HRM Experiments

Using MeltDoctor™ HRM Reagents and High Resolution Melt Software v3.0

User Guide

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About This Guide

Purpose

This guide is designed to help you quickly learn to perform high resolution melting (HRM) analysis using MeltDoctor™ HRM Reagents and High Resolution Melt Software v3.0. This guide provides step-by-step procedures on:

- How to calibrate the StepOnePlus™ Real-Time PCR System, StepOne™ Real-Time PCR System, or 7500 Fast Real-Time PCR System to use the MeltDoctor™ HRM Dye
- How to perform a general HRM experiment using the MeltDoctor™ HRM Positive Control Kit
- How to perform HRM genotyping, HRM mutation scanning, and HRM methylation studies using MeltDoctor™ HRM Reagents and High Resolution Melt Software v3.0: How to design the experiment, prepare the reactions, run the reactions, and review the HRM data

For more information on HRM, go to **www.appliedbiosystems.com/hrm**.

Prerequisites

This guide assumes that you have working knowledge of the:

- Microsoft® Windows® XP operating system
- Instrument system software for your real-time PCR system:
  - StepOne™ Software v2.2 or later for the StepOne and StepOnePlus systems
  - 7500 Software v2.0 or later for the 7500 Fast System

Safety information

Safety alert words

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

IMPORTANT! – Indicates information that is necessary for proper instrument operation 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. This signal word is to be limited to the most extreme situations.

Except for IMPORTANTs, each safety alert word in an Applied Biosystems document appears with an open triangle figure that contains a hazard symbol.

SDSs

The Safety Data Sheets (SDSs) for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining SDSs, see "SDSs" on page 101.

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

1

Calibrate the Instrument

The first time that you perform high resolution melting experiments, you must calibrate your instrument for the HRM dye that you plan to use. Life Technologies recommends that you calibrate each instrument for each HRM dye that you are using or for each significant change in master mix composition.

This chapter describes how to calibrate a StepOnePlus™ Real-Time PCR System, StepOne™ Real-Time PCR System, or 7500 Fast Real-Time PCR System for the MeltDoctor™ HRM Dye. To use a different HRM dye, see page 89 for instructions and guidelines.

IMPORTANT! Amplify the DNA, calibrate the instrument, and perform a melt curve to generate the HRM calibration file on the same day.

Perform a background calibration

1.Prepare the background calibration plate (page 10)
2.Run the background calibration plate (page 10)
3.Review the background calibration results (page 10)

⬇

Amplify the DNA in the HRM calibration plate

4.Prepare the MeltDoctor™ HRM Calibration Plate (page 11)
5.Run the HRM calibration plate to amplify the DNA (page 11)
6.Verify that the HRM calibration samples amplified (page 13)

⬇

Calibrate the instrument to use the MeltDoctor™ HRM Dye

7.Run the HRM calibration plate for the custom dye calibration (page 13)
8.Review the custom dye calibration results (page 14)

⬇

Perform a melt curve to generate the HRM calibration file

9.Run the HRM calibration plate for the HRM calibration (page 14)
10.Verify that the Melt Curve contains only one Tm peak (page 16)

Perform a background calibration

IMPORTANT! Before you can run the HRM dye calibration plate on your instrument, you must perform a background calibration. The background calibration ensures that the background signal is even across the reaction plate and that variation is at a minimum.

Required materials for background calibration

- Appropriate reaction plate for your reaction block:
  - MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL
  - MicroAmp® Fast Optical 48-Well Reaction Plate
- MicroAmp® Optical Adhesive Film
- Deionized water
- Pipettors and pipette tips
- Centrifuge

Prepare the background calibration plate

1. Add 20 µL deionized water into each well of the reaction plate.
2. Seal the reaction plate with optical adhesive film, then spin the plate.

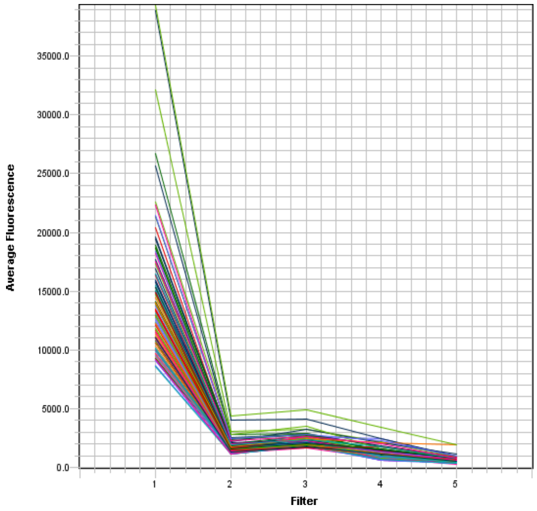
Run the background calibration plate

1. In the instrument software, select **Instrument ▸ Instrument Maintenance Manager**, then in the navigation pane, select **Background**.
2. Click **Start Calibration**, then follow the instructions in the Setup screen using the background calibration plate you prepared above.
3. In the Run screen, click **START RUN**.

   When the run is complete, the Analysis screen is automatically displayed.

Review the background calibration results

1. In the Analysis screen, verify that the background calibration passed.



2. Finish the background calibration and close the Instrument Maintenance Manager. The software saves the background calibration file.

3. Unload the background calibration plate.

Note: If the background calibration failed, refer to the procedures for cleaning the sample block in your instrument guide. The background calibration must pass before you can perform a custom dye calibration.

Amplify the DNA in the HRM calibration plate

Required materials for HRM calibration

- Appropriate calibration plate for your reaction block:
  - MeltDoctor™ HRM Calibration Plate, Fast 96-Well
  - Prepare your own 48-well HRM calibration plate
- Centrifuge

Note: To prepare your own HRM calibration plate using the MeltDoctor™ HRM Master Mix and MeltDoctor™ HRM Calibration Standard, follow the procedure in Appendix B on page 90.

Prepare the MeltDoctor™ HRM Calibration Plate

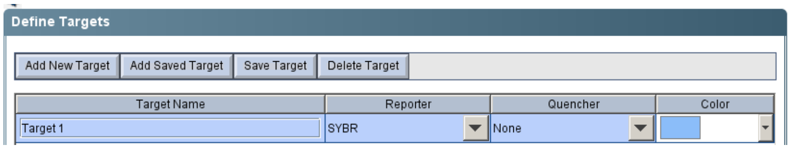
1. Remove the Fast 96-Well MeltDoctor™ HRM Calibration Plate from the freezer, then allow it to thaw.

2. Spin the plate briefly.

Run the HRM calibration plate to amplify the DNA

1. In the instrument software, create a new experiment for the amplification:
   - Experiment Name – Use the convention: **Amplification_<*today's date*>**
   - Experiment type – **Quantitation - Standard Curve**
   - Reagents – **SYBR® Green Reagents**
   - Ramp speed – **Standard (~ 2 hours to complete a run**)

2. In the Plate Setup ▸ Define Targets and Samples tab, define the calibration target as **Target 1** for the target name and **SYBR** for the reporter.

