USER GUIDE

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by life technologies™

Applied Biosystems High Resolution Melt Software for QuantStudio™ 12K Flex Real-Time PCR System

GETTING STARTED GUIDE

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life technologies™

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6/12/12

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About This Guide

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Purpose

This guide is designed to help you quickly learn to perform High Resolution Melt experiments with QuantStudio™ 12K Flex Software.

This guide provides step-by-step procedures for:

- Calibrating an Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System 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 QuantStudio™ 12K Flex Software

Prerequisites

This guide assumes that you have working knowledge of the:

- Microsoft® Windows® XP or Windows® 7 operating system
- Software for your QuantStudio™ 12K Flex System
- General techniques for handling DNA samples and preparing them for PCR

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 example experiment data file provided in the ViiA™ 7 Software
- Tips for running your own experiments

Note: First-time users of the QuantStudio™ 12K Flex System, please read *Getting Started with the* QuantStudio™ 12K Flex System *Multi-Well Plates and Array Card Experiments* it is the first booklet of the Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System *Multi-Well Plates and Array Card Experiments User Guide* (Part no. 4470050).

User attention words

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

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.

1 Experiment Overview

About HRM experiments

High Resolution Melt (HRM) analysis is a new, 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™ 12K Flex System consists of the QuantStudio™ 12K Flex Instrument, QuantStudio™ 12K Flex Software, computer, and associated devices.

The QuantStudio™ 12K Flex System can perform:

- **Mutation scanning experiments** – Screen DNA samples for new single-base changes, insertions/deletions, or other unknown mutations.
- **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™ 12K Flex Software compares the melt curves of the unknown samples to the melt curves of the positive controls and assigns each unknown a variant call. If the unknown matches a positive control the variant call is the name of the control. If the unknown does not match to any positive control, the variant call is “variant X”, where X is a number.

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. The variant call is either "wild type" or "variant X".
- **Methylation studies** – Methylated DNA standards that contain from 0% to 100% methylated DNA. The variant calls are the % of methylation.
- **Genotyping experiments** – Three samples: one homozygous AA (Allele A/A), one homozygous GG (Allele G/G), and one heterozygous for both alleles AG (Allele A/G). The variant calls are the genotypes. (There is a fourth allele, AAG, in the example experiment, but it will be omitted from analysis).

  Note: The example experiment shows a basic genotyping experiment. For information on mutation and methylation experiments, see the *Applied Biosystems High Resolution Melting Getting Started Guide* (Part no. 4393102), *Perform an HRM Methylation Study Quick Reference Card* (Part no.4457856), and *Perform an HRM Mutation Scanning Experiment Quick Reference Card* (Part no. 4457855).

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 Experiment" on page 17.

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About the HRM example experiment

To illustrate how to perform HRM experiments, the software installs an example genotyping 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™ 12K Flex System.

To view the example experiment in the QuantStudio™ 12K Flex Software:

Select **Open** and then browse to:

C:\Program Files\Applied Biosystems\QuantStudio 12K Flex Software\examples\Melt Curve\96-Well Fast (Standard) HRM 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 postitive control samples:
  - AA (Allele A/A)
  - GG (Allele G/ G)
  - AG (Allele A/G)
- The 3 positive samples each use 3 wells of the 96-well plate.
- There are 3 wells for NTC samples which serve as negative controls.

Perform the example experiment with the MeltDoctor™ HRM Positive Control Kit.

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.

2 Calibrate the QuantStudio™ 12K Flex System

About HRM calibration

This chapter describes how to calibrate an Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System for either the MeltDoctor™ HRM Dye or for an alternative HRM dye.

During HRM calibration, the Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System 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 perform a separate dye calibration. 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

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 62.

IMPORTANT! Before you can perform an HRM calibration on your QuantStudio™ 12K Flex System, the region of interest, background, and uniformity calibrations must be current. For more information, see *Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Maintenance and Administration* (Part no. 4470689).

Required materials for HRM calibration

- Centrifuge
- Powder-free gloves
- Safety goggles
- Life Technologies MeltDoctor™ HRM Calibration Plate, 384-Well (Part no. 4425559)

  or

  Life Technologies MeltDoctor™ HRM Calibration Plate, 96-Well Fast (Part no. 4425618)

Prepare the MeltDoctor™ HRM calibration 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.

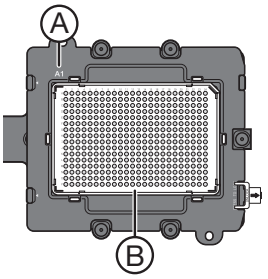
1. Remove the MeltDoctor™ HRM Calibration Plate from the freezer, then allow it to thaw. Use it immediately after it thaws. Do not leave the plate at room temperature.
2. Spin the plate briefly to collect liquid at the bottom of the wells.
3. Verify that the liquid in each of the wells of the MeltDoctor™ 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

1. In the QuantStudio™ 12K Flex Instrument Console, select your instrument from the list of instruments on the network, then click **Add to My Instruments**.

   Note: You must add an instrument to your list before you can manage it.
2. Double-click the instrument icon to open the **Instrument Manager**.
3. 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
4. Click **Start Calibration**.

5. Follow the instructions of the QuantStudio™ 12K Flex 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 bar code 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.

6. Start the calibration:
   a. In the Setup tab, select **Check the box when the HRM calibration plate has been loaded**, 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™ 12K Flex Instrument is in operation.

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

7. 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™ 12K Flex Instrument does not eject the plate, remove the plate as explained in "QuantStudio™ 12K Flex Instrument: Instrument does not eject the plate" on page 70.

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.

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

3 Design an HRM 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 60-250 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 57. For a list of HRM reagents, see "Ordering Information" on page 57.

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 for each expected variant 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.
- At least one wild type control (for mutation scanning experiments).

  Run 3-5 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.

Define the experiment properties

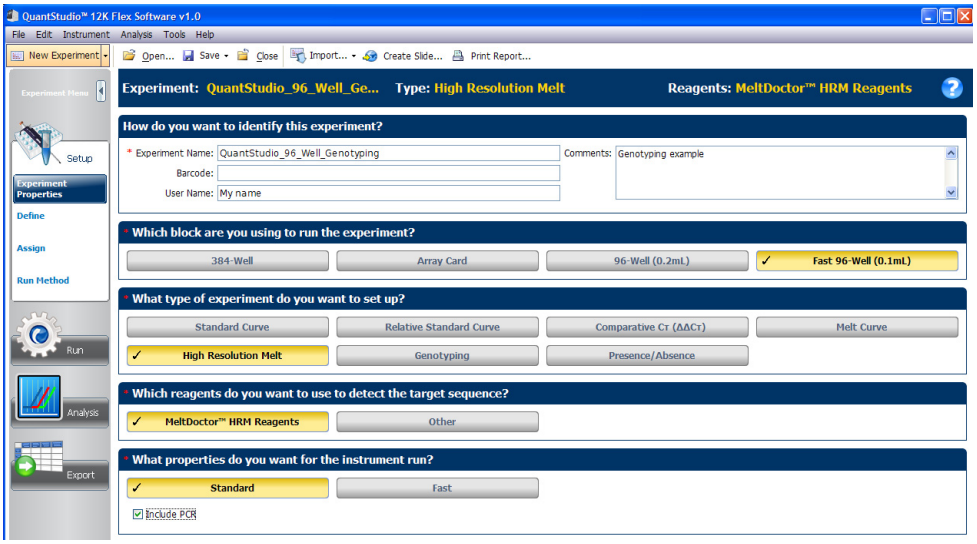
The following table lists the experiment properties used in the example experiment. For your own experiment, enter properties as appropriate.

To view the example experiment, select **Open** and then browse to:

C:\Program Files\Applied Biosystems\QuantStudio 12K Flex Software\examples\Melt Curve\96-Well Fast (Standard) HRM Example.eds

To create a new experiment, access the QuantStudio™ 12K Flex Software: **New Experiment ▸ Experiment Setup**. Enter:

Field	Entry
Experiment Name	QuantStudio_96_Well_Genotyping (Using a different name than the example experiment to avoid saving over it)
Barcode	Leave field empty
User Name	Example User
Comments	Genotyping example
Block	96-Well Block
Experiment Type	High Resolution Melt
Reagents	MeltDoctor™ HRM Reagents
Instrument Run properties (Ramp speed)	Standard
Include PCR checkbox	Check to perform PCR on the QuantStudio™ 12K Flex Instrument



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
Gene A	MELTDOCTOR	None	

2. Samples

Sample Name	Color
AA (Allele A/A)	
GG (Allele G/G)	
AG (Allele A/G)	
AGG (Allele A/GG) (Omitted)	

3. Define Controls

Control Name	Color
Control 1	

Tips for defining controls in your own HRM experiment

If you are creating your own HRM experiment, include controls for each target sequence in your HRM experiment:

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

Run 3-5 replicates for each expected variant 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.

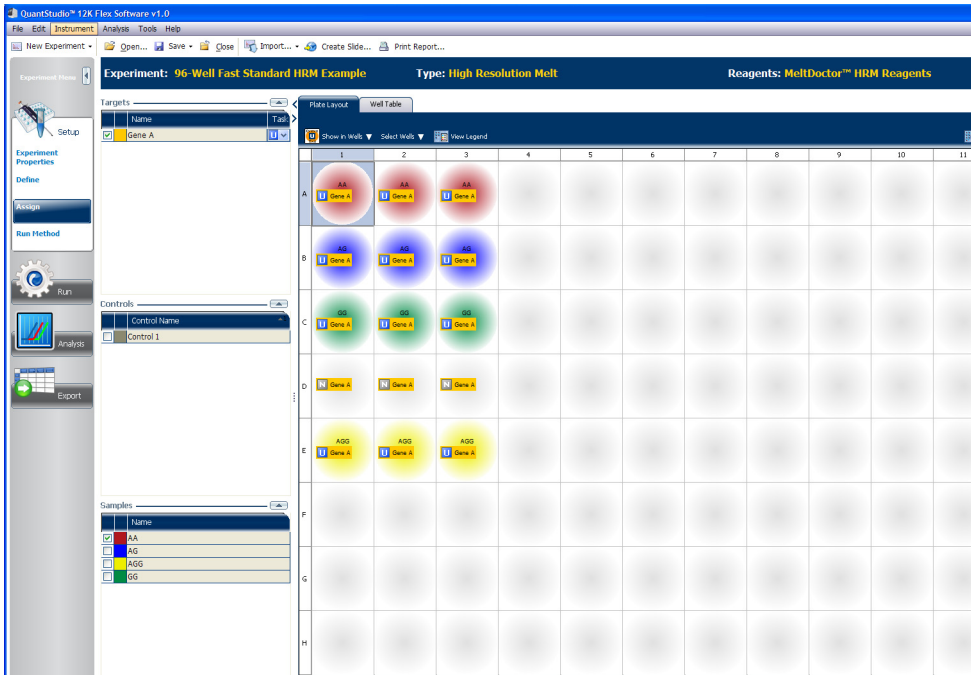
If you wish to use a passive reference dye, select it from the drop-down menu at the bottom of the Define screen.

