic DNA (20 ng/µL)	1.0 µL	3.30 µL	2.5 µL	8.25 µL
Deionized water	6.6 µL	21.78 µL	16.5 µL	54.45
Total reaction volume	**20 µL**	**66 µL**	**50 µL**	**165 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Life Technologies recommends an excess volume of at least 10%.

3. Vortex the reactions to mix, then spin the tubes briefly.
4. Prepare a reaction plate appropriate for your instrument:
   a. Pipet each reaction replicate to the appropriate wells of the optical reaction plate:

Reaction plate	Reaction volume
Fast 384-well plate	20 µL
Fast 96-well plate	20 µL
Standard 96-well plate	50 µL

   b. Seal the reaction plate with optical adhesive film, then spin the reaction plate.
   c. Confirm that the liquid is at the bottom of the wells in the reaction plate.

Note: If you plan to wait more than 24 hours before running the plate, store the plate at 4°C. Allow the plate to warm to room temperature, then spin the plate briefly before running it.

Amplify and melt the DNA

After you have prepared the reaction plate, run the plate to amplify and melt the DNA and to generate HRM fluorescence data using the following settings:

Stage	Step	Temp	Time
Holding	Enzyme activation	95°C	10 min
Cycling (40 cycles)	Denature	95°C	15 sec
	Anneal/extend	60°C	1 min
Melt curve/dissociation	Denature	95°C	10 sec
	Anneal	60°C	1 min
	High resolution melting	95°C	15 sec
	Anneal	60°C	15 sec

Note: Adjust the annealing temperature during the amplification to increase or decrease the extent of the PCR bias.

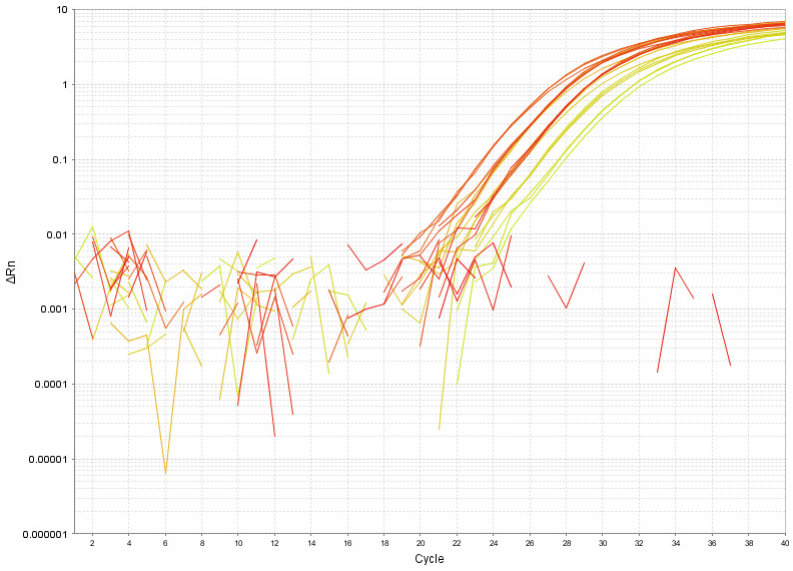
Note: See Chapter 5, "Run an HRM Genotyping Experiment" on page 33 for information on running an HRM experiment.

Verify that the samples amplified

Review the Amplification Plot

Review the Amplification Plot for normal characteristics:

- Fluorescence levels that exceed the threshold between cycles 8 and 35
- An exponential increase in fluorescence



Note the following:

- Outlier wells with C_T values that differ from replicates by more than 2.
- The outliers may produce erroneous HRM results.
- If the Amplification Plot looks abnormal, refer to Appendix C, "Troubleshooting HRM Experiments" on page 95 to identify and resolve the problem.