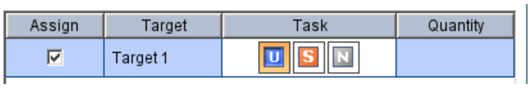


Target Name	Reporter	Quencher	Color
Target 1	SYBR	None	

3. In the Plate Setup ▸ Assign Targets and Samples tab, assign the Target 1 target to the wells in the plate grid:

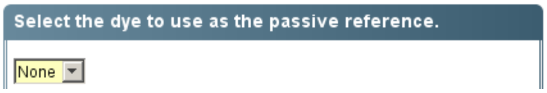
   a. Select all the wells in the plate grid.

b. Select the **Assign** checkbox for Target 1, then select U (**Unknown**) as the Task.



Assign	Target	Task	Quantity
☑	Target 1	U S N	

c. Select **None** as the passive reference.



Select the dye to use as the passive reference.

None

4. In the **Setup ▸ Run Method** tab, set the thermal cycler conditions:
   - Reaction Volume Per Well: **20** µL
   - Thermal profile:

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

5. Load the HRM calibration plate into the instrument, then start the run.

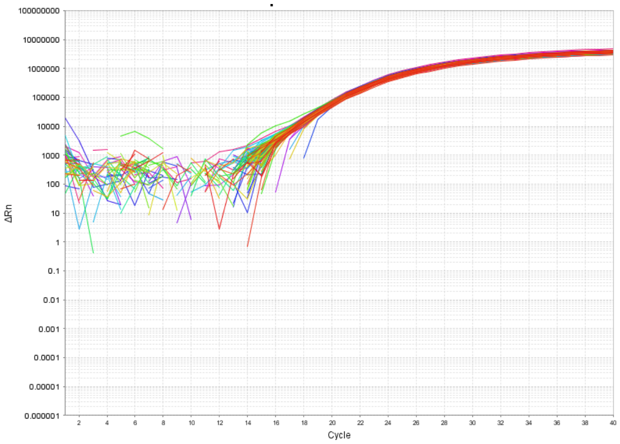
6. At the prompt, save the amplification file:
   - Location: Create a folder called **HRMCalibrationFiles**.
   - File name: Use the convention **Amplification_*<today's date>***

   When the run is complete, the Analysis screen is automatically displayed.

7. Unload the HRM calibration plate.

Verify that the HRM calibration samples amplified

1. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence
   - Standard deviation of C_T values ≤0.25.



Note: If the Amplification Plot looks abnormal, contact an Applied Biosystems representative to identify and resolve the problem.

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2. Save and close the file.

Calibrate the instrument to use the MeltDoctor™ HRM Dye

Perform a custom dye calibration for the MeltDoctor™ HRM Dye using the same HRM calibration plate that you amplified on page 11.

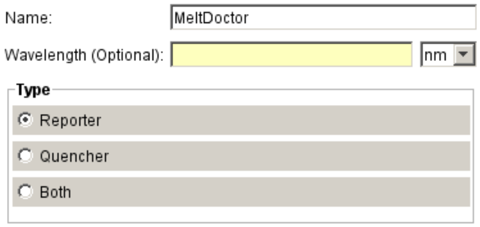
Run the HRM calibration plate for the custom dye calibration

1. In the instrument software, select **Instrument ▸ Instrument Maintenance Manager**, then in the navigation pane, select **Custom Dye Calibration**.

2. Click **Start Calibration**, then follow the instructions in the Setup screen:

   a. In step 1, for the custom dye calibration plate, use the amplified HRM calibration plate.

   b. Add the HRM dye to the Dye Library: Click **New Dye**, then enter **MeltDoctor** for the name, select **Reporter** as the dye type, and click **OK**.

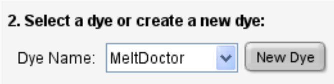


Name: MeltDoctor
Wavelength (Optional): nm

Type

● Reporter
○ Quencher
○ Both

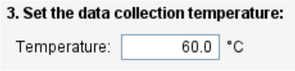
c. In step 2, select **MeltDoctor** as the Dye Name.



2. Select a dye or create a new dye:

Dye Name: MeltDoctor

d. In step 3, set the temperature to **60 °C (default)**.



3. Set the data collection temperature:

Temperature: 60.0 °C

e. Spin the HRM calibration plate briefly, load the plate into the instrument, select the checkbox **The custom dye plate is loaded into the instrument**, then click **Next**.

3. In the Run screen, click **START RUN**.

4. When the run is complete, unload the HRM calibration plate, then click **Next** to view the Analysis screen.

Review the custom dye calibration results

1. In the Analysis screen, verify that the custom dye calibration passed.

Note: If the custom dye calibration failed, refer to your instrument guide. You cannot complete the HRM calibration until the custom dye calibration passes.

2. Finish the custom dye calibration and close the Instrument Maintenance Manager. The software saves the custom dye calibration file.

Perform a melt curve to generate the HRM calibration file

Use the HRM calibration plate a third time to perform a melt curve and generate an HRM calibration file for the HRM Software.

Run the HRM calibration plate for the HRM calibration

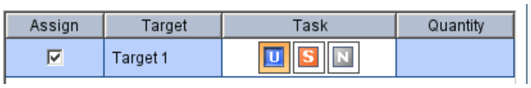
1. In the instrument software, create a new experiment for the HRM calibration:
   - Experiment Name – Use the convention **HRMCalibration_MeltDoctorDye_<*instrument info*>_<*today's date*>**
   - Experiment type – **Melt Curve**
   - Reagents – **Other**
   - Ramp speed – **Fast**

2. In the Plate Setup ▸ Define Targets and Samples tab, define the calibration target as **Target 1** for the target name and **MeltDoctor** for the reporter.

3. In the Plate Setup ▸ Assign Targets and Samples tab, assign Target 1 to the wells in the plate grid:

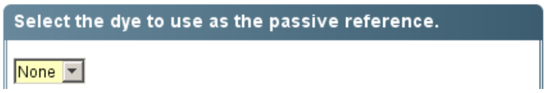
   a. Select all the wells in the plate grid.

b. Select the **Assign** checkbox for Target 1, then select U (**Unknown**) as the Task.



Assign	Target	Task	Quantity
☑	Target 1	U S N	

c. Select **None** as the passive reference.



Select the dye to use as the passive reference.

None

4. Set the thermal cycler protocol in the **Instrument ▸ Thermal Profile** tab:
   - Reaction Volume Per Well – **20** µL
   - Thermal profile:

Stage	Step	Temp	Time
Melt curve	Denature	95 °C	10 sec
	Anneal	60 °C	1 min
	High resolution melt	95 °C	15 sec
	Anneal	60 °C	15 sec

   - Ramp mode and rate (StepOne™ and StepOnePlus™ systems) – Select the **Continuous** ramp mode, then set the ramp rate to 0.3%.
   - Expert Mode (7500 system) – Select the **Expert Mode** checkbox.
   - Filters (7500 system) – Click **Select/View Filters**, then select only **Filter-1**.