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 row A, columns 1-3 in the Plate Layout and click in the checkbox for Sample AA.
2. Select row B for Sample AG and row C for Sample GG.



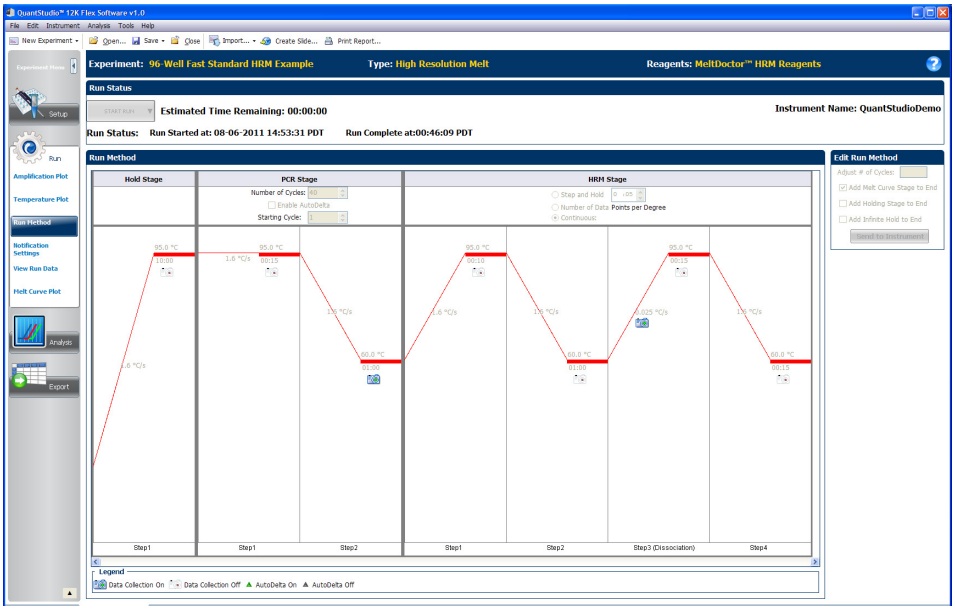
Assign controls

1. Select row D, columns 1-3 in the Plate Layout and assign Control 1.

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™ 12K Flex Software Help** (click ? or press **F1**).

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

4 Export Analysis Results

How to export analysis results

This procedure shows how to export the HRM example experiment to a .txt file.

1. Select **Open** and then browse to:

   C:\Program Files\Applied Biosystems\QuantStudio 12K Flex Software\examples\Melt Curve\96-Well Fast (Standard) HRM 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 checkbox during experiment setup or before running the experiment. Auto Export is unchecked for the example experiment.

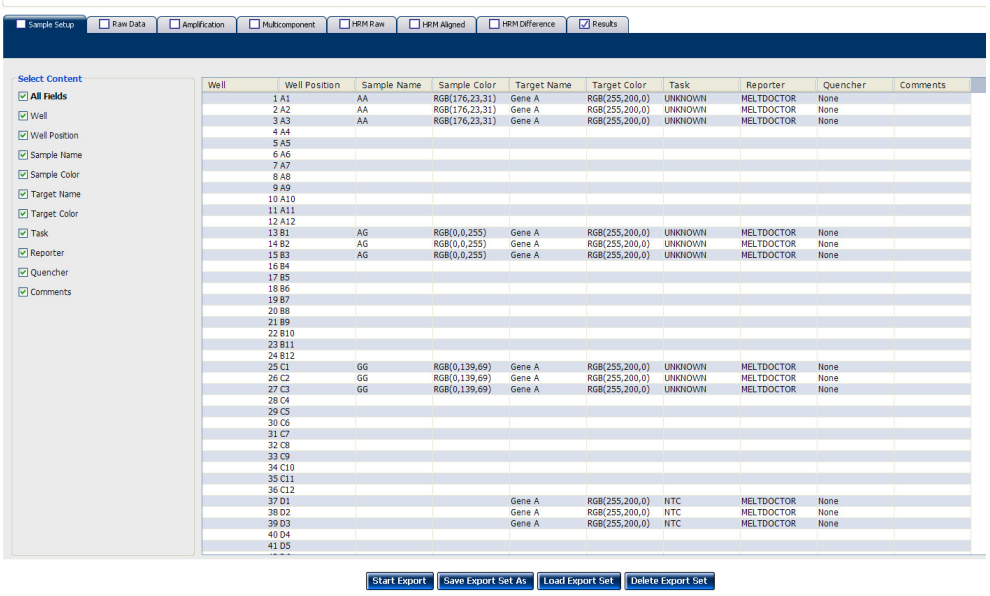
3. For Format, from the drop-down menu, select 'QuantStudio12KFlex'.

4. Complete the Export dialog box as shown below:

Field or Selection	Entry
Select Data to export/ Select Content	All Fields
Export Data To	One File
Export File Name	QuantStudioHRMExport
File Type	*.txt
Export file Location	C:\Applied Biosystems\QuantStudio 12K Flex Software\User Files\experiments is the default location. You can save 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.

Your export screen should look like the following image:



Well	Well Position	Sample Name	Sample Color	Target Name	Target Color	Task	Reporter	Quencher	Comments
1	A1	AA	RGB(176,23,31)	Gene A	RGB(255,200,0)	UNKNOWN	MELTDOCTOR	None	
2	A2	AA	RGB(176,23,31)	Gene A	RGB(255,200,0)	UNKNOWN	MELTDOCTOR	None	
3	A3	AA	RGB(176,23,31)	Gene A	RGB(255,200,0)	UNKNOWN	MELTDOCTOR	None	
4	A4								
5	A5								
6	A6								
7	A7								
8	A8								
9	A9								
10	A10								
11	A11								
12	A12								
13	B1	AG	RGB(0,0,255)	Gene A	RGB(255,200,0)	UNKNOWN	MELTDOCTOR	None	
14	B2	AG	RGB(0,0,255)	Gene A	RGB(255,200,0)	UNKNOWN	MELTDOCTOR	None	
15	B3	AG	RGB(0,0,255)	Gene A	RGB(255,200,0)	UNKNOWN	MELTDOCTOR	None	
16	B4								
17	B5								
18	B6								
19	B7								
20	B8								
21	B9								
22	B10								
23	B11								
24	B12								
25	C1	GG	RGB(0,139,69)	Gene A	RGB(255,200,0)	UNKNOWN	MELTDOCTOR	None	
26	C2	GG	RGB(0,139,69)	Gene A	RGB(255,200,0)	UNKNOWN	MELTDOCTOR	None	
27	C3	GG	RGB(0,139,69)	Gene A	RGB(255,200,0)	UNKNOWN	MELTDOCTOR	None	
28	C4								
29	C5								
30	C6								
31	C7								
32	C8								
33	C9								
34	C10								
35	C11								
36	C12								
37	D1			Gene A	RGB(255,200,0)	NTC	MELTDOCTOR	None	
38	D2			Gene A	RGB(255,200,0)	NTC	MELTDOCTOR	None	
39	D3			Gene A	RGB(255,200,0)	NTC	MELTDOCTOR	None	
40	D4								
41	D5								

Your exported file should look like the following image when opened in Notepad:

```
* Barcode = NA
* Block Type = Fast 96-Well Block (0.1mL)
* Calibration  is expired  = No
* Calibration  performed on  = 08-06-2011 14:25:07 PM PDT
* Calibration Background is expired  = Yes
* Calibration Background performed on  = 08-04-2011 16:39:12 PM PDT
* Calibration FAM is expired  = Yes
* Calibration FAM performed on  = 08-04-2011 17:01:42 PM PDT
* Calibration MELTDOCTOR is expired  = Yes
* Calibration MELTDOCTOR performed on  = 08-06-2011 14:25:05 PM PDT
* Calibration ROI is expired  = Yes
* Calibration ROI performed on  = 08-04-2011 16:28:09 PM PDT
* Calibration ROX is expired  = Yes
* Calibration ROX performed on  = 08-05-2011 10:12:02 AM PDT
* Calibration SYBR is expired  = Yes
* Calibration SYBR performed on  = 08-06-2011 12:14:28 PM PDT
* Calibration Uniformity is expired  = Yes
* Calibration Uniformity performed on  = 08-04-2011 16:51:32 PM PDT
* Calibration VIC is expired  = Yes
* Calibration VIC performed on  = 08-04-2011 17:11:19 PM PDT
* Chemistry = MELT_DOCTOR
* Comment = NA
* Date Created = 03-20-2012 13:58:27 PM PDT
* Experiment File Name = C:\Program Files\Applied Biosystems\QuantStudio 12K Flex Software\examples\Melt Curve\96-Wel
* Experiment Name = 96-Well Fast Standard HRM Example
* Experiment Run End Time = 08-07-2011 00:46:09 AM PDT
* Experiment Type = High Resolution Melt
* Instrument Name = QuantStudioDemo
* Instrument Serial Number = QuantStudioDemo
* Instrument Type = QuantStudio 12K Flex
* Number of Cluster for target Gene A = 4
* Passive Reference =
* Quantification Cycle Method = Ct
* Signal Smoothing On = true
* Stage/ Cycle where Analysis is performed = Stage 2, Step 2
* User Name = NA

[Results]
Well    Well Position   Sample Name Target Name Task    Reporter    Quencher    Method  Variant Calls   Confidence Va
1   A1  AA  Gene A  UNKNOWN MELTDOCTOR  None    Auto    variant1        25.747  25.644  0.097   79.471          1.000
2   A2  AA  Gene A  UNKNOWN MELTDOCTOR  None    Auto    variant2        25.555  25.644  0.097   79.489          1.000
3   A3  AA  Gene A  UNKNOWN MELTDOCTOR  None    Auto    variant2        25.631  25.644  0.097   79.471          1.000
13  B1  AG  Gene A  UNKNOWN MELTDOCTOR  None    Auto    variant3        24.835  24.855  0.021   78.650  79.908      2
14  B2  AG  Gene A  UNKNOWN MELTDOCTOR  None    Auto    variant3        24.854  24.855  0.021   78.632  79.927      2
15  B3  AG  Gene A  UNKNOWN MELTDOCTOR  None    Auto    variant3        24.877  24.855  0.021   78.650  79.927      2
25  C1  GG  Gene A  UNKNOWN MELTDOCTOR  None    Auto    variant4        23.821  23.815  0.033   80.000          1.000
26  C2  GG  Gene A  UNKNOWN MELTDOCTOR  None    Auto    variant4        23.844  23.815  0.033   79.981          1.000
```