Review the peaks in the melt curve

1. Verify that the Dissociation Curve/Melt Curve shows no unexpected T_m peaks:

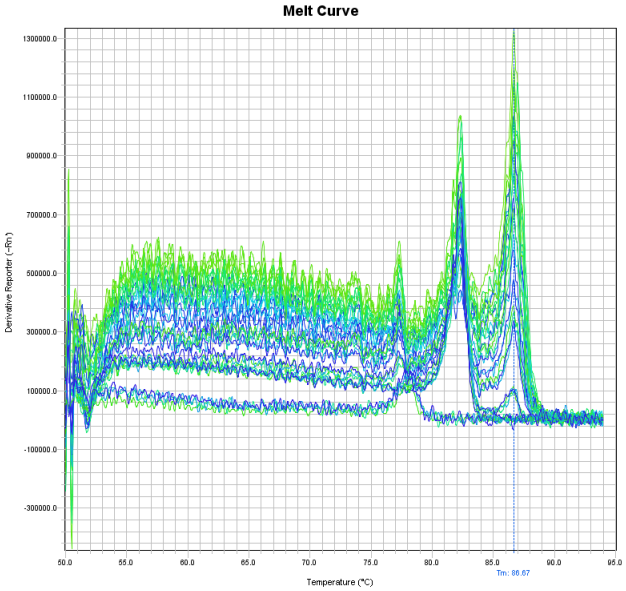
   With methylation experiments, you will likely see multiple peaks. The number of peaks in the melt curve is correlated with the number of methylation sites in the amplicon.

   Note the following:
   Unexpected peaks may indicate:

   - Contamination
   - Primer dimers, or
   - Non-specific amplification

   Note the following:
   The data appear noisy because:

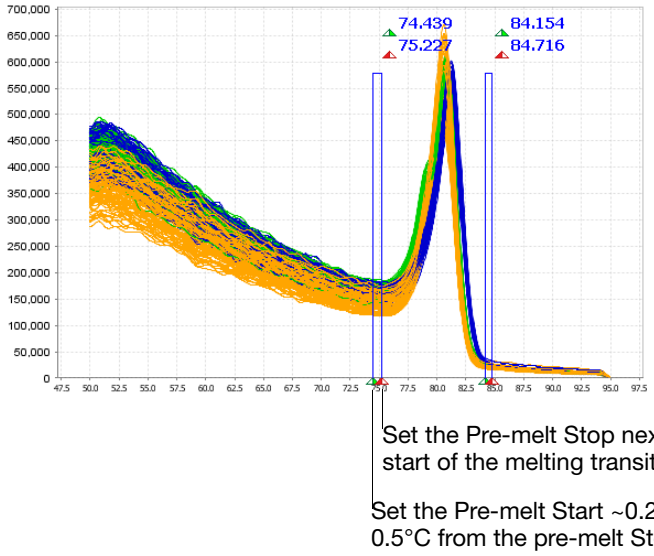
   - More data is collected during a high resolution melt curve than during a standard melt curve
   - The extra data are required for analysis with the High Resolution Melt Module for the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software

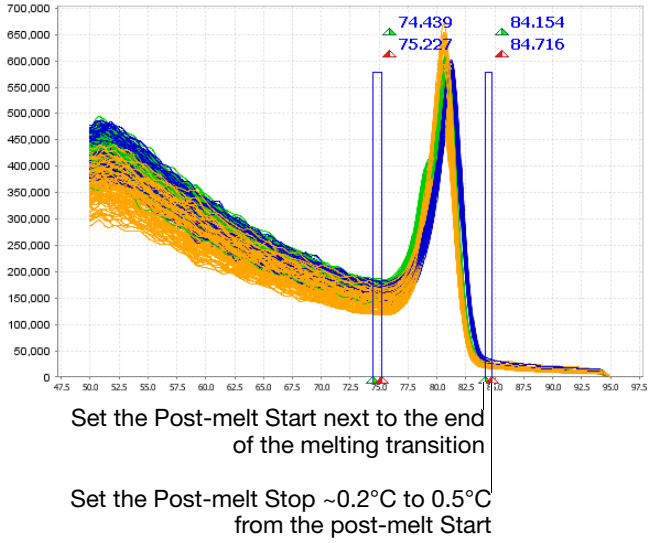
   The low methylation example above shows multiple T_m peaks because of the large differences between the samples with low methylation and the wild type samples. Notice that there are no large Tm peaks at the lower temperatures.

2. In the Derivative Melt Curves plot, review and adjust the Pre- and Post-melt regions to optimize your separation and variant calls. For most experiments, set the Pre- and Post-melt regions as close as possible to the melting transition region:

- The Pre-melt Start and Stop temperature lines (green and red arrows on the left) should be approximately 0.2°C to 0.5°. apart from each other.



- The Post-melt Start and Stop temperature lines (green and red arrows on the right) should be approximately 0.2°C to 0.5°C apart from each other.



Review the high-resolution melting data

This section provides tips and guidelines to review the high-resolution data for a methylation study experiment.

After you create, run, and analyze the *.eds file using the QuantStudio™ 6 or 7 Instrument with the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software, use the HRM module for the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software to perform high resolution melting analysis of the data and review the methylation data.