5. Spin the HRM calibration plate briefly, load the plate into the instrument, then start the run.

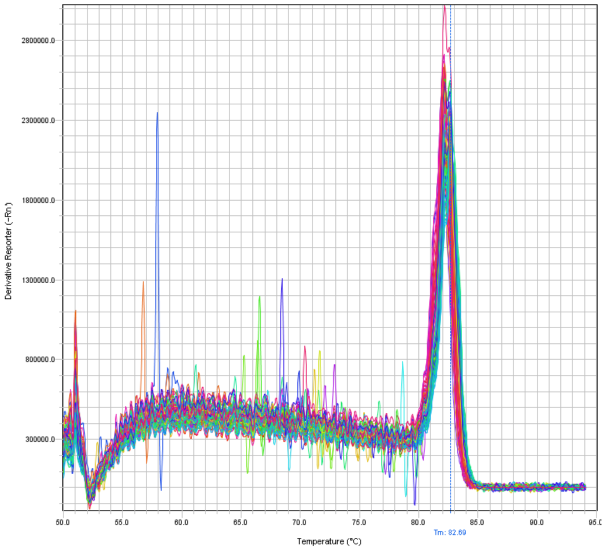
6. At the prompt, save the HRM calibration file:
   - Location: HRMCalibrationFiles folder that you created when you ran the HRM calibration plate to amplify the DNA (see page 11).
   - File name: Use the convention:
     HRMCalibration_MeltDoctorDye_*<instrument info>*_*<today's date>*

IMPORTANT! Make sure you include instrument information (instrument model number, plate type, and software version) in the file name so you can easily verify that the HRM calibration file is appropriate to assign to the HRM experiment file. If you have multiple instruments, include information to identify the instrument (for example, an instrument name).

When the run is complete, the Analysis screen is automatically displayed.

Verify that the Melt Curve contains only one Tm peak

1. Verify that the Melt Curve contains only 1 Tm peak, as in the example below.



Note: If the Melt Curve contains more than 1 Tm Peak, more than one PCR product was produced. Contact an Applied Biosystems representative to identify and resolve the problem.

2. Save and close the file, then unload the HRM dye calibration plate.

IMPORTANT! When you open an experiment file in the HRM Software for the first time, you will be prompted to select the default HRM calibration file. Select the file that you created using the convention: **HRMCalibration_MeltDoctorDye_<*instrumentinfo*>_<*today's date*>**.

2

Perform an HRM Experiment

Use MeltDoctor™ HRM Reagents and High Resolution Melt Software v3.0 to generate and analyze high-resolution melting curves from HRM reactions run on your StepOne™, StepOnePlus™, or 7500 Fast Real-Time PCR System.

This chapter provides general instructions for performing an HRM experiment, using the MeltDoctor™ HRM Positive Control Kit as an example.

Perform an HRM Experiment

Design the HRM experiment (below)
⬇
Prepare the HRM reactions (page 18)
⬇
Amplify and melt the DNA (page 21)
⬇
Review the high-resolution melting data (page 25)

Design the HRM experiment

Design and order the primers

Using Primer Express® Software v3.0 or later, design the primers to amplify the sequence of interest. Order the primers from the Applied Biosystems Store.

If you are using the MeltDoctor™ HRM Positive Control Kit, the kit contains primers designed to amplify the alleles in the positive control DNA. You do not need to design primers to use the Positive Control Kit.

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

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

2. Go to **www.appliedbiosystems.com**, then log into the Applied Biosystems Store if you have an account; register if you are a new user. For more instructions, see "Order custom primers" on page 92.

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

Prepare the HRM reactions

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

With the MeltDoctor™ HRM Positive Control Kit, combine positive control Allele DNA with the MeltDoctor™ HRM Master Mix and MeltDoctor™ HRM Primer Mix to amplify the alleles.

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

Required materials

- Microcentrifuge tubes
- Optical reaction plate appropriate for your Real-Time PCR instrument
- MicroAmp® Optical Adhesive Film
- For the example experiment using the MeltDoctor™ HRM Positive Control Kit, components from the kit:
  - MeltDoctor™ HRM Primer Mix (20X)
  - MeltDoctor™ HRM Allele A DNA (20X)
  - MeltDoctor™ HRM Allele G DNA (20X)
  - MeltDoctor™ HRM Allele A/G DNA (20X)
- For your own HRM experiments:
  - Forward and reverse primers (5 µM each)
  - DNA samples
- MeltDoctor™ HRM Master Mix
- Deionized water
- Pipettors and pipette tips
- Vortexer
- Centrifuge

Prepare the HRM reactions

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

To prepare HRM reactions for your own HRM experiments:

HRM experiment type	See page
HRM genotyping experiments	page 37
HRM mutation scanning experiments	page 50
HRM methylation studies	page 66

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

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

Components	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10 µL	33.0 µL
MeltDoctor™ HRM Primer Mix (20X)	1 µL	3.3 µL
Deionized water	9 µL	29.7 µL
Total volume	**20 µL**	**66 µL**

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

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

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
One type of allele DNA: • MeltDoctor™ HRM Allele A DNA (20X) • MeltDoctor™ HRM Allele G DNA (20X) • MeltDoctor™ HRM Allele A/G DNA (20X)	1 µL	3.3 µL
MeltDoctor™ HRM Primer Mix (20X)	1 µL	3.3 µL
Deionized water	8 µL	26.4 µL
Total volume	**20 µL**	**66 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume 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 each reaction replicate to 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.

Amplify and melt the DNA

After you have prepared the reaction plate, run the plate to amplify and melt the DNA and to generate HRM fluorescence data.

Create and set up a new experiment file for the HRM run

1. In the instrument software, create a new experiment to amplify and melt the DNA, then set up the file in the Experiment Properties screen:
    - Experiment Name – Enter a unique name for your experiment
    - Instrument – Select the appropriate instrument type
    - Experiment type – **Quantitation - Standard Curve**
    - Reagents – **Other**, then select the **Include Melt Curve** checkbox
    - Ramp speed – **Standard (~ 2 hours to complete a run)**

2. Define each target sequence and each sample in the reaction plate in the Plate Setup ▸ Define Targets and Samples tab:
    - For each target sequence in the reaction plate, add a corresponding target to the experiment: Click the **Target Name** cell, enter a target name, then select **MeltDoctor** from the Reporter dropdown menu.
    - For each sample in the reaction plate, add a sample name: Click **Add New Sample**, then enter a sample name.

3. Assign targets and samples to wells in the plate grid to match the layout of your reaction plate in the Plate Setup ▸ Assign Targets and Samples tab:
   - Set up the negative controls: Select the negative control wells in the plate grid, then select the **Assign** checkbox next to your target and select the N (**Negative Control**) task.

     Note: Negative controls are needed to control for the amplification, but they are not appropriate for HRM analysis. Make sure you assign the Negative Control task for negative controls so that the HRM Software will automatically omit the negative controls from the HRM analysis.

   - Set up the unknowns: Select the wells containing DNA samples in the plate grid, then select the **Assign** checkbox next to your target. The U Unknown task is selected by default.

     Note: Set up your variant controls as unknowns during experiment setup. You will set up the variant controls when you set up the analysis using the HRM Software.

   - For each sample, select the wells that contain a sample, then select the **Assign** checkbox next to the corresponding sample name.

   Note: If you are running multiple assays, make sure you define each target sequence in the plate and assign the targets to the appropriate wells. The HRM Software will automatically separate the wells into different assays according to the target assigned to the well.

4. Set the Passive Reference to **None**.

5. In the Run Method screen, set the thermal cycler conditions:
   - Reaction Volume Per Well: **20** µL
   - Thermal profile:

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	Denature	95 °C	10 sec
	Anneal	60 °C	1 min
	High resolution melt	95 °C	15 sec
	Anneal	60 °C	15 sec

   - Ramp mode and rate (StepOne™ and StepOnePlus™ systems): Select the **Continuous** ramp mode, then set the ramp rate to 0.3%.
   - Expert Mode (7500 system): Select the **Expert Mode** checkbox.
   - Filters (7500 system): Click **Select/View Filters**, then select only **Filter-1**.