5 Prepare HRM 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 96/384-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 (Part no. 4410126), components from the kit:
  - MeltDoctor™ HRM Primer Mix (20×)
  - MeltDoctor™ HRM Allele A DNA (20×)
  - MeltDoctor™ HRM Allele G DNA (20×)
  - MeltDoctor™ HRM Allele A/G DNA (20×)
- MeltDoctor™ HRM Master Mix (Part no. 4415440)

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 63.

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" on page 27.

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

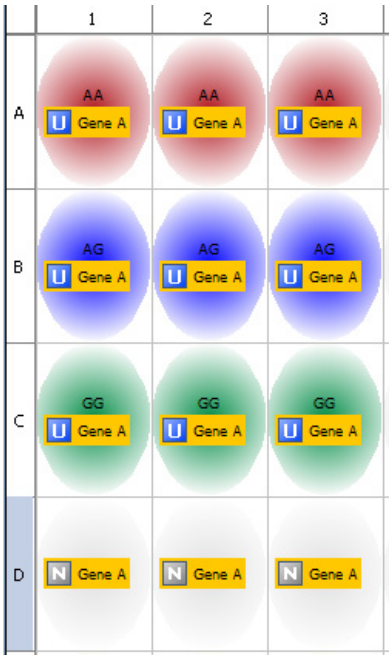
1. Prepare the reactions using the Positive Control Kit in separate appropriately sized, labeled tubes:

Components	96-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 (20×) • MeltDoctor™ HRM Allele G DNA (20×) • MeltDoctor™ HRM Allele A/G DNA (20×)	1 µL	3.3 µL
MeltDoctor™ HRM Primer Mix (20×)	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 as shown in the following image.



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 *Getting Started with the* QuantStudio™ 12K Flex System *Multi-Well Plates and Array Card Experiments* it is the first booklet of the Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System *Multi-Well Plates and Array Card Experiments User Guide* (Part no. 4470050).

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™ 12K 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 (20×)	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 read *Getting Started with the* QuantStudio™ 12K Flex System *Multi-Well Plates and Array Card Experiments* it is the first booklet of the Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System *Multi-Well Plates and Array Card Experiments User Guide* (Part no. 4470050)).

Note: For information about using the MeltDoctor™ HRM Reagent Kit to optimize your reactions, see "Optimizing the reaction conditions" on page 64.

6 Run an HRM Experiment

Prepare for the run

Open the HRM experiment

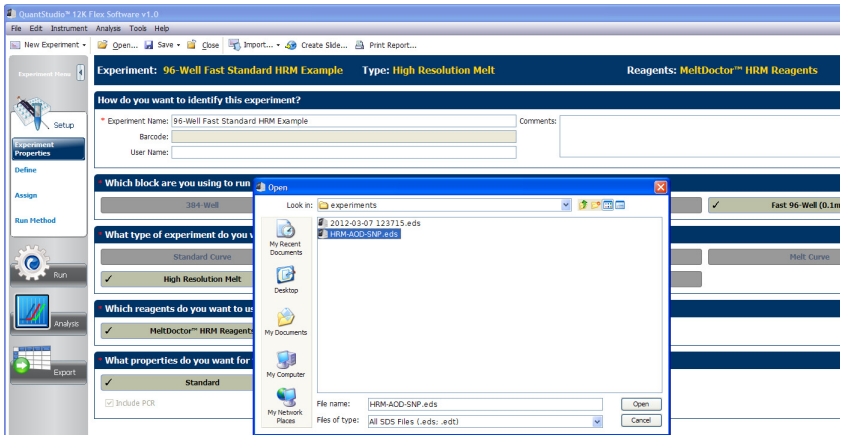
From the Home screen, open one of the following:

- The example experiment:

  Select **Open** and then browse to:

  C:\Program Files\Applied Biosystems\QuantStudio 12K Flex Software\examples\Melt Curve\96-Well Fast (Standard) HRM Example.eds
- Your experiment:

  Open ▸ 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.

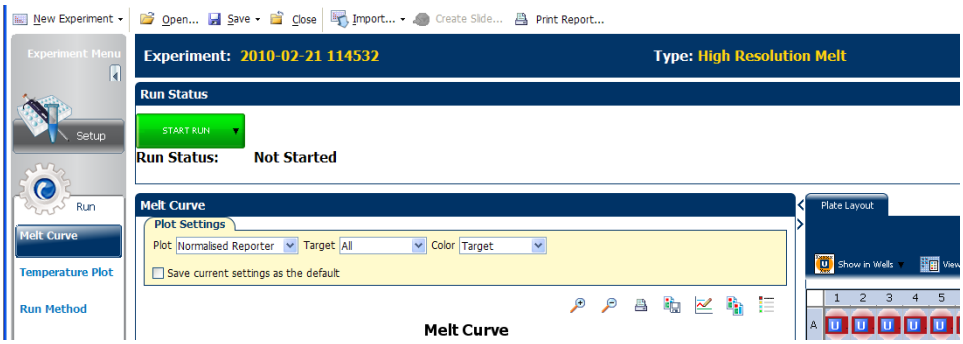
1. In the Home screen of the QuantStudio™ 12K Flex Software, click **Instrument Console**.
2. Select your instrument from the list of instruments.
3. If your instrument is in the "On the Network" group, add it to **My Instrument**.
4. Click **Open Door** in the Instrument Console tool bar.
5. Load your 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 selecting **Start Run** in the Melt Curve window.



Monitor the run

You can view the progress of the run in real-time as described below. During the run, periodically view all three plots available from the QuantStudio™ 12K Flex Software for potential problems.

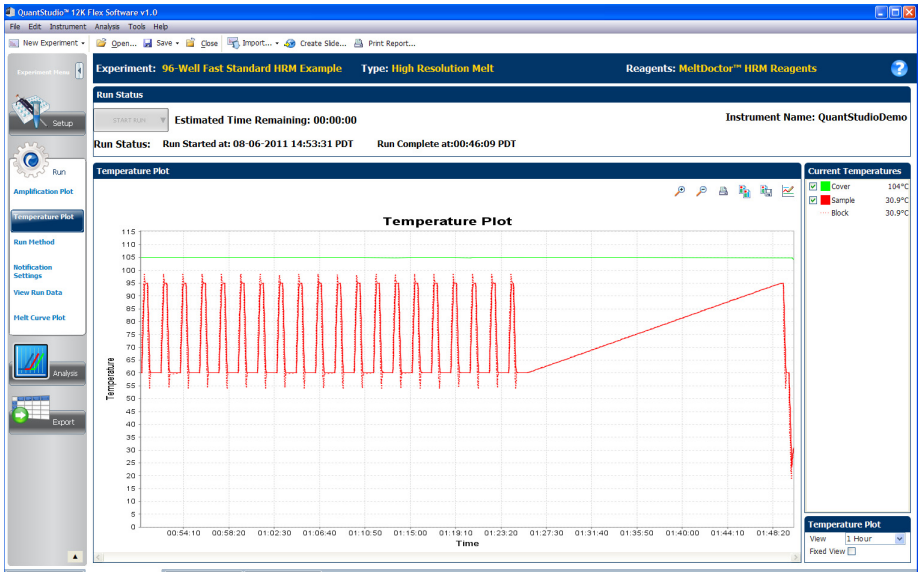
To...	Perform the following action
View temperature data for the run in real-time	Select **Temperature**
View progress of the run in the Run Method screen	Select **Run Method**
Enable/disable the Notification Settings	Select or deselect **Notification Settings**
Stop the run	1. In the QuantStudio™ 12K Flex Software, click **STOP RUN**. 2. In the Stop Run dialog, click one of the following: • **Stop Immediately** to stop the run immediately. • **Stop after Current Cycle/Hold** to stop the run after the current cycle or hold. • **Cancel** to continue the run.

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...	Perform the following 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 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	Select **Fixed View**.

The following figure shows 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.