About the melting profiles

The melting profile of a PCR product depends on its GC content, length, sequence, and heterozygosity. High-resolution melting analysis calls variants based on the differences in the shape of the melt curves and the differences in the Tm values.

In methylation studies, the shape of the melt curve and the Tm values vary according to the number of C residues converted to U after the bisulfite treatment.

Review the populations in the Aligned Melt Curves plot

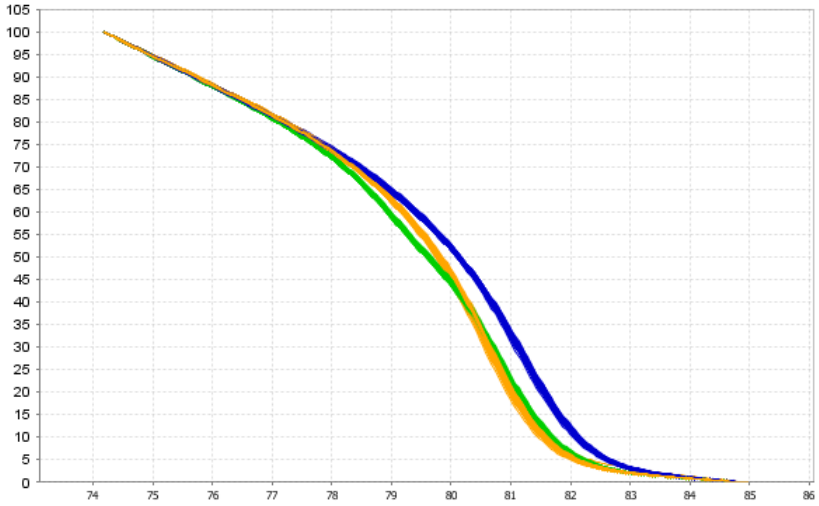
The Aligned Melt Curves plot displays the melt curves as % melt (0-100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions that you set (see page 79).

1. In the Analyzed Data pane, select the **Aligned Melt Curves** tab.
2. Review:
   - Methylated DNA standards – Do the melt curves for the methylated DNA standards cluster well? Are there any outliers?
   - Define methylation range for unknowns – Which methylated standard melt curves are above and below the melt curves for the unknowns? For example, if the melt curve for an unknown sample lies between the melt curves for the 5% and 10% methylated standards, the unknown sample contains between 5% and 10% methylated nucleotides.

Aligned Melt Curves example

In the following example, there are 8 distinct variant groups, 1 for each methylation standard.

Note: The colors represent three different variants.



Review the Difference Plot for outliers

The Difference Plot displays the aligned data as the difference in fluorescence between the melt curve for a reference sample and the other melt curves. You can select a control or any well as a reference. After you select the reference, the software subtracts the reference curve from the other curves.

The Difference Plot allows you to more easily see small differences between curves and identify outliers.

1. In the Analyzed Data pane, select the **Aligned Data - Difference Plot** tab.

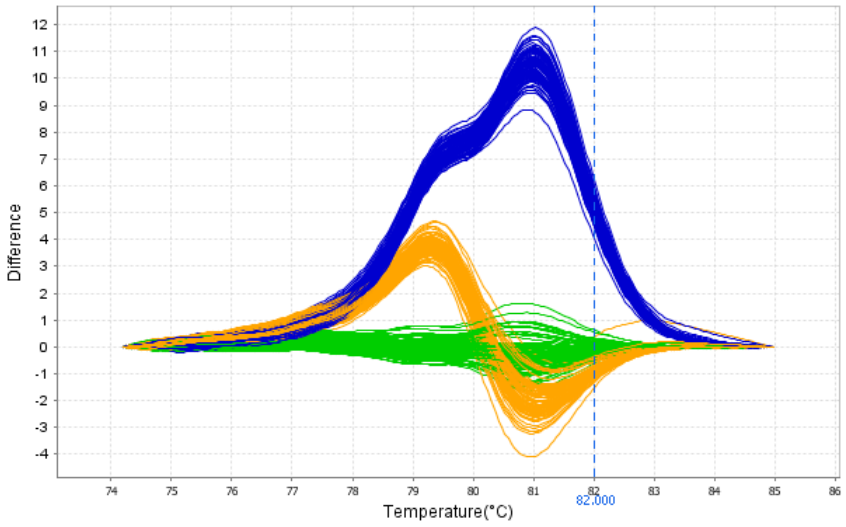
2. From the **Reference** drop-down menu, select a control or any well as the reference, then review:
   - Variant clusters – How many distinct clusters are displayed?
   - Outliers – How tight are the curves within each variant cluster?