Run the plate to amplify and melt the DNA

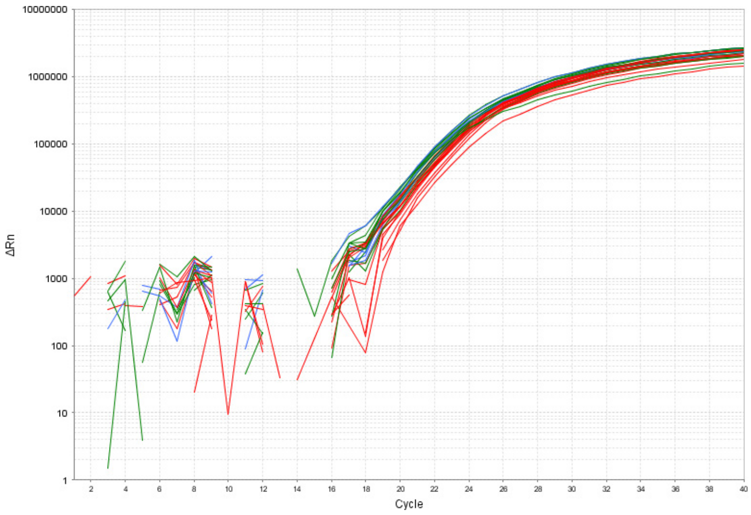
1. In the navigation pane, select **Run**, then load the reaction plate into the instrument, then click **START RUN**.

   Note: You may receive a message recommending SYBR® Green reagents for melt curve experiments. Click **OK** to close the message.

2. At the prompt, save the file to a desired save location.

Verify that the samples amplified and review the Tm peaks

1. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence



Note: Note which wells are outliers with C_T values that differ from replicates by more than 2. The outliers may produce erroneous HRM results.

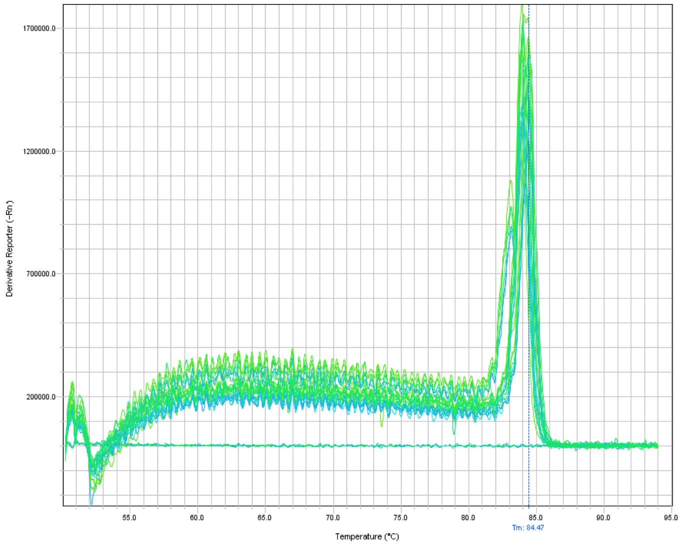
Note: If the Amplification Plot looks abnormal, refer to "Troubleshooting HRM Experiments" on page 79 to identify and resolve the problem.

2. Verify that the Melt Curve shows no unexpected Tm peaks:

   If the sequence you amplified contains more than 1 variant or a more complex mutation, you may see more than 1 Tm peak because of the large differences between the different sequence types.

   Note: Unexpected peaks at low temperatures may indicate contamination, primer dimers, or non-specific amplification.

   Note: The data may appear noisy because more data is collected during a high resolution melt curve than during a standard melt curve. The extra data are required for analysis with the HRM Software.



3. Save and close the file.

Review the high-resolution melting data

After you create, run, analyze, and save the *.eds experiment file on your real-time PCR system, use the High Resolution Melt Software v3.0 (HRM Software) to perform high resolution melting analysis of the data and review the variants.

Start the software

You can start the HRM Software two ways:

- From the desktop: Double-click (HRM Software v3.0 or later)

  or
- From the Start menu: Select **Start** ▶ **All Programs** ▶ **Applied Biosystems** ▶ **HRM Software** ▶ **HRM Software v3.0** (or later)

Access the Help system

The HRM Software has a Help system that describes how to use each feature of the software. You can access the Help system three ways:

- From the HRM Software window or dialog box: Click ?.
- From the HRM Software menu: Select **Help** ▶ **High Resolution Melt Software Help**.
- From your keyboard: Press **F1**.

About the HRM calibration file

The HRM calibration file is the *.eds file that you created, ran, and analyzed on your real-time PCR system during the HRM calibration (see Chapter 1, Calibrate the Instrument). When you analyze an HRM experiment file in the HRM Software, the software requires an HRM calibration file to assign to the HRM experiment file. The data from the HRM calibration file are used in the HRM analysis.

HRM calibration file requirements

You need to have an HRM calibration file for each instrument that you are using to run the HRM reactions.

IMPORTANT! The HRM calibration file must be:

- Run on the same instrument system (the same instrument and the same software version) as the run file.
- Generated using the same reagents (HRM dye and master mix) as the reactions in your HRM experiment plate.
- Run using the same run conditions (the same ramp mode and ramp increment) as the run file.

For each instrument type, the first time that you open a file from that instrument type, the software will prompt you to select a default HRM calibration file to assign to HRM experiment files from that instrument type. If you have multiple instruments of the same instrument type, you need to create an HRM calibration file for each instrument, but you can select only one default HRM calibration file for each instrument type.

Note: The HRM Software can distinguish between calibration files from two different instrument types but cannot distinguish between two different instruments of the same type.

One recommendation for managing the HRM calibration file is to select the HRM calibration file from the most commonly used instrument as your default calibration file. When you analyze new HRM experiments, consistently verify that the HRM calibration file and the HRM experiment were run on the same instrument. If the HRM experiment file was run on a different instrument, change the calibration file.

IMPORTANT! Make sure you include instrument information (instrument name, instrument model number, plate type, and software version) in the HRM calibration file name so you can easily verify that the HRM calibration file is appropriate for the HRM experiment file.

Example HRM experiments

Example HRM experiment files are located in *X*:\Applied Biosystems\HRM\Examples, where *X* is the drive where you installed the HRM Software.

- Genotyping Example-HRM.eds
- Class 4 SNP StepOnePlus Example -HRM.eds
- Low Percent Methylation Titration-HRM.eds
- Mutation Scanning Example
- Multiple Assay StepOnePlus Example-HRM.eds

To follow the example HRM experiment used in this chapter, use the Genotyping Example-HRM.eds file.

Note: The example files installed with the software are HRM experiment files and already have the appropriate HRM calibration files assigned to them.

Open the HRM experiment

In the HRM Software, open the *.eds experiment file from your real-time PCR system.

The HRM Software uses the default analysis settings to automatically assign a variant call to each sample. The software determines the variant calls by melt curve characteristics – melt curve shapes and Tm values. Before you assign the controls, the software labels each variant call *variant1*, *variant2*, *variant3*, and so on.

1. Using the HRM Software, select **File ▸ Open** from the menu bar *or* click Open... in the toolbar.
2. Browse to and select the *.eds file to undergo HRM analysis.