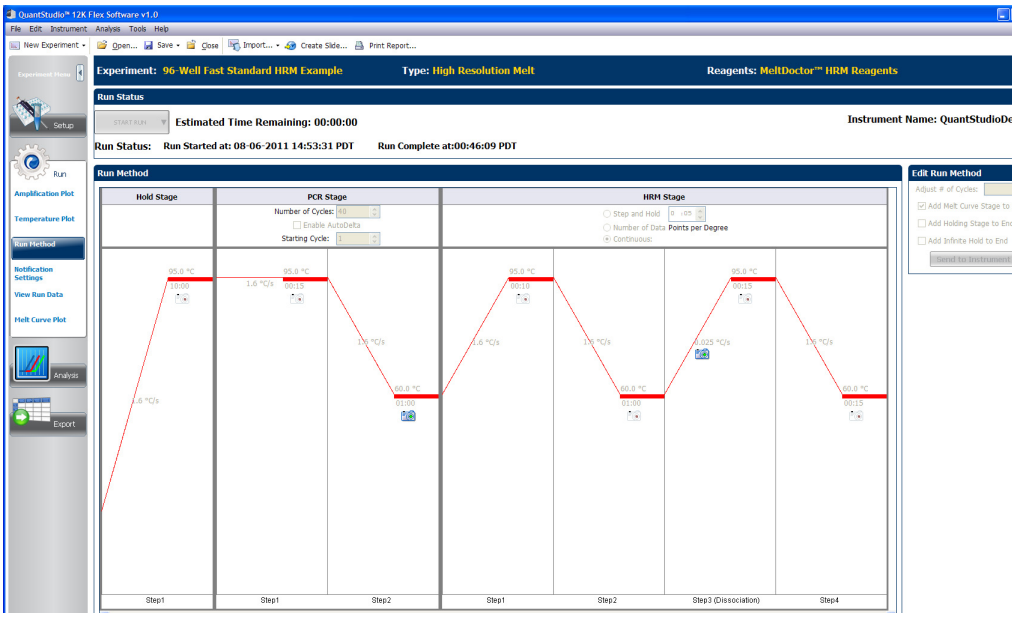
If you notice an abnormal temperature plot, troubleshoot the error as explained in the QuantStudio™ 12K Flex Software Help (click or press F1).

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

To...	Perform the following 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**.



If an alert appears, click the error for more information and troubleshoot the problem as explained in the QuantStudio™ 12K Flex Software Help (click ? or press F1).

7 Review 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 36), the Plate Layout (see page 39), and the Well Table (see page 41) to evaluate the genotype calls made by the QuantStudio™ 12K Flex Software.
2. Perform a thorough review of the QC Summary (see page 43) to evaluate the samples that triggered QC flags. Review the raw data (see page 44) and amplification data (see page 47) for the samples that exhibit abnormal amplification.
3. If necessary, define the analysis settings (see page 50) or modify the calls manually (see page 53).

After evaluating the results, you can publish the results as explained in "Publish the data" on page 55.

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.

- The HRM example experiment:

  Select **Open** and then browse to:

  C:\Program Files\Applied Biosystems\QuantStudio 12K Flex Software\examples\Melt Curve\96-Well Fast (Standard) HRM 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**.
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.

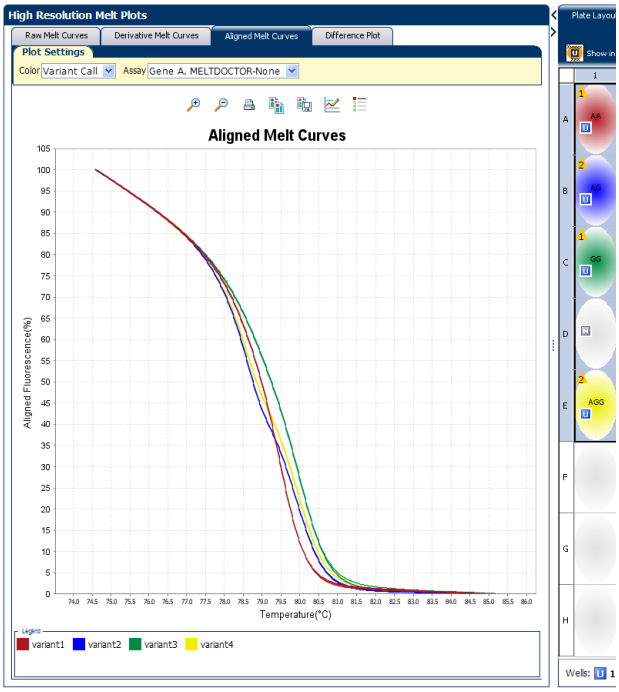
About melt curves

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:

- Variant 2 has a different curve shape compared to Variant 1 and Variant 3. The shape of the melt curve is an indicator of heteroduplex formation.
- Variant 1 and Variant 3 are both homozygous, but are distinguished from each other by the differences in Tm values.



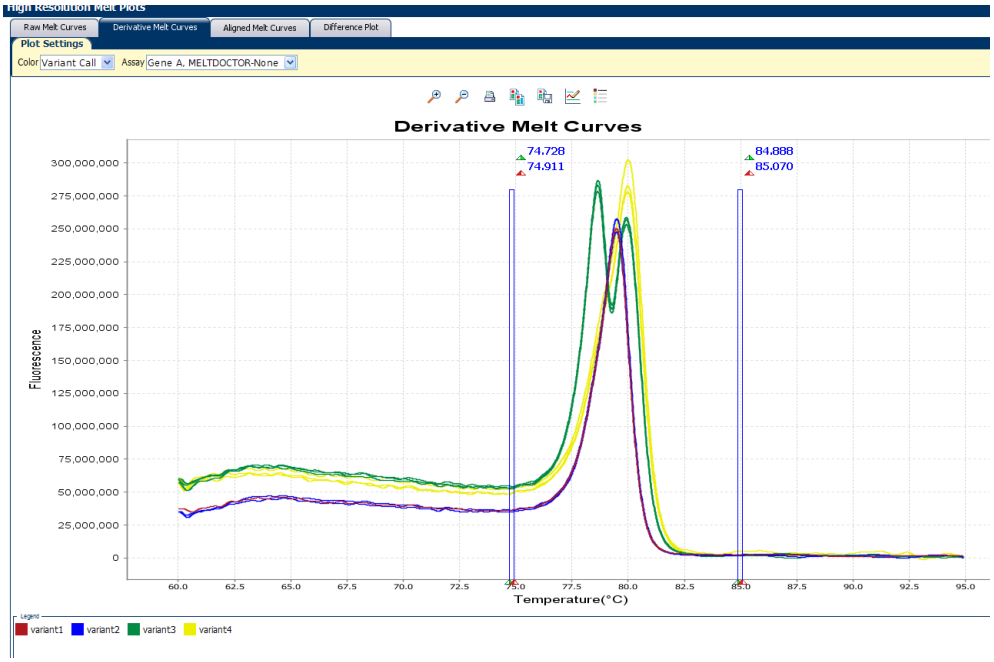
About the Pre- and Post-melt regions

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.

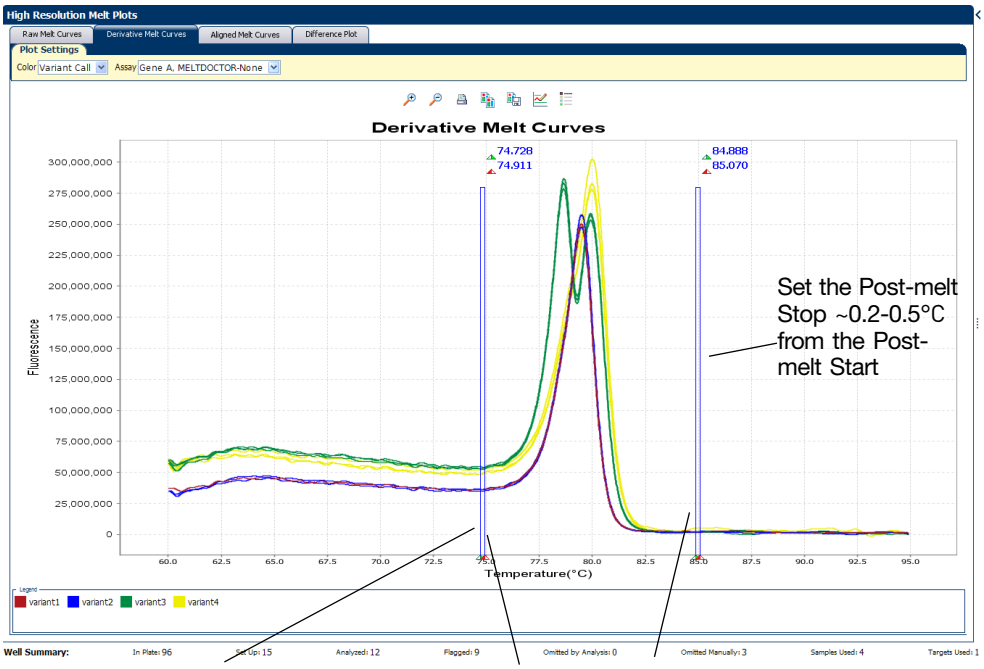


Review and adjust the Pre- and Post-melt regions

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 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 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.

   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.

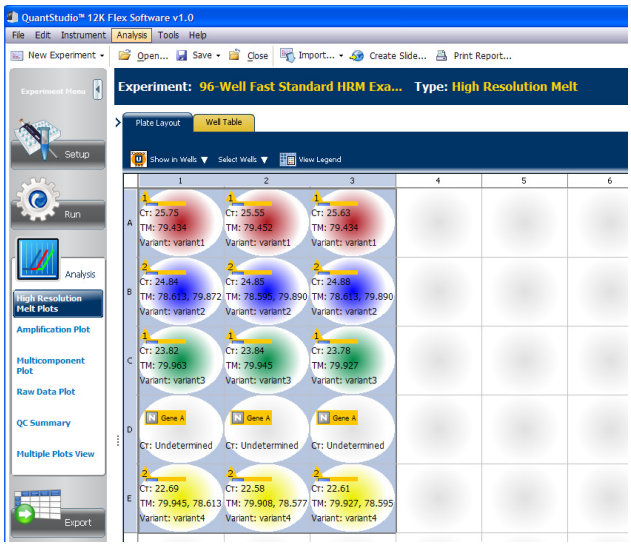
   For information on saving these settings, see "Apply custom Pre- and Post-melt settings to an assay" on page 52.

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 the ‹ icon 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™ 12K Flex Software called:

- 3 samples as variant1
- 3 samples as variant2
- 3 samples as variant3
- 3 samples as C_T: Undetermined (control)
- 3 samples as variant4

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

For descriptions of QC flags ( ), select QC Summary in the Experiment Menu.