   Note: Try selecting different reference samples to find the optimal display of the clusters.

Difference Plot example

In the following example, there are 8 distinct variant groups, 1 for each methylation standard.

Note: The colors represent three different variants.



Review the software calls

The QuantStudio™ 6 and 7 Flex Real-Time PCR System Software automatically makes a call for each sample according to the shape of the aligned melt curves and the T_m. Review the software calls, then omit outliers or change calls.

1. In the Results pane, click the **Well** column header to sort the results according to the well position.
2. For the methylation standard controls, review:
   - Variant Call column – Do all of the methylation standard controls have the correct call?
   - Confidence column – Are there any outliers within the replicate group? Do the values for the replicate group differ from the confidence values for the other replicate groups in the plate?

   Note: If any of the controls are outliers, omit them from the HRM analysis, then reanalyze.
3. To view the fluorescence data for certain wells, select the rows in the Results table.

Omit outliers or change calls

After you review the software calls, you can omit outliers or change calls. Remember to click Analyze to reanalyze the data after you omit outliers or change calls.

For more detailed instructions on the following, see page 39:

- Omit outliers from analysis

- Change calls made by the software
- Revert selected manual calls to the software Auto call
- Revert all manual calls to the software Auto call

(Optional) Sequence the variants

After you identify the samples that contain methylated C residues in the amplified region, dilute or purify the PCR product from the HRM reactions, then sequence the variants.

A Ordering Information

How to order

You can order materials accessories directly from the Life Technologies store over the internet.

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

- Confirm that your computer has an Internet connection.
- We recommend the following browsers and Adobe® Acrobat® Reader® Software versions to use the Life Technologies website:

Operating system	Microsoft® Internet Explorer®	Apple® Safari®	Mozilla® Firefox®	Adobe® Acrobat® Reader®
Microsoft® Windows®	v6.x or later	Macintosh® application only	v2.x or later	v4.0 or later
Macintosh®	None†	v2.0.4 or later		

† Browser not available for this platform.

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

How to order HRM products from the Life Technologies website

1. Go to **www.lifetechnologies.com**
2. In the search field, either enter the part number of the product you are interested in or enter search terms (HRM, QuantStudio, and so forth). Alternatively, go to **Products ▸ Real-Time PCR** and browse by category.

   Note: For a list of part numbers see, "Materials and equipment for HRM experiments" on page 86.
3. Select the desired components and complete the order as instructed.

How to order custom primers

1. Go to **www.lifetechnologies.com**, then log into the Life Technologies Store if you have an account; register if you are a new user.
2. Go to **Products and Services ▸ Oligos, Primers, Probes, Nucleotides ▸ Applied Biosystems® Custom TaqMan® Probes and Primers ▸ Sequence Detection Primers**.

3. In the Ordering Information tab, select the check box next to the quantity of primers to order, then click **Configure**.
4. Follow the instructions on the web page to configure the primers:
   a. Select purification and formulation options.
   b. Enter or upload the primer names and sequences.
   c. Review the oligos to order.

   Note: If any of the oligos are invalid, follow the instructions on the web page to edit the sequence information.
5. Click **Add to Basket**.
6. Follow the link to your Shopping Basket, then follow the instructions on the web page to place your order.

Reagents and consumables for HRM calibration

Item	Life Technologies Part Number
MeltDoctor™ HRM Calibration Plate, Fast 96-Well	4425618
MeltDoctor™ HRM Calibration Plate, 384-Well	4425559
MeltDoctor™ HRM Calibration Standard (20X), 1-mL	4425562
MeltDoctor™ HRM Master Mix, 5-mL bottle	4415440
MeltDoctor™ HRM Master Mix, 5 x 5-mL bottle	4415452
MeltDoctor™ HRM Master Mix, 10 x 5-mL bottle	4415450
MeltDoctor™ HRM Master Mix, 50-mL bottle	4409535
MeltDoctor™ HRM Reagent Kit: • AmpliTaq Gold® 360 DNA Polymerase • AmpliTaq Gold® 360 Buffer • 360 GC Enhancer • GeneAmp® dNTP Blend • MeltDoctor™ HRM Dye (20X)	4425557
AmpliTaq Gold® 360 Master Mix, 1-mL	4398876
AmpliTaq Gold® 360 Master Mix, 5-mL	4398881
AmpliTaq Gold® 360 Master Mix, 10 x 5-mL	4398901
AmpliTaq Gold® 360 Master Mix, 50-mL	4398886