3. Select or check the HRM calibration file:
    - If this is the first time to open an *.eds file for a particular instrument type, browse to and select the HRM calibration file to use as the default HRM calibration file.
    - If the default HRM calibration file was already selected for the instrument type, go to the Experiment Properties screen and verify that the HRM calibration file is correct.

IMPORTANT! Use the HRM calibration file name to verify that the HRM calibration file that is assigned to the HRM experiment is correct. The HRM calibration file must be:

- Run on the same instrument system (the same instrument, the same block type, and the same software version) as the run file
- Run using the same HRM dye and master mix used in the HRM calibration plate
- Run using the same run conditions (the same ramp mode and ramp increment) as the run file.

Note: To change the HRM calibration file for a selected experiment or to change the default HRM calibration file for subsequent new HRM experiments, see page 95.

Assign controls

For each positive control sample, enter information about that control in the HRM software and assign the control to the appropriate wells.

Note: Negative control samples that were designated as Negative Controls using the instrument software are automatically omitted from analysis in the HRM Software.

1. Add controls to the experiment in the Controls section of the Define screen:
    - To create a new control: Click **New** in the toolbar above the control table, then define the control.
    - To add one or more controls from the Control Library: Click **Add Saved** in the toolbar above the control table, select one or more controls, then click **Add Selected Controls**.

2. Assign controls to wells on the Assign screen:
    a. Select wells using the plate layout or the well table.

b. In the Controls list, select the checkbox next to the control you want to assign to the selected wells.

> **Note:** You can assign only 1 control to a well. If the selected wells contain mixed assignments (indicated by a −), remove existing control assignments before you make the new control assignment.

Note: After you reanalyze the experiment, the variant call for the controls is named using the convention Control-<*name*>, where <*name*> is the name you entered when you set up the controls.

About the melting profiles

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

With the MeltDoctor™ HRM Positive Control Kit:

- The heterozygotes have a different curve shape compared to the homozygotes. The shape of the melt curve is an indicator of heteroduplex formation.
- The two homozygotes are distinguished from each other based on the difference in Tm values.

About the pre- and post-melt regions

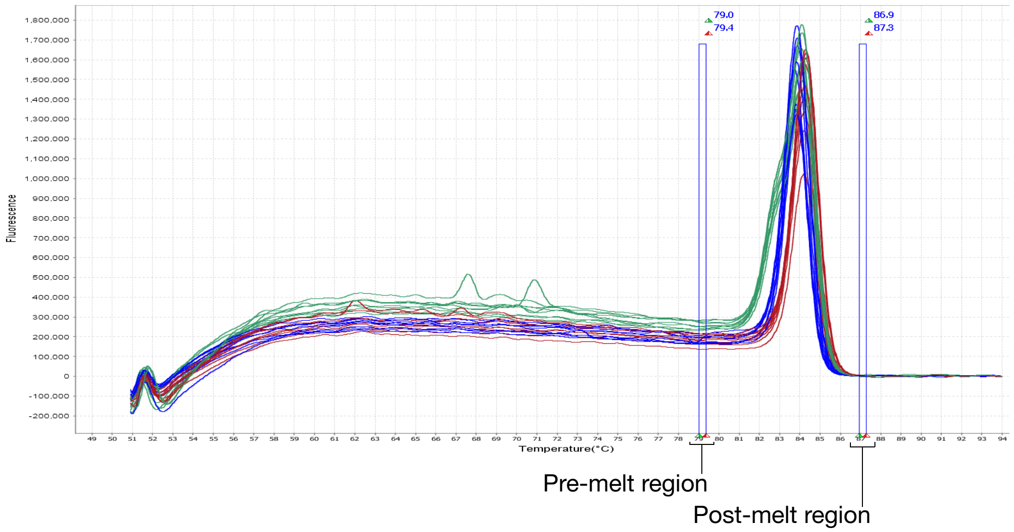
In the Derivative Melt Curves plot and the Raw Melt Curves plot, there are two sets of lines before and after the data peak. These lines define the pre-melt and post-melt regions. The pre- and post-melt regions are used to scale the data in the Aligned Melt Curves and Difference Plot.

- Pre-melt region: The set 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 correspond to 100% fluorescence.
- Active melt region: The data peak indicates the active melt region of the plot. For each sample, the change in fluorescence to the right of the 100% fluorescence point correspond to the true fluorescence change. Data from the active melt region are used to plot the Aligned Melt Curves.

  Note: In methylation studies, the Derivative Melt Curves plot typically displays multiple peaks.
- 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.



Specify the analysis settings

Specify the analysis settings for each assay in the plate:

1. In the navigation pane, select to view the **High Resolution Melt Plots** screen, click **Analysis Settings** (top right corner), then select an assay from the table of assays on the left.

2. Specify the settings for the pre-melt and post-melt regions:
   - Select the checkbox for the software to automatically set the pre- and post-melt regions for each well (default setting).
   - Deselect the checkbox to manually define the pre- and post-melt regions for all assay wells:
     - **Pre-melt region** – Pre-melt stop temperature is next to the start of the melt transition region and pre-melt start temperature is 0.5 to 1 °C lower than the pre-melt stop temperature.
     - **Post-melt region** – Post-melt start temperature next to the end of the melt transition region and post-melt stop temperature 0.5 to 1 °C higher than the pre-melt stop temperature.

3. For the number of variant groups, make sure the checkbox is selected so that the software will automatically determine the number of variant groups (default setting).

   IMPORTANT! If you assigned controls, you need to select the checkbox for the software to automatically set the number of variant groups. The algorithms that assign variant calls according to an entered number of variant groups are incompatible with the algorithms that assign variant calls according to the controls that are assigned. If you assign controls and enter the number of variant groups, you will get unpredictable results.

4. Click **Apply Analysis Settings** to close the analysis settings and reanalyze the experiment.

5. After the reanalysis is complete, save the experiment.

   Note: When you save the HRM experiment the first time, "-HRM" is added to the file name to indicate that the experiment run file is associated with an HRM calibration file and contains HRM data. You cannot open an HRM experiment file using the instrument software. The original experiment run file is maintained. Keep the "-HRM" in the HRM experiment file name to distinguish between the *.eds files.

Manually adjust the pre- and post-melt regions (optional)

Review and adjust the pre- and post-melt regions to optimize the separation and variant calls. For most experiments, set the pre- and post-melt regions as close as possible to the melting transition region.

This procedure describes how to manually adjust the pre- and post-melt regions from the plot.

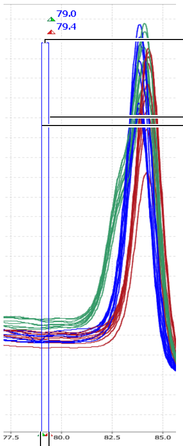
1. In the plot pane, select the **Derivative Melt Curves** tab.

2. Press **Ctrl+A** to select all the wells.

3. Set the pre-melt region:

a. Move the pre-melt stop and start temperature lines together by clicking the horizontal line that joins the lines at the top then dragging the lines so that the pre-melt stop temperature line is next to the start of the melting transition region.

b. If necessary, click and drag the pre-melt start temperature line approximately 0.2 to 0.5 °C from the pre-melt stop temperature line.