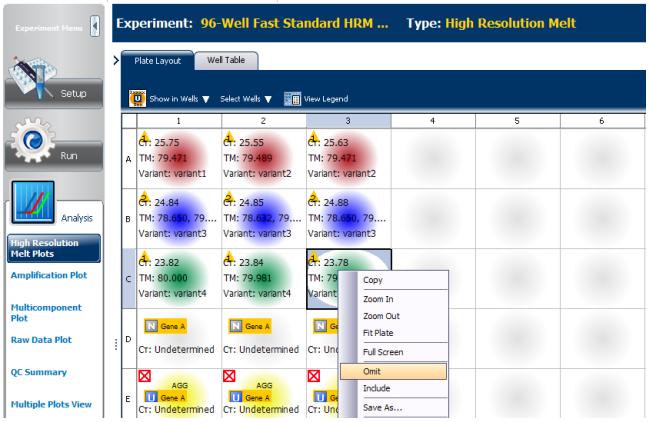
Analysis guidelines for the plate layout view

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 then 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

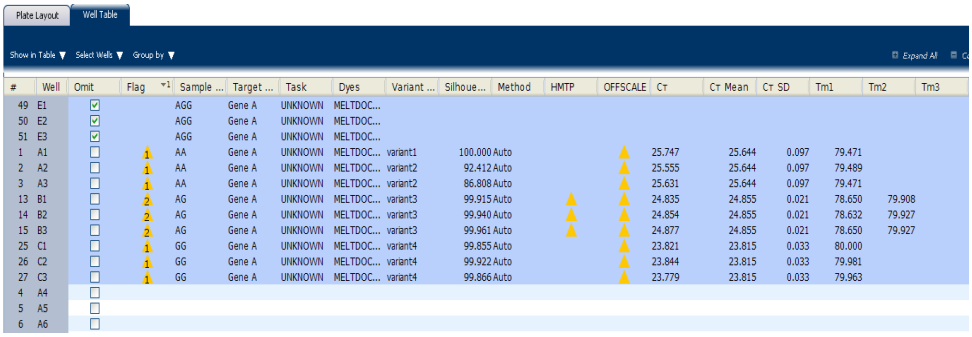
Review the details of the experiment results in the well table and identify flagged wells. The well table displays the assay-specific setup and analysis properties for the experiment in a table format.

Example experiment well data

If you are running the example experiment, you can see descriptions of QC flags (⚠) by selecting QC Summary in the Experiment Menu.

Review the well table

1. Select the Well Table tab.



2. Click the Flag column header to sort the data so that the wells that triggered flags appear at the top of the table.
3. Confirm the integrity of the controls:
   a. From the Group By menu, select Task to organize the table rows by their function on the reaction plate.
   b. Confirm that each of the controls do not display flags (1).
   c. Click the "–" 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 1 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.
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 52.
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™ 12K Flex 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.

Column	Description
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.

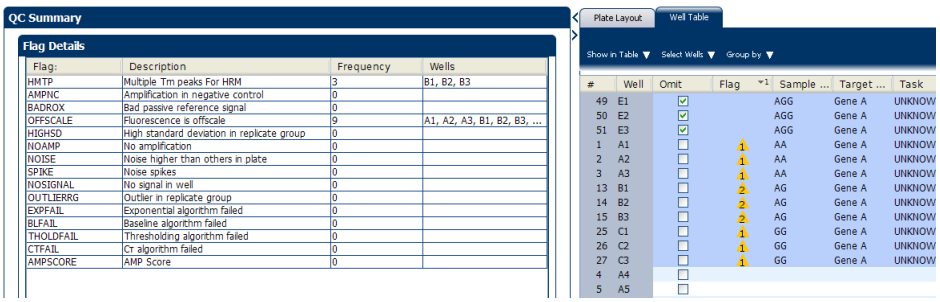
   Note: You must reanalyze the experiment each time you omit or include a well.

View the QC Summary

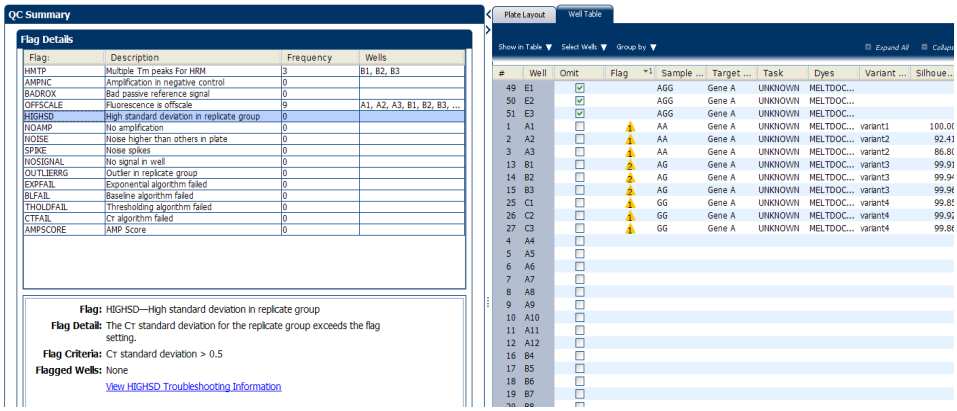
Review the summary of the QC flags triggered by the experiment data and troubleshoot the flags. The QC summary displays a frequency and location of all QC flags. If a flag does not appear in the experiment, its frequency is 0. If the frequency is not 0, that flag appears at the well position listed in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

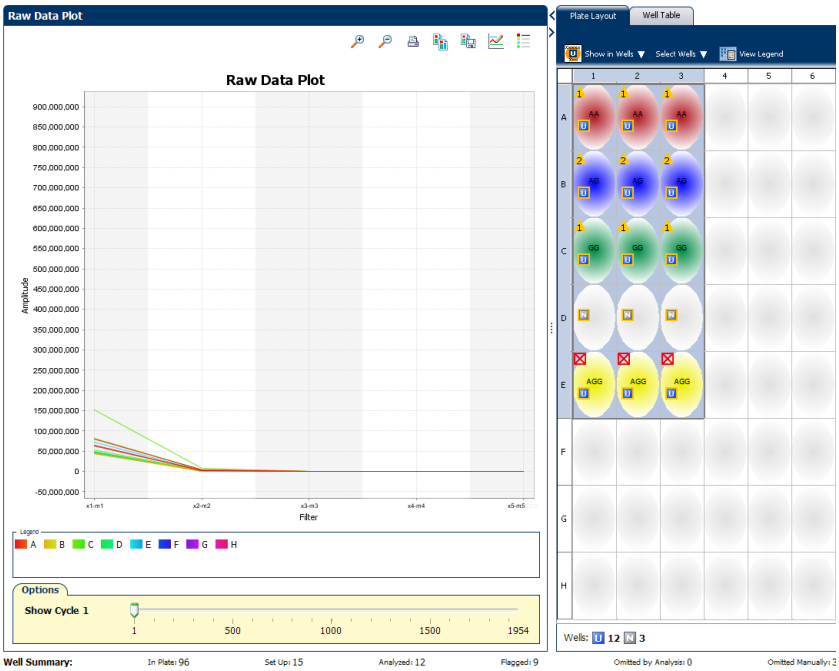
4. (*Optional*) For those flags with frequency >0, click the troubleshooting link to view information on correcting the flag.

View the Raw Data Plot

The Raw Data Plot displays the amplitude of the raw fluorescence collected 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. Drag the Show Cycle slider to view temporal changes in each filter of the raw data profile. (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). The filters are:

Filter set	Color	Filter wavelength (nm)†		Supported dyes
		Excitation	Emission	
x1-m1	Blue	470±15	520±15	FAM™ and SYBR® Green dyes
x2-m2	Green	520±10	558±12	VIC®, JOE™, TET™, and HEX™ dyes
x3-m3	Yellow	549.5±10	586.5±10	TAMRA™ and NED™ dyes
x4-m4	Orange	580±10	623±14	ROX™ dye
x5-m5	Red	640±10	682±14	LIZ™ dye
x6-m6	Deep red	662±10	711±12	None‡

† The central wavelengths are the optimized wavelengths.
‡ No Life Technologies supported dye currently available.

View the Multicomponent Plot

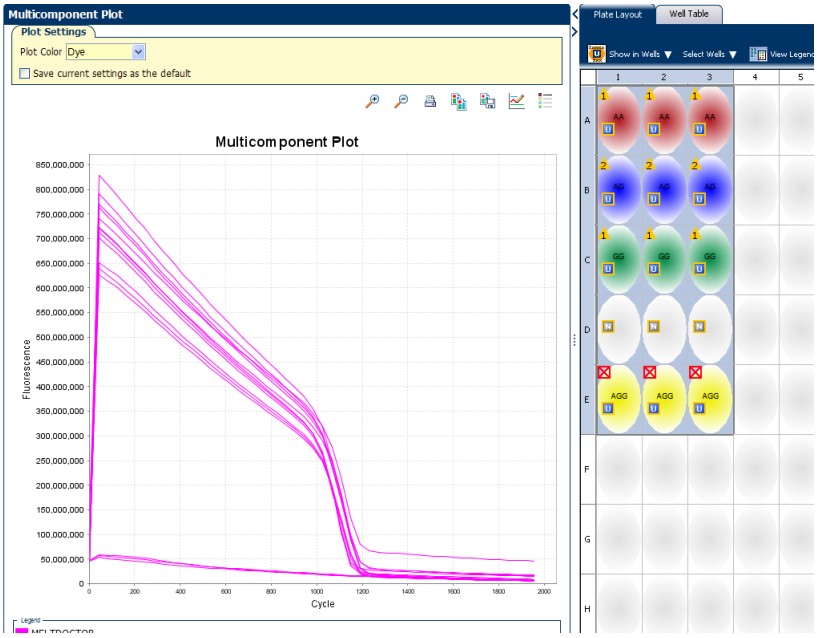
The Multicomponent Plot displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Review the Multicomponent Plot for:

- Any dye you have included as a passive reference
- Mediator™ 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 a time and check for amplification. If amplification has taken place, there may be contamination in the wells.

   In the HRM example experiment, the negative control wells contain reactions with no DNA template.