Materials and equipment for HRM experiments

MeltDoctor™ HRM reagents

Item	Life Technologies Part Number
MeltDoctor™ HRM Master Mix, 5-mL bottle	4415440

Item	Life Technologies Part Number
MeltDoctor™ HRM Master Mix, 5 x 5-mL bottle	4415452
MeltDoctor™ HRM Master Mix, 10 x 5-mL bottle	4415450
MeltDoctor™ HRM Master Mix, 50-mL bottle	4409535
MeltDoctor™ HRM Positive Control Kit: • MeltDoctor™ HRM Allele A DNA (20X), 150-µL • MeltDoctor™ HRM Allele G DNA (20X), 150-µL • MeltDoctor™ HRM Allele A/G DNA (20X), 150-µL • MeltDoctor™ HRM Primer Mix (20X), 500-µL	4410126
MeltDoctor™ HRM Reagent Kit: • AmpliTaq Gold® 360 DNA Polymerase • AmpliTaq Gold® 360 Buffer • 360 GC Enhancer • GeneAmp® dNTP Blend • MeltDoctor™ HRM Dye (20X)	4425557
AmpliTaq Gold® 360 Master Mix, 1-mL	4398876
AmpliTaq Gold® 360 Master Mix, 5-mL	4398881
AmpliTaq Gold® 360 Master Mix, 10 x 5-mL	4398901
AmpliTaq Gold® 360 Master Mix, 50-mL	4398886

Note: For information on reagents for the Methylation and Mutation Scanning experiments, refer to the respective Application Notes (see "Documentation and Support" on page 99).

Software

Item	Source	Quantity	Part Number
QuantStudio™ 6 and 7 Flex Real-Time PCR System Software (HRM license)	Shipped or installed with the QuantStudio™ 6 and 7 Instruments	5 licenses	Contact Life Technologies
		1 license	
Primer Express® Software v3.0 or later	Life Technologies	—	
Methyl Primer Express® Software v1.0	Life Technologies	—	

General-use materials and consumables

Item	Source
Centrifuge with plate adapters	Major laboratory suppliers (MLS)
Lab equipment	
Microcentrifuge	
Microcentrifuge tubes	
Pipettors and pipette tips	
Vortexer	

General Purpose Laboratory Equipment (GPLE)

Item		Life Technologies Part Number
MicroAmp® Optical 96-Well Reaction Plate (0.2-mL)	10 plates	N8010560
MicroAmp® Optical 96-Well Reaction Plate with Barcode (0.2-mL)	10 plates	4306737
MicroAmp® Fast Optical 96-Well Reaction Plate (0.1-mL)	10 plates	4346907
MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1-mL)	10 plates	4346906
MicroAmp® Optical Adhesive Film	25 films	4481193
MicroAmp® Adhesive Film Applicator	5 applicators	4481196

B Supplemental Information and Procedures

This appendix contains supplemental information and procedures for preparing and running HRM reactions and for using the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software.

HRM dyes and MeltDoctor™ HRM dye

The melt profile of a PCR product is best obtained with high-resolution melt dyes (HRM dyes). HRM dyes are double-stranded DNA(dsDNA)-binding dyes that have high fluorescence when bound to dsDNA and low fluorescence in the unbound state. HRM analysis uses dsDNA-binding dyes that are brighter than those previously used, and they do not inhibit PCR at high-dye concentrations. With traditional dyes (for example, SYBR® Green I dye), only limited concentrations of the dye can be used before the dye inhibits the PCR.

Of all the dyes that are pre-installed in the Dye Library, only the MeltDoctor™ HRM Dye is valid for HRM.

Custom HRM dyes

This getting started guide describes procedures for calibrating your instrument and performing HRM experiments using the MeltDoctor™ HRM Dye.

If you choose to use a different HRM dye, calibrate your instrument for that dye. Follow the procedures provided, but substitute for the MeltDoctor™ HRM Dye with your HRM dye and prepare your own calibration plate. See “Prepare a custom HRM calibration plate” on page 91.

You should also optimize your reactions for the HRM dye that you choose, because each dye interacts uniquely with all other reaction components.

Prepare a 96-well 0.2-mL HRM calibration plate

This procedure is for preparing a 96-well 0.2-mL HRM calibration plate using the MeltDoctor™ HRM Master Mix and MeltDoctor™ HRM Calibration Standard.

IMPORTANT! The HRM calibration plate should be prepared fresh and used immediately. It is important to perform the custom dye calibration and HRM calibration on the same day that the HRM calibration plate is prepared.