Click and drag the top bar to move the start and stop temperatures together

Click and drag one temperature line to move each temperature individually

Set the pre-melt stop next to the start of the melting transition

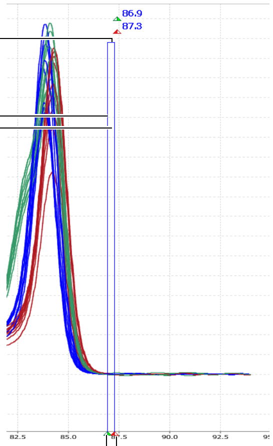
Set the pre-melt start ~0.2 to 0.5 °C from the pre-melt stop

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. Move the post-melt stop and start temperature lines together by clicking the horizontal line that joins the lines at the top then dragging the lines so that the post-melt start temperature line is next to the end of the melting transition region.

b. If necessary, click and drag the post-melt stop temperature line approximately 0.5 to 1 °C from the post-melt start temperature line.



Click and drag the top bar to move the start and stop temperatures together

Click and drag one temperature line to move each temperature individually

Set the post-melt start next to the end of the melting transition

Set the post-melt stop ~0.5 to 1 °C from the post-melt start

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

---

5. Click **Analyze**, then save the changes.

   The software reanalyzes the data using the new pre- and post-melt regions. In all of the high resolution melt plots, the color of the melt curves changes to reflect the new results.

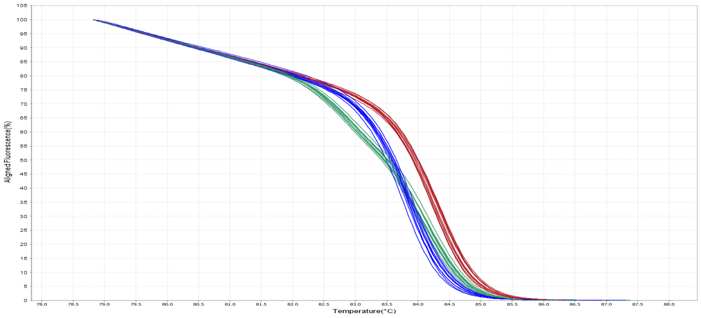
Review the populations in the Aligned Melt Curves plot

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

1. In the navigation pane, select to view the **High Resolution Melt Plots** screen, then select the **Aligned Melt Curves** tab.
2. Review:
   - Variant groups (different colors) – How many different variant groups are displayed? Does this number correspond to the number of variant groups you were expecting?
   - Outliers – Are there any curves within a variant group that do not cluster tightly with the other samples in that group?

Aligned Melt Curves example

In the example experiment, there are 3 distinct variant groups, 1 for each genotype. Notice how the curves differ for the variants: The green curves have a different shape, and the red and blue curves have a similar shape but different Tm.



Review the Difference Plot for outliers

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

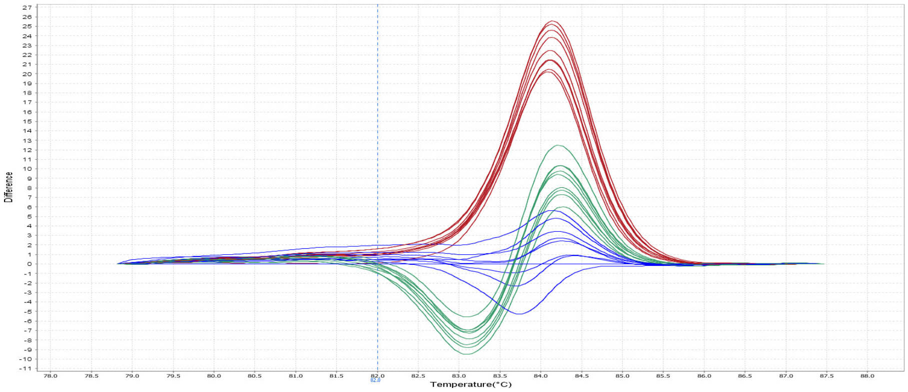
The Difference Plot allows you to more easily see small differences between curves and identify outliers.

1. In the High Resolution Melt Plots screen, select the **Difference Plot** tab.
2. From the **Reference** dropdown menu (above the plot), select a control or any well as the reference, then review:
   - Variant groups – How many distinct clusters are displayed?
   - Outliers – How tight are the curves within each variant group?

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

Difference Plot example

In the example experiment, there are 3 distinct variant groups, 1 for each genotype. The wild type control (heterozygote) is selected as the reference (blue curves):



Review the software calls

The HRM Software automatically makes a call for each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. In the Results pane, click the **Well** column header to sort the results according to the well position.
2. For the positive controls, review:
   - Variant Call column – Do all of the positive control replicates have the correct call?
   - Silhouette Score column – Are the silhouette scores close to 1.0 (0.8 to 1.0)?

   Note: For wells with low silhouette scores (below 0.8), review the data.
3. For each replicate group, review:
   - Variant Call column – Do all replicates have the same call?
   - Silhouette Score column – Are the silhouette scores close to 1.0 (0.8 to 1.0)?

   Note: For wells with low silhouette scores (below 0.8), review the data.
4. To view the fluorescence data for certain wells, select the rows in the Results table.
5. In the Results pane, click the **Variant Call** column header to sort the results according to the variant call. For each variant call, review the samples that were assigned that call.

Omit outliers from analysis

After you review the data, omit outliers from the analysis. You can omit outliers in a high resolution melt plot, the plate layout, or the well table.

Note: Try omitting outliers from the Difference Plot, where the variance is easily visualized.

1. Select the outliers, then omit the wells:
   - **High resolution melt plot** – Click and drag to draw a box around the outliers, right-click in the box, then select **Omit Wells**.
   - **Plate layout** – Right-click the wells, then select **Omit**.
   - **Well table** – Select one or more wells in the table, right-click, then select **Omit**.
2. Click **Analyze** to omit data from the selected wells and reanalyze the remaining data.

Change calls made by the software

If you do not agree with the call automatically made by the software (Auto call), you can manually change the call in a high resolution melt plot or the plate layout.

1. Select samples, then manually change the call:

Location	Procedure
High resolution melt plot	1. Click and drag to draw a box around the samples, right-click in the box, then select **Manual Call**. 2. To change the call to: • A call that has already been made for other samples: Select the appropriate call from the dropdown menu. • A new call: Select **New**, enter a name for the new call, then select a color.
Plate layout	1. Double-click one well. 2. To change the call to: • A call that has already been made for other samples: Select the appropriate call from the dropdown menu. • A new call: Select **New custom call**, enter a name for the new call, then select a color.

For each sample that received a manual call, *Manual Call* appears in the Comments column.

2. Click **Analyze** to reanalyze the data using your manual calls.

Revert selected manual calls to the software Auto call

If you want to remove a manual call for selected samples, you can revert the manual call to the call automatically made by the software (Auto call).

1. Select samples, then revert the manual call:

Pane	Procedure
High resolution melt plot	Click and drag to draw a box around the samples, right-click in the box, then select **Auto Call**.
Plate layout	Double-click one well, then select **Auto call** from the dropdown menu.

2. Click **Analyze** to reanalyze the data using the software Auto call for the selected samples.

Revert all manual calls for an assay

If you want to remove all manual calls for all samples in an assay, you can revert all manual calls to the call automatically made by the software.