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:

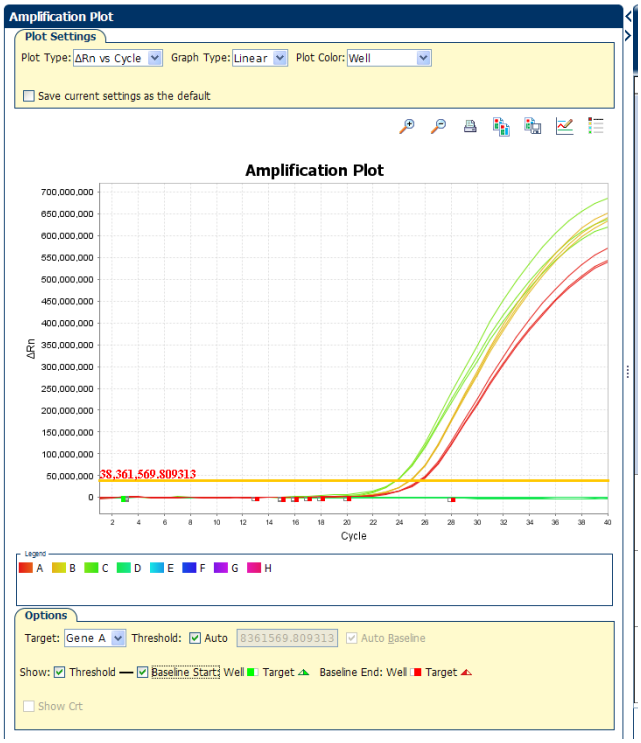
- **ΔRn vs Cycle** – This plot displays ΔR_n as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle** – This plot displays Rn as a function of cycle number. You can use this plot to identify 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 **Baseline 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.

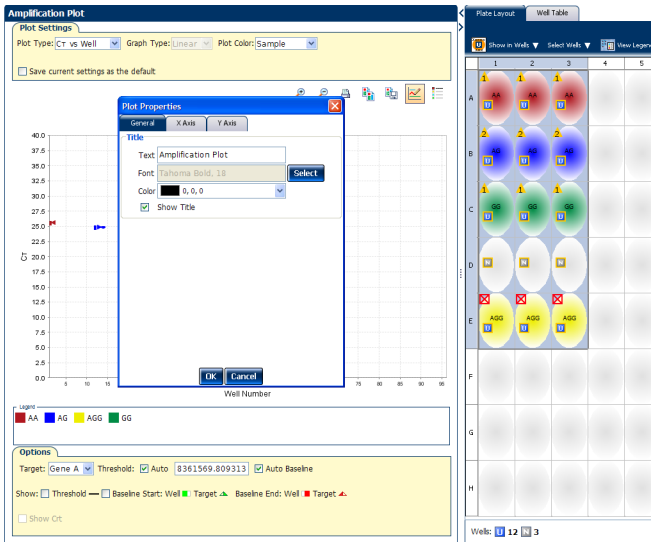
      In the example experiment, the threshold is in the exponential phase.

6. Repeat steps 2-5 for all targets.

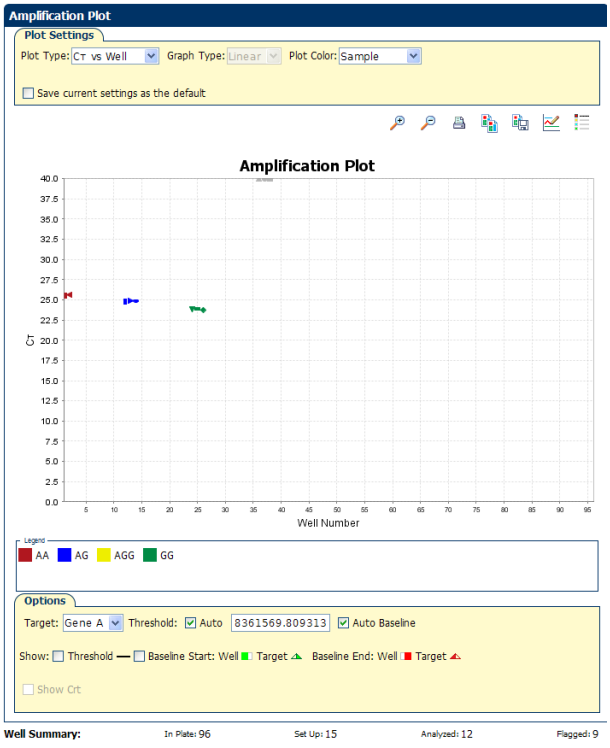
7. From the Plot Type drop-down menu, select **CT vs Well** and from the Plot Color drop-down menu, select **Sample**.

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 on **Plot Properties** icon , select **Y axis** tab, change Y axis to **40**, and **Save**.



9. Confirm that the replicate wells have achieved similar amplification.



Analysis guidelines for Amplification Plot

When you analyze the Amplification Plot, look for:

- **Outliers**
- **A typical amplification plot** – The QuantStudio™ 12K Flex 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 "Troubleshooting HRM Experiments" on page 67.

Modify the Analysis Settings

If you are dissatisfied with how the QuantStudio™ 12K Flex 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

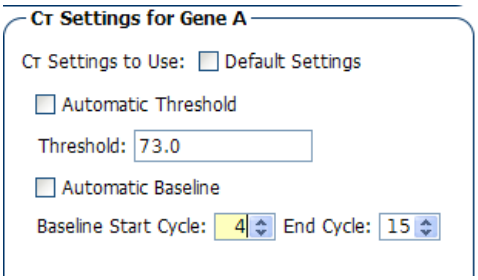
The C_T Settings are available only for experiments that include amplification data.

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: The Relative Threshold algorithm has no user-defined settings. Use Baseline Threshold if you wish to adjust parameters.

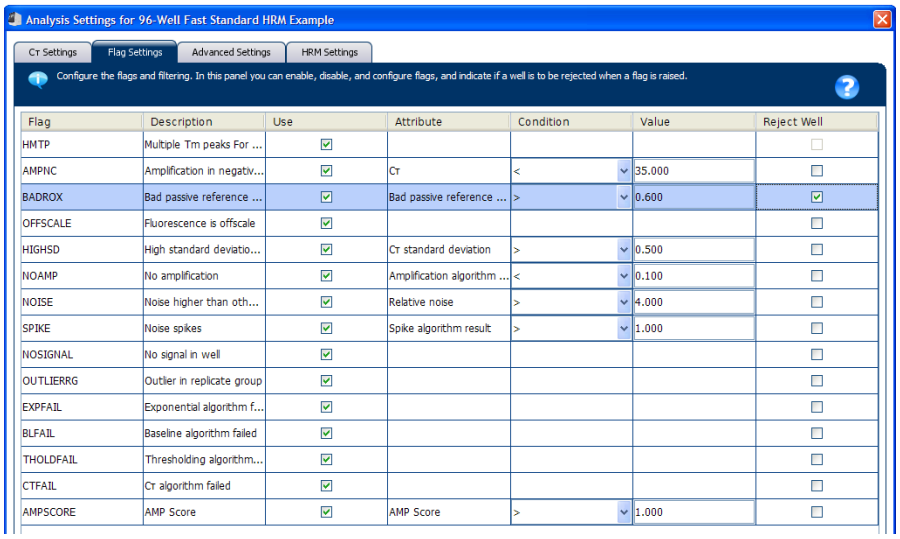
2. Select a target in the **Select a Target** list. (Gene A is the only target in the example experiment.)
3. In the **C_T Settings for Gene A** section, uncheck **Default Settings.**
4. Uncheck **Automatic Threshold**, then enter a new threshold value.
5. Uncheck **Automatic Baseline**, then enter new 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
HMTP	Multiple Tm peaks For ...	☑				☐
AMPNC	Amplification in negativ...	☑	Cт	<	35.000	☐
BADROX	Bad passive reference ...	☑	Bad passive reference ...	>	0.600	☑
OFFSCALE	Fluorescence is offscale	☑				☐
HIGHSD	High standard deviatio...	☑	Cт standard deviation	>	0.500	☐
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	☑				☐
EXPFAIL	Exponential algorithm f...	☑				☐
BLFAIL	Baseline algorithm failed	☑				☐
THOLDFAIL	Thresholding algorithm...	☑				☐
CTFAIL	Cт algorithm failed	☑				☐
AMPSCORE	AMP Score	☑	AMP Score	>	1.000	☐

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

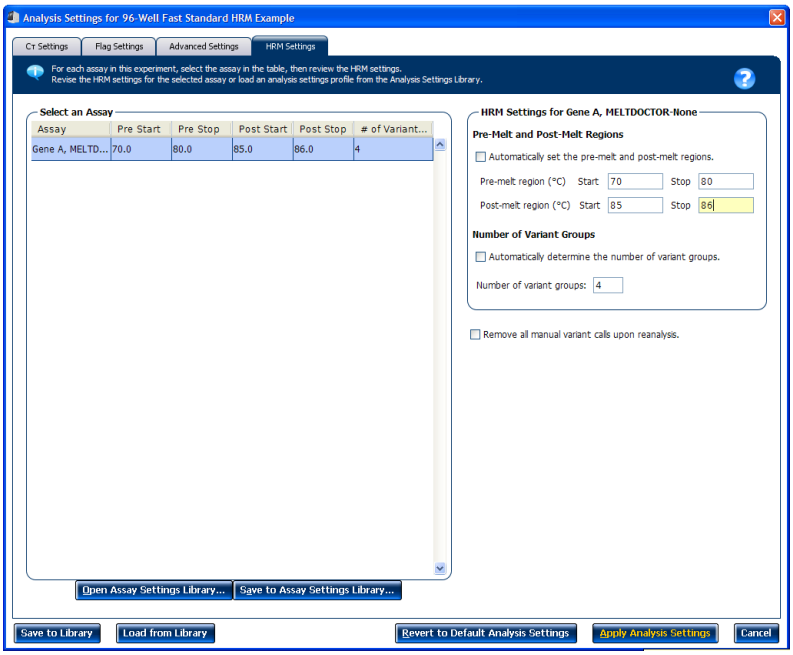
Adjust Pre- and Post-melt call settings to automatic or manual

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™ 12K Flex 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

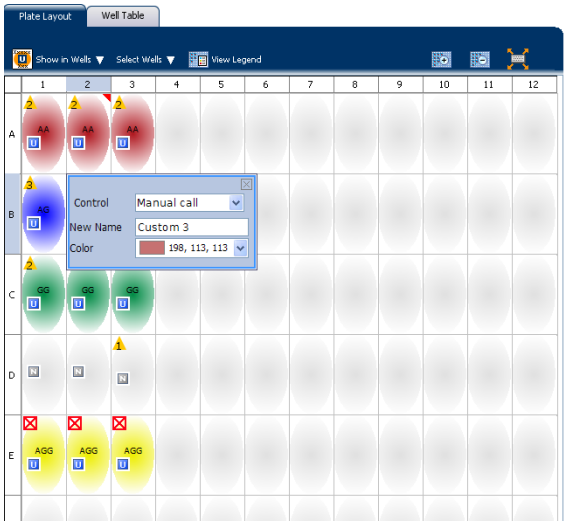
Perform manual calls when you want to manually assign a sample to a variant group.