Note: If you are using the MeltDoctor™ HRM Reagent Kit instead of the MeltDoctor™ HRM Master Mix, use the same component volumes in the HRM calibration plate that you are using in your HRM reactions.

Required materials

- MeltDoctor™ HRM Master Mix
- MeltDoctor™ HRM Calibration Standard
- MicroAmp® Optical Adhesive Film
- Deionized water
- 96-well 0.2-mL reaction plate

Prepare the 96-well 0.2-mL HRM calibration plate

1. Add the required volumes of each component to an appropriately sized tube:

Component	Volume (µL)	
	1 reaction	110 reactions (includes 10% excess)
MeltDoctor™ HRM Master Mix	10µL	1100µL
MeltDoctor™ HRM Calibration Standard (20X)	1µL	110µL
Deionized water	9µL	990µL
Total volume	**20µL**	**2200µL**

2. Cap the tube, then vortex to mix.
3. Spin the tube briefly.
4. Pipet the HRM calibration reactions to each well of an appropriate reaction plate for your instrument.

   IMPORTANT! Accurate pipetting is required for proper calibration.

5. Inspect the plate to make sure all wells contain liquid.

   IMPORTANT! Empty wells may cause the calibration to fail.

6. Seal the reaction plate with optical adhesive film, then spin the reaction plate.

7. Verify that the liquid in each of the wells of the HRM calibration plate is at the bottom of the well. If not, centrifuge the plate again at a higher rpm and for a longer period of time.

Prepare a custom HRM calibration plate

This procedure is for preparation of a custom HRM calibration plate.

IMPORTANT! A custom dye must fluoresce within the 520 to 650 nm spectral range measured by the QuantStudio™ 6 and 7 Instruments.

You will need to perform a custom dye calibration before you can use the custom dye in the HRM calibration plate.

For information on calibrating a custom dye, refer to Booklet 1, *Getting Started with QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Experiments* in the *QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide* (Pub. no. 4489822).

For component volumes, refer to the dye manufacturer's instructions.

IMPORTANT! The HRM calibration plate should be prepared fresh and used immediately. It is important to perform the custom dye calibration and HRM calibration on the same day that the HRM calibration plate is prepared.

Required materials

- AmpliTaq Gold® 360 Master Mix, 1-mL (Pub. no. 4398876) or your master mix of choice
- Your custom dye
- MeltDoctor™ HRM Calibration Standard
- MicroAmp® Optical Adhesive Film
- Deionized water
- Appropriate reaction plate

Prepare the custom HRM calibration plate

1. Add the required volumes of each component to an appropriately sized tube.

Components	Volume (µL)		
	1 reaction	110 reactions (96-well) (includes 10% excess)	425 reactions (384-well) (includes 10% excess)
AmpliTaq Gold® 360 Master Mix	10µL	1100µL	4250µL
Custom dye (20x) (typical dye concentration 0.1 µM	1µL	110µL	425µL
MeltDoctor™ HRM Calibration Standard (20X)	1µL	110µL	425µL
Deionized water	8µL	880µL	3400µL
Total volume	**20µL**	**2200µL**	**8500µL**

2. Cap the tube, then vortex to mix.
3. Spin the tube briefly.
4. Pipet the HRM calibration reactions to each well of an appropriate reaction plate for your instrument.

   IMPORTANT! Accurate pipetting is required for proper calibration.

5. Inspect the plate to make sure all wells contain liquid.

   IMPORTANT! Empty wells may cause the calibration to fail.

6. Seal the reaction plate with optical adhesive film, then spin the reaction plate.
7. Verify that the liquid in each of the wells of the HRM calibration plate is at the bottom of the well. If not, centrifuge the plate again at a higher rpm and for a longer period of time.

Prepare the DNA templates

1. Purify all the DNA samples in an HRM experiment using the same method. Watch out for salt carryover because it will subtly change the thermodynamics of the DNA melting transition.
2. Perform agarose gel electrophoresis and spectrophotometry to make sure the DNA template is intact and is not contaminated with other DNAs, RNAs, proteins, or organic chemicals. Proteins and organic chemicals may inhibit the PCR amplification, and contaminating DNAs and RNAs may result in sub-optimal PCR performance or increased change of non-specific amplification.
3. Determine the quantity of DNA using spectrophotometry. If too little DNA template is added to the reaction, the fluorescence signal may not be sufficient for successful HRM analysis. If too much DNA template is added to the reaction, the PCR may be inhibited.