1. In an Analysis screen, click **Analysis Settings** (top right corner).
2. Select an assay from the table of assays on the left.
3. Select the **Remove manual calls upon reanalysis** checkbox, then click **Apply Analysis Settings**.

The software reanalyzes the data for all samples in the selected assay.

3

Perform an HRM Genotyping Experiment

Perform an HRM genotyping experiment to determine the genotype of a DNA sample.

Perform an HRM Genotyping Experiment

Design the HRM experiment (below)
↓
Prepare the HRM reactions (page 38)
↓
Amplify and melt the DNA (page 40)
↓
Review the high-resolution melting data (page 43)

Design the HRM experiment

Design and order the primers

Using Primer Express® Software v3.0 or later, design the primers to amplify the genomic DNA that contains the single nucleotide polymorphism (SNP) of interest. Order the primers from the Applied Biosystems Store.

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

Design attribute	Design guidelines
Amplicon	• Length is less than 250 basepairs • Contains only 1 SNP
Primer length	~20 bases each
Tm	58 °C to 60 °C (Optimal Tm is 59 °C)
% GC content	30–80% GC content in each primer
3′ end	No more than 2 G+C residues in the last 5 nucleotides at the 3′ end
Repeating oligonucleotides	Avoid consecutive identical nucleotides. If you are unable to avoid consecutive identical nucleotides, make sure that each primer contains fewer than 4 consecutive Gs.

2. Go to **www.appliedbiosystems.com**, then log into the Applied Biosystems Store if you have an account; register if you are a new user. For more instructions, see "Order custom primers" on page 92.

Plan to use controls

Include controls for each SNP sequence in your HRM genotyping experiment:

- At least one negative control
- At least one positive control to represent each expected genotype

  Run 3 to 5 replicates for each expected genotype 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.

Prepare the HRM reactions

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

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

Required materials

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

Prepare the HRM reactions

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

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

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

Components	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL
Deionized water	7.6 µL	25.08 µL
Total reaction volume	**20.0 µL**	**66.00 µL**

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

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

Components	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL
Genomic DNA (20 ng/µL)	1.0 µL	3.30 µL
Deionized water	6.6 µL	21.78 µL
Total reaction volume	**20 µL**	**66 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume 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 each reaction replicate to 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.

Amplify and melt the DNA

After you have prepared the reaction plate, run the plate to amplify and melt the DNA and to generate HRM fluorescence data. This chapter contains brief instructions. For detailed instructions, see page 21.

Create and set up a new experiment file for the HRM run

Using the real-time PCR instrument software, open and set up the HRM experiment run file:

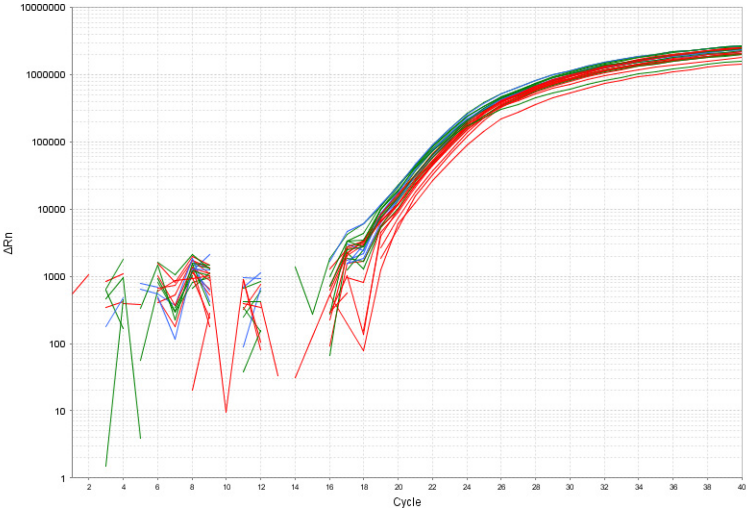
Setup	Setting
Experiment properties	• Experiment type: **Quantitation - Standard Curve** • Reagents: **Other**, then select the **Include Melt Curve** checkbox • Ramp speed: **Standard (~ 2 hours to complete a run)**
Target properties	• Reporter: **MeltDoctor** • Quencher: **None**
Plate layout	• Task for negative control wells: N • Passive Reference: **None**
Run method	• Reaction Volume Per Well: **20** µL • Ramp mode and rate (StepOne™ and StepOnePlus™ systems): Select **Continuous**, then set the ramp rate to 0.3% • Expert Mode (7500 systems): Select the checkbox • (7500 systems) Click **Select/View Filters**, then select only **Filter-1**

Run the plate

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	Denature	95 °C	10 sec
	Anneal	60 °C	1 min
	High resolution melting	95 °C	15 sec
	Anneal	60 °C	15 sec

Verify that the samples amplified and review the Tm peaks

1. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence



Note: Note which wells are outliers with C_T values that differ from replicates by more than 2. The outliers may produce erroneous HRM results.

Note: If the Amplification Plot looks abnormal, refer to "Troubleshooting HRM Experiments" on page 79 to identify and resolve the problem.

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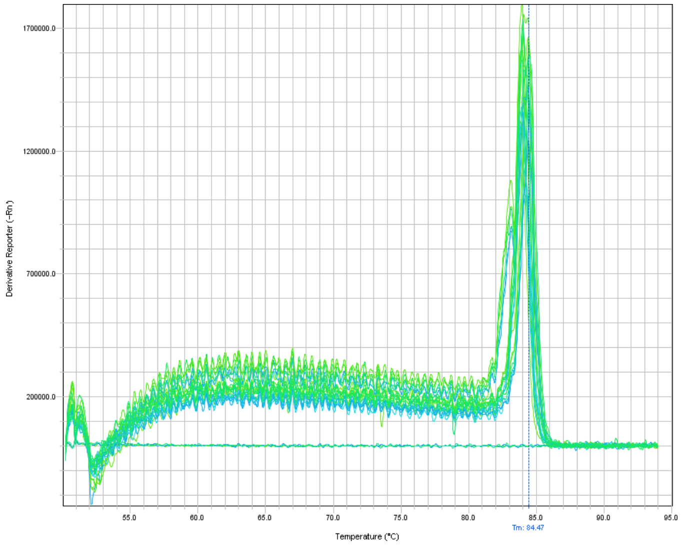
2. Verify that the Melt Curve shows no unexpected Tm peaks:

   If the sequence you amplified contains more than 1 SNP or a more complex mutation, you may see more than 1 Tm peak because of the large differences between the different sequence types.

   Note: Unexpected peaks at low temperatures may indicate contamination, primer dimers, or non-specific amplification.

   Note: The data may appear noisy because more data is collected during a high resolution melt curve than during a standard melt curve. The extra data are required for analysis with the HRM Software.



Review the high-resolution melting data

After you create, run, analyze, and save the *.eds experiment file on your real-time PCR system, use the High Resolution Melt Software v3.0 (HRM Software) to perform high resolution melting analysis of the data and make genotype calls.

Example HRM experiments

Example HRM experiment files are located in *X*:\Applied Biosystems\HRM\Examples, where *X* is the drive where you installed the HRM Software.

- Genotyping Example-HRM.eds
- Class 4 SNP 7500 Fast Example-HRM.eds
- Class 4 SNP StepOnePlus Example -HRM.eds

To follow the example HRM genotyping experiment used in this chapter, use the Genotyping Example-HRM.eds file.