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 close the dialog box.
   - **A new variant call** - Click Manual, enter a name for the new call, select a color, then close the dialog box..

     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.



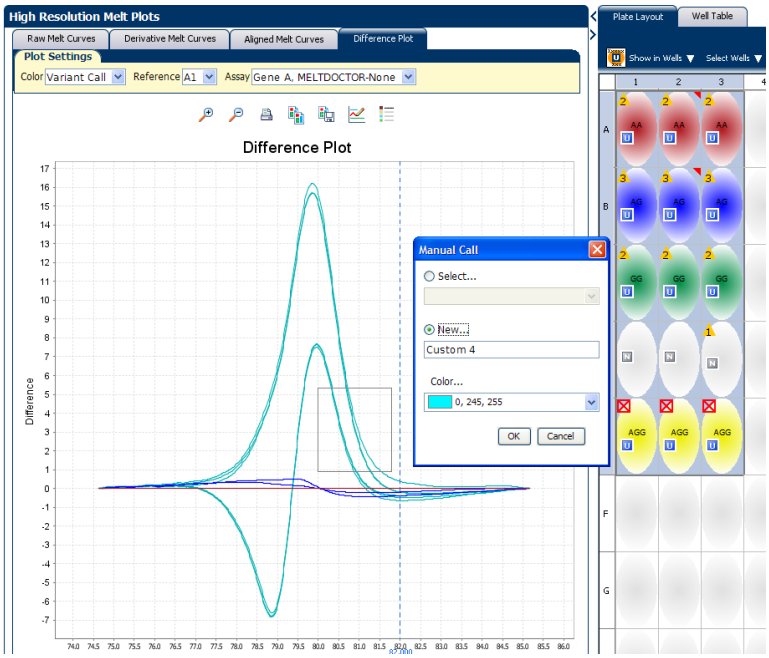
3. Repeat steps 1 and 2 to assign more manual calls.
4. Click **Analyze**. The software reanalyzes the data using the manual calls.

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 "Export Analysis Results" on page 23)

A

Ordering Information

How to order

You can order materials accessories from the Life Technologies website.

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

Note: Make sure that cookies and Java Script 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**, then log into the Life Technologies Store if you have an account; register if you are a new user.
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, select **Products & Services** ▸ **Real-Time PCR** and browse by category.

   Note: For a list of part numbers see, "Materials and equipment for HRM calibration and HRM experiments" on page 58.
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. Select **Products & Services** ▸ **Oligos, Primers, Probes & Nucleotides**. Select **Get Started** under **Applied Biosystems® Custom TaqMan® Probes and Primers**, .
3. 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.
4. Click **add to cart**.
5. Follow the link to your Shopping Basket, then follow the instructions on the web page to place your order.

Materials and equipment for HRM calibration and HRM experiments

MeltDoctor™ HRM reagents

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 × 5 mL bottle	4415452
MeltDoctor™ HRM Master Mix, 10 × 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), 150 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

Equipment and software

Item	Source
Primer Express® Software v3.0 or later	Life Technologies
Centrifuge with plate adapters	Major laboratory suppliers (MLS)
Lab equipment	MLS
Microcentrifuge	MLS
Microcentrifuge tubes	MLS
Pipettors and pipette tips	MLS
Vortexer	MLS

Supplies

Item	Source
Appropriate reaction plate for your instrument: • MicroAmp® Optical 384-Well Reaction Plate with Barcode, 50 plates • MicroAmp® Optical 384-Well Reaction Plate with Barcode, 500 plates • MicroAmp® Optical 384-Well Reaction Plate with Barcode, 1000 plates • MicroAmp® Optical 96-Well Reaction Plate with Barcode, 0.1 mL, 20 plates • MicroAmp® Optical 96-Well Reaction Plate with Barcode, 0.1 mL, 200 plates • MicroAmp® Optical 96-Well Reaction Plate with Barcode, 0.2 mL • MicroAmp® Optical 96-Well Reaction Plate , 0.2 mL	Applied Biosystems • Part no. 4309849 • Part no. 4326270 • Part no. 4343814 • Part no. 4346906 • Part no. 4366932 • Part no. 4306737 • Part no. N8010560
MicroAmp® Optical Adhesive Film: • 25 covers • 100 covers	Applied Biosystems • Part no. 4360954 • Part no. 4311971
Microcentrifuge tubes	MLS
Pipettors and pipette tips	MLS

B Supplemental Information and Procedures

This appendix contains supplemental information and procedures for preparing and running HRM reactions and for using the QuantStudio™ 12K Flex Software.

HRM dyes and MeltDoctor™

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 63.

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	10	1100
MeltDoctor™ HRM Calibration Standard (20X)	1	110
Deionized water	9	990
Total volume	**20**	**2200**

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 520-650 nm spectral range measured by the QuantStudio™ 12K Flex System.

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, see Appendix C, Creating a custom dye plate for calibration in the *Applied Biosystems QuantStudio 12K Flex Real-Time PCR System Maintenance and Administration Guide* (Part no. 4470689).

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 (Part 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	10	1100	4250
Custom dye (20x) (typical dye concentration 0.1 µM	1	110	425
MeltDoctor™ HRM Calibration Standard (20X)	1	110	425
Deionized water	8	880	3400
Total volume	**20**	**2200**	**8500**

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. Check the samples 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, 10X	2 µL	1X	1X
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.appliedbiosystems.com/troubleshoot**
- Life Technologies *Guide to High Resolution Melting (HRM) Analysis* (Stock number O-081740-0509)

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 68 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 time 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.

Possible causes	Recommended action
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(single-nucleotide polymorphism) (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*.

QuantStudio™ 12K Flex Instrument: Instrument does not eject the plate

Possible cause	Recommended action
The adhesive cover may have adhered the plate to the heated cover within the instrument.	1. Power off the QuantStudio™ 12K Flex Instrument. 2. Wait for 15 minutes, then power on the QuantStudio™ 12K Flex Instrument and eject the plate. 3. If the plate does not eject, power off and unplug the cord of the QuantStudio™ 12K Flex Instrument, then open the access door. 4. While wearing powder-free gloves, reach into the QuantStudio™ 12K Flex Instrument and remove the plate from the heated cover, then close the access door. 5. Perform a background calibration to confirm that the sample block has not been contaminated.

D

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Symbols on this instrument

Symbols may be found on the instrument to warn against potential hazards or convey important safety information. In this document, the symbol is used along with user attention words described in the "About This Guide" section to highlight important safety information. The following table gives the meaning of these symbols.

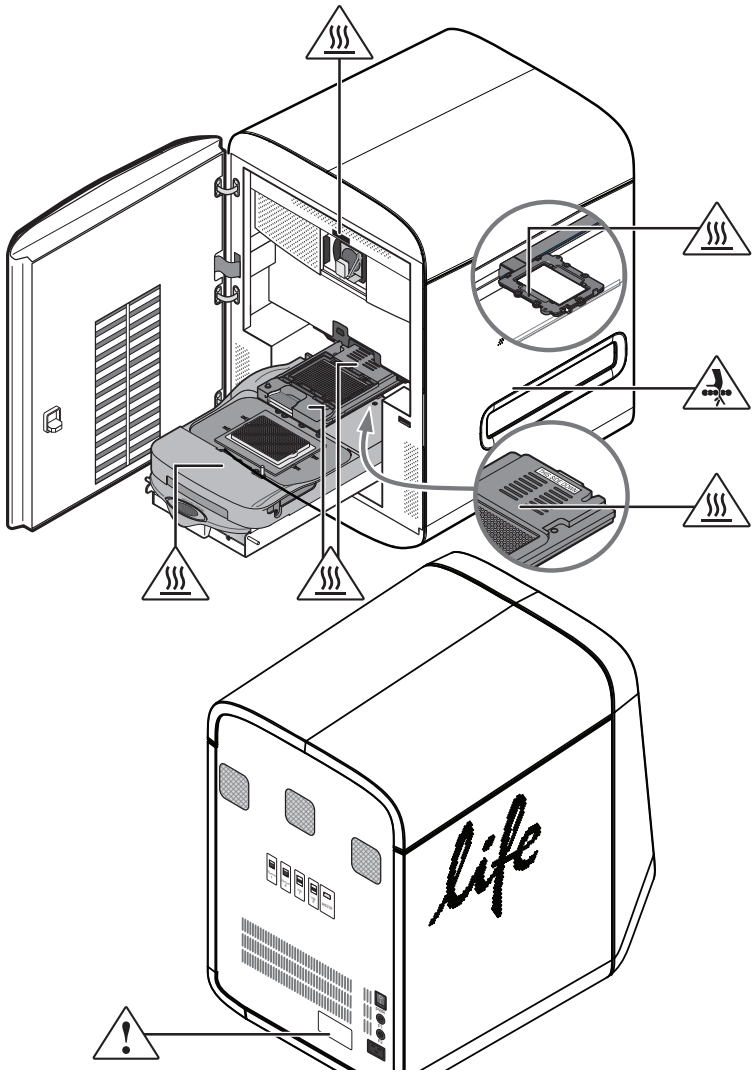
Symbol	English	Français
	Caution, risk of danger Consult the manual for further safety information.	Attention, risque de danger Consulter le manuel pour d'autres renseignements de sécurité.
	Caution, hot surface	Attention, surface chaude
	Caution, risk of electrical shock	Attention, risque de choc électrique
	Laser radiation	Rayonnement laser
 	Moving parts	Parties mobiles
	Potential biohazard	Danger biologique potentiel