4. (*Optional*) Dilute the DNA to 20 ng/µL.

Optimizing the reaction conditions

If you want to optimize the reaction conditions, use the MeltDoctor™ HRM Reagent Kit.

For more information on optimizing your HRM reactions, refer to *A Guide to High Resolution Melting (HRM) Analysis* (Stock number O-081740 0509).

Recommended reaction component volumes using the MeltDoctor™ HRM Reagent Kit

Components	Volume for one 20-µL reaction	Final concentration	Acceptable concentration range
AmpliTaq Gold® 360 Buffer, 10×	2 µL	1×	1×
25 mM Magnesium Chloride	1.6 µL	2 mM	1.5 to 3.5 mM
GeneAmp® dNTP Blend, 10 mM	0.4 µL	200 µM each	100 to 300 µM each
Primer 1 (5 µM)	1.2 µL	0.3 µM	0.2 to 0.5 µM
Primer 2 (5 µM)	1.2 µL	0.3 µM	0.2 to 0.5 µM
MeltDoctor™ HRM Dye (20X)	1.0 µL	1X	0.5X to 2X
AmpliTaq Gold® 360 DNA Polymerase (5 U/µL)	0.4 µL	0.1 U/µL	0.05 to 0.15 U/µL
Human gDNA (20 ng/µL)	1 µL	1 ng/µL	10 pg/µL to 10 ng/µL
Deionized water	11.2 µL		
Total volume	**20 µL**		

C Troubleshooting HRM Experiments

Problems with HRM experiments are usually evidenced by abnormal amplification plots or by abnormal HRM curves.

For more guidance on troubleshooting, refer to:

- Life Technologies Real-Time PCR Troubleshooting Tool:
  www.lifetechnologies.com/troubleshooting
- Life Technologies *Guide to High Resolution Melting (HRM) Analysis* (Stock number O-081740-0509)
- The instrument user guide for more information on troubleshooting the QuantStudio™ 6 and 7 Instruments.

Late amplification: C_T value >30 for a majority of samples

The amplification reaction may not reach the plateau phase. HRM resolution may be affected by the lower increase in fluorescence.

Possible causes	Recommended action
Poor DNA quality.	Re-extract the DNA.
Amount of DNA added to the HRM reactions is too low.	Perform PCR optimization, and increase sample input or increase the number of amplification cycles.

Some late amplification: C_T value >30 for some samples

Sample outliers with C_T values that are greater than those for the replicates also have a Tm shift in the HRM curve. The resulting Tm shift may affect the variant call.

Possible causes	Recommended action
Reaction volume for the outlier is visibly greater than or less than the reaction volume for the replicates.	Repeat the HRM reactions, and make sure that you add the correct volumes to each well. Also, after you seal the plate, spin the plate briefly.
Amount of DNA added to the HRM reactions is too low.	Repeat the HRM reactions with more DNA in each reaction.
PCR inhibition.	If the amplification curve also has a low slope and all replicates for a sample are affected, see page 96 to troubleshoot PCR inhibition in your HRM reactions.

PCR inhibition: Amplification curve with low slope and CT values higher than expected

The amplification curve has a low slope and the amplification reaction may not reach the plateau phase. HRM resolution may be affected by the lower increase in fluorescence.

Possible causes	Recommended action
DNA sample contains contaminants that inhibit PCR.	Dilute the samples 1:10 or 1:100, then repeat the HRM reactions.
Incorrect salt concentration.	Perform a $MgCl_2$ titration to find the optimal salt concentration for each reaction.
Reaction does not contain sufficient enzyme.	Optimize the reaction using the MeltDoctor™ HRM Reagent Kit. You can add up to 0.15 U/µL AmpliTaq Gold® 360 DNA Polymerase to each reaction.
Reaction does not contain sufficient primer.	Optimize the reaction using the MeltDoctor™ HRM Reagent Kit. You can add up to 0.5 µM of each primer to each reaction.
Amplicon is greater than 200 bp.	Increase the extension times during the amplification reaction.
Primers are amplifying multiple targets.	Perform a BLAST search to ensure primer specificity. If the primers are not specific, design new primers.
	Reduce the number of amplification cycles.

Nonspecific amplification: Decreased PCR efficiency and multiple amplicons

Decreased PCR efficiency and multiple amplicons may affect the melting behavior of the true target amplicons.

Possible causes	Recommended action
Incorrect salt concentration.	Perform a $MgCl_2$ titration to find the optimal salt concentration for each reaction.
Primers are amplifying multiple targets.	Perform a BLAST search to ensure primer specificity. If the primers are not specific, design new primers.
	Reduce the number of amplification cycles.
	After PCR amplification, consider running some of the PCR product on a gel to make sure that it contains a single band.

Replicates are widely spread: Sample replicates show a wide spread in HRM curves

A wide spread within a population leads to difficulties in assessing true sequence differences, particularly between two different homozygous populations.

Possible causes	Recommended action
Population spread	Use multiple controls for HRM analysis to help you define the population spread.
Incorrect salt concentration.	Perform a $MgCl_2$ titration to find the optimal salt concentration for each reaction.
DNA starting concentrations vary widely between samples.	Make sure that the starting DNA concentrations are similar for the samples that you are testing.
Low PCR efficiencies.	Ensure efficient PCR.

Multiple melt regions: Complex melt curves with multiple melting regions

Complex melt curves are difficult to interpret. If the amplicon is too long, the melt curve may have multiple melt regions because of the regional sequence context of the amplicon.

Possible causes	Recommended action
The amplicon contains more than one SNP (genotyping experiments only).	Sequence the PCR product to confirm whether the amplicon contains more than 1 SNP. If the sequencing reveals more SNPs, redesign the primers so that the amplicon contains only 1 SNP.
The amplicon is too long.	Redesign the primers to reduce the amplicon size.

More than three different variant calls (HRM genotyping experiments only)

If the target contains unknown SNPs, multiple heterozygous and homozygous amplicons can be produced. If the amplicon is too long, the melt curve may have multiple melt regions even without a SNP because of the regional sequence context of the amplicon.

Possible causes	Recommended action
The amplicon contains more than 1 SNP.	Sequence the PCR product to confirm whether the amplicon contains more than 1 SNP. If the sequencing reveals more SNPs, redesign the primers so that the amplicon contains only 1 SNP.
The amplicon is too long.	Redesign the primers to reduce the amplicon size.

Messy HRM curves: Diagonal wavy curves below heterozygous clusters

HRM data from negative controls and unamplified samples skew the Pre- and Post-melt curve settings and interfere with the variant calls.

Possible cause	Recommended action
Negative controls and unamplified samples are included in the HRM analysis.	Omit negative controls and unamplified samples from the HRM analysis. Refer to the Life Technologies *High Resolution Melting Software Help*.

Documentation and Support

Related documentation

The following related documents are shipped with the instrument:

Document	Pub. no.	Description
QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide	4489822	Provides some information on how to use the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software. Contains seven individual booklets that explain how to perform the six different experiments using QuantStudio™ 6 and 7 Flex Real-Time PCR System Software on the QuantStudio™ 6 and 7 Instruments. 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 QuantStudio™ 6 and 7 Flex Real-Time PCR System Software. • A guide for your own experiments. Intended for laboratory staff and principal investigators who perform experiments using the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software.
QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Programming Supplement	4489825	Provides IT administrative personnel with sufficient information to integrate the instrument and software with a LIS/LIMS. Intended to be used with the *QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide*.
Applied Biosystems® Twister® Robot Automation Accessory Quick Reference	4468146	Provides abbreviated instructions for operating an Applied Biosystems® Twister® Robot Automation Accessory that has been installed with the QuantStudio™ 6 or 7 Flex Real-Time PCR System. Intended for laboratory staff and principal investigators who perform experiments using the QuantStudio™ 6 or 7 Flex Real-Time PCR System.

Document	Part Number	Description
QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Help	NA	Explains how to use the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software to: • Set up, run, analyze, audit, sign, export, and print experiments. • Monitor a networked QuantStudio™ 6 or 7 Instrument. • Intended for: – Laboratory staff and principal investigators who perform experiments using the QuantStudio™ 6 and 7 Flex Real-Time PCR System Software.

Note: For additional documentation, see "How to obtain support" on page 100.

How to obtain support

For the latest services and support information for all locations, go to:

www.lifetechnologies.com/support

At the Life Technologies web site, you can:

- Access worldwide telephone and fax numbers to contact Life Technologies Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Life Technologies 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 QuantStudio™ 6 and 7 Flex Real-Time PCR System 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 QuantStudio™ 6 and 7 Flex Real-Time PCR System Software window.
- Select **Help ▸ QuantStudio™ 6 and 7 Flex Real-Time PCR System 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

Limited Product Warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
For support visit lifetechnologies.com/support or email techsupport@lifetech.com

lifetechnologies.com

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