Symbol	English	Français
	Ultraviolet light	Rayonnement ultraviolet
	Potential slipping hazard	Danger de glisser potentiel
	On	On (marche)
	Off	Off (arrêt)
	On/Off	On/Off (marche/arrêt)
	Standby	En attente
	Earth (ground) terminal	Borne de (mise à la) terre
	Protective conductor terminal (main ground)	Borne de conducteur de protection (mise à la terre principale)
	Terminal that can receive or supply alternating current or voltage	Borne pouvant recevoir ou envoyer une tension ou un courant de type alternatif
	Terminal that can receive or supply alternating or direct current or voltage	Borne pouvant recevoir ou envoyer une tension ou un courant continu ou alternatif
	Do not dispose of this product in unsorted municipal waste **CAUTION!** To minimize negative environmental impact from disposal of electronic waste, do not dispose of electronic waste in unsorted municipal waste. Follow local municipal waste ordinances for proper disposal provision and contact customer service for information about responsible disposal options.	Ne pas éliminer ce produit avec les déchets usuels non soumis au tri sélectif. **CAUTION!** Pour minimiser les conséquences négatives sur l'environnement à la suite de l'élimination de déchets électroniques, ne pas éliminer ce déchet électronique avec les déchets usuels non soumis au tri sélectif. Se conformer aux ordonnances locales sur les déchets municipaux pour les dispositions d'élimination et communiquer avec le service à la clientèle pour des renseignements sur les options d'élimination responsable.

Conformity mark	Description
	Indicates conformity with safety requirements for Canada and U.S.A.
	Indicates conformity with European Union requirements for safety and electromagnetic compatibility.
	Indicates conformity with Australian standards for electromagnetic compatibility.

Locations of safety labels on instruments

The QuantStudio™ Instrument contains warnings at the following locations:



Safety alerts on this instrument

Additional text may be used with one of the symbols described above when more specific information is needed to avoid exposure to a hazard. See the following table for safety alerts found on the instrument.

English	French translation
CAUTION! Hazardous chemicals. Read the Safety Data Sheets (SDSs) before handling.	**ATTENTION! Produits chimiques dangereux.** Lire les fiches signalétiques (FS) avant de manipuler les produits.

English	French translation
CAUTION! Hazardous waste. Refer to SDS(s) and local regulations for handling and disposal.	**ATTENTION! Déchets dangereux.** Lire les fiches signalétiques (FS) et la réglementation locale associées à la manipulation et à l'élimination des déchets.
CAUTION! Class 2(II) visible and/or invisible laser radiation present when open Do not stare directly into the beam or view directly with optical instruments.	**ATTENTION!** Rayonnement laser visible ou invisible de classe 2 (II) présent en position ouverte . Ne pas regarder directement dans le faisceau ou avec des instruments optiques.

Instrument safety

General

CAUTION! Do not remove instrument protective covers. If you remove the protective instrument panels or disable interlock devices, you may be exposed to serious hazards including, but not limited to, severe electrical shock, laser exposure, crushing, or chemical exposure.

Physical injury

CAUTION! Moving and Lifting Injury. The instrument is to be moved and positioned only by the personnel or vendor specified in the applicable site preparation guide.

Improper lifting can cause painful and permanent back injury.

Things to consider before lifting or moving the instrument or accessories:

- Depending on the weight, moving or lifting may require two or more persons.
- If you decide to lift or move the instrument after it has been installed, do not attempt to do so without the assistance of others, the use of appropriate moving equipment, and proper lifting techniques.
- Ensure you have a secure, comfortable grip on the instrument or accessory.
- Make sure that the path from where the object is to where it is being moved is clear of obstructions.
- Do not lift an object and twist your torso at the same time. Keep your spine in a good neutral position while lifting with your legs.
- Participants should coordinate lift and move intentions with each other before lifting and carrying.
- For smaller packages, rather than lifting the object from the packing box, carefully tilt the box on its side and hold it stationary while someone else slides the contents out of the box.

CAUTION! Moving Parts. Moving parts can crush, pinch and cut. Keep hands clear of moving parts while operating the instrument. Disconnect power before servicing.

Electrical

WARNING! Fuse Installation. Before installing the instrument, verify that the fuses are properly installed and the fuse voltage matches the supply voltage. Replace fuses only with the type and rating specified for the unit. Improper fuses can damage the instrument wiring system and cause a fire.

WARNING! Ensure appropriate electrical supply. For safe operation of the instrument:

- Plug the system into a properly grounded receptacle with adequate current capacity.
- Ensure the electrical supply is of suitable voltage.
- Never operate the instrument with the ground disconnected. Grounding continuity is required for safe operation of the instrument.

WARNING! Power Supply Line Cords. Use properly configured and approved line cords for the power supply in your facility.

WARNING! Disconnecting Power. To fully disconnect power either detach or unplug the power cord, positioning the instrument such that the power cord is accessible.

Cleaning and decontamination

CAUTION! CLEANING AND DECONTAMINATION.

- If hazardous materials are spilled onto the instrument, the instrument should be appropriately decontaminated.
- Using cleaning or decontamination methods other than those recommended by the manufacturer may compromise the safety or quality of the instrument.
- Care should be taken to not use decontamination or cleaning agents which would cause a hazard as a result of a reaction with parts of the equipment or with material contained in it.
- For the protection of others, ensure the instrument is properly decontaminated prior to having the instrument serviced at your facility or before sending the instrument for repair, maintenance, trade-in, disposal, or termination of a loan. Decontamination forms may be requested from customer service.

Laser

CAUTION! LASER HAZARD, Bar Code Scanner. The bar code scanner included with the instrument system is a Class 2 laser. To avoid damage to eyes, do not stare directly into the beam or point into another person's eyes.

Safety and electromagnetic compatibility (EMC) standards

The instrument design and manufacture complies with the standards and requirements for safety and electromagnetic compatibility as noted in the following table:

Safety

Reference	Description
EU Directive 2006/95/EC	European Union "Low Voltage Directive"
IEC 61010-1 EN 61010-1 UL 61010-1 CSA C22.2 No. 61010-1	*Safety requirements for electrical equipment for measurement, control, and laboratory use – Part 1: General requirements*
IEC 61010-2-010 EN 61010-2-010	*Safety requirements for electrical equipment for measurement, control and laboratory use – Part 2-010: Particular requirements for laboratory equipment for the heating of materials*
IEC 61010-2-081 EN 61010-2-081	*Safety requirements for electrical equipment for measurement, control and laboratory use – Part 2-081: Particular requirements for automatic and semi-automatic laboratory equipment for analysis and other purposes*

EMC

Reference	Description
Directive 2004/108/EC	European Union "EMC Directive"
EN 61326-1	*Electrical Equipment for Measurement, Control and Laboratory Use – EMC Requirements – Part 1: General Requirements*
FCC Part 18 (47 CFR)	U.S. Standard "Industrial, Scientific, and Medical Equipment"
AS/NZS 2064	*Limits and Methods of Measurement of Electromagnetic Disturbance Characteristics of Industrial, Scientific, and Medical (ISM) Radiofrequency Equipment*
ICES-001, Issue 3	*Industrial, Scientific and Medical (ISM) Radio Frequency Generators*

Environmental design

Reference	Description
Directive 2002/96/EC	European Union "WEEE Directive" – Waste electrical and electronic equipment
Directive 2002/95/EC	European Union "RoHS Directive" – Restriction of hazardous substances in electrical and electronic equipment

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Documentation and Support

Related documentation

The following related documents are shipped with the QuantStudio™ System:

Document	Purpose and audience
Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Quick Reference (PN 4470688)	Explains how to perform genotyping and gene expression experiments using the QuantStudio™ System. Intended for laboratory staff and principal investigators who perform experiments using the QuantStudio™ System.
Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Maintenance and Administration Guide (PN 4470689)	Explains how to calibrate, maintain, network, and secure the QuantStudio™ 12K Flex System. Intended for laboratory staff and principal investigators who maintain the QuantStudio™ 12K Flex System.
Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments (PN 4470050)	Explains how to perform experiments on the QuantStudio™ 12K Flex System using multi-well plates and array cards. The guide functions as both a: • Tutorial, using example experiment data provided with the QuantStudio™ 12K Flex Software. • Guide for your own experiments. Intended for laboratory staff and principal investigators who perform experiments using the QuantStudio™ 12K Flex System.
Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: OpenArray® Experiments User Guide (PN 4470935)	Explains how to perform experiments on the QuantStudio™ 12K Flex System using OpenArray® plates. The guide functions as both a: • Tutorial, using example experiment data provided with the QuantStudio™ 12K Flex Software. • Guide for your own experiments. Intended for laboratory staff and principal investigators who perform experiments using the QuantStudio™ 12K Flex System.
Applied Biosystems QuantStudio™ Real-Time PCR System *Robotics User Guide* (PN 4470693) (Only ships if you have ordered the robot)	Explains how to integrate a robotic plate handler with the QuantStudio™ 12K Flex System. Intended for engineering personnel who are responsible for integrating a robotic plate handler with the QuantStudio™ 12K Flex System.

Portable document format (PDF) versions of the Quick Reference Guide, User Guide, and Getting Started Guide are available on the QuantStudio™ 12K Flex Software CD.

Note: To open the user documentation included on the Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Software CD, use the Adobe® Acrobat® Reader® software available from **www.adobe.com**

Obtaining information from the Help system

The QuantStudio™ 12K Flex System 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™ 12K Flex Software window.
- Select **Help** ▸ **ViiA 7 Software Help**.
- Press **F1**.

You can use the Help system to find topics of interest by:

- Reviewing the table of contents
- Searching for a specific topic

Obtaining support

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

www.lifetechnologies.com

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.

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