Note: The example files installed with the software are HRM experiment files and already have the appropriate HRM calibration files assigned to them.

Open and set up the HRM experiment

For more detailed instructions on how to open and set up an HRM experiment, see pages 26 through 30.

1. Open the *.eds experiment file that was run on your real-time PCR system.

   Note: If there is no default HRM calibration file selected for the instrument type, browse to and select the default HRM calibration file. To change the HRM calibration file for a selected experiment or for all subsequent HRM experiments, see page 95.

   IMPORTANT! Use the HRM calibration file name to make sure that the HRM calibration file that is assigned to the HRM experiment is correct. The HRM calibration file must be:

   - Run on the same instrument system (the same instrument, the same block type, and the same software version) as the run file
   - Run using the same reagents (HRM dye and master mix) as the reactions in your HRM experiment plate
   - Run using the same run conditions (the same ramp mode and ramp increment) as the run file.

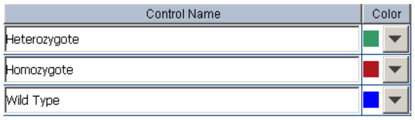
   The HRM Software cannot distinguish between all calibration files. For example, it cannot distinguish between two different 7500 v2.0 instruments or between a StepOnePlus system and a StepOne system.

2. Set up and assign the controls (optional, only if you do not manually define the number of variant groups):

   IMPORTANT! For hard-to-detect SNPs, you need to either assign controls to the experiment or manually enter the number of variant groups in the analysis settings.

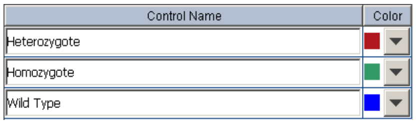
   a. Add controls to the experiment in the Controls section of the Define screen.

      - Controls in Genotyping Example-HRM.eds:



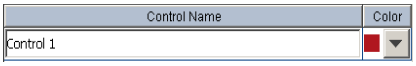
Control Name	Color
Heterozygote	🟩
Homozygote	🟥
Wild Type	🟦

      - Controls in Class 4 SNP StepOnePlus Example -HRM.eds:



Control Name	Color
Heterozygote	🟥
Homozygote	🟩
Wild Type	🟦

      - Controls in Class 4 SNP 7500 Fast Example-HRM.eds:



Control Name	Color
Control 1	🟥

      Note: For control names, do not use the convention *variantN*, where *N* is any number (for example, *variant1*, *variant2*, and so on). The HRM Software uses the convention *variantN* when automatically assigning the variant calls.

   b. Assign controls to wells on the Assign screen.

      Note: You can assign only one control to a well. To change the control assigned to a well, remove the existing control assignment first, then make the new assignment.

3. For each assay in the plate, specify the analysis settings:

   a. View an analysis screen, click **Analysis Settings** (top right corner), then select an assay from the table of assays on the left.

b. Specify the settings for the pre-melt and post-melt regions:

- Select the checkbox for the software to automatically set the pre- and post-melt regions for each well (default setting).
- Deselect the checkbox to manually define the pre- and post-melt regions for all assay wells:
  - **Pre-melt region** – Pre-melt stop temperature is next to the start of the melt transition region and pre-melt start temperature is 0.5 to 1 °C lower than the pre-melt stop temperature.
  - **Post-melt region** – Post-melt start temperature next to the end of the melt transition region and post-melt stop temperature 0.5 to 1 °C higher than the pre-melt stop temperature.

c. Specify the settings for the number of variant groups:

- If you defined and assigned controls, make sure the checkbox is selected so that the software will automatically determine the number of variant groups based on the melt curve differences for the selected assay (default setting).
- If you did not define and assign controls, deselect the checkbox, then enter the number of variant groups to call for the selected assay.

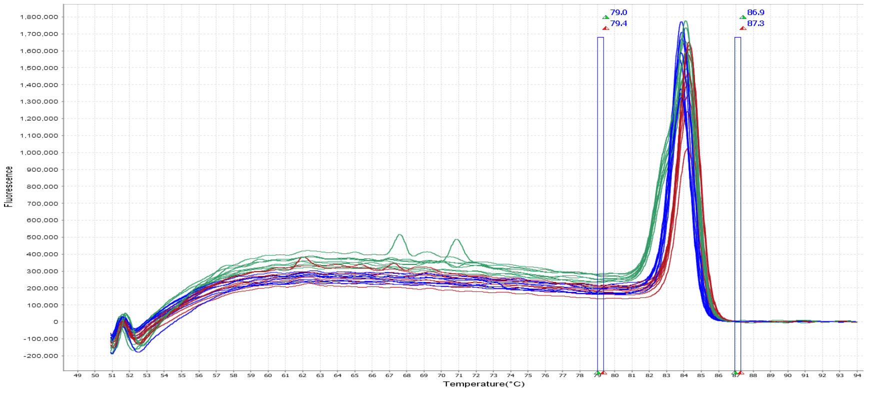
IMPORTANT! The algorithms that assign variant calls according to an entered number of variant groups are incompatible with the algorithms that assign variant calls according to the controls that are assigned. If you assign controls and enter the number of variant groups, you will get unpredictable results.

d. Click **Apply Analysis Settings** to close the analysis settings and reanalyze the experiment.

e. After the reanalysis is complete, save the experiment.

Note: When you save the HRM experiment the first time, "-HRM" is added to the file name to indicate that the experiment run file is associated with an HRM calibration file and contains HRM data. You cannot open an HRM experiment file using the instrument software. The original experiment run file is maintained. Keep the "-HRM" in the HRM experiment file name to distinguish between the *.eds files.

4. (Optional) View the Derivative Melt Curves, set the pre- and post-melt regions as close as possible to the melting transition region, as in the example below, click **Analyze**, then save the changes.



About the melting profiles

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

In genotyping experiments:

- The heterozygotes have a different curve shape compared to the homozygotes. The shape of the melt curve is an indicator of heteroduplex formation.
- The two homozygotes are distinguished from each other based on the difference in Tm values.

Review the populations in the Aligned Melt Curves plot

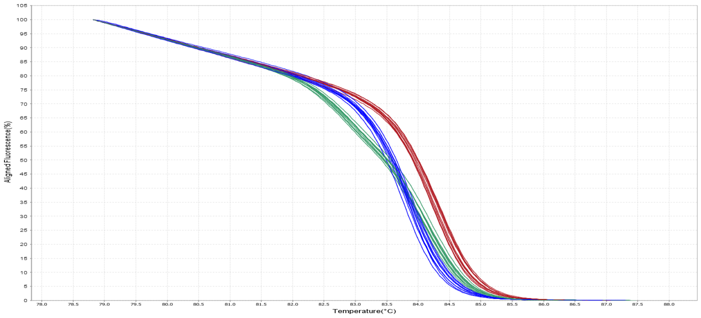
The Aligned Melt Curves plot displays the melt curves as % melt (0–100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions.

1. In the navigation pane, select to view the **High Resolution Melt Plots** screen, then select the **Aligned Melt Curves** tab.

2. Review:
   - Variant groups (different colors) – How many different variant groups are displayed? Does this number correspond to the number of variant groups you were expecting?
   - Outliers – Are there any curves within a variant group that do not cluster tightly with the other samples in that group?

Aligned Melt Curves example

In the example experiment, there are 3 distinct variant groups, 1 for each genotype. Notice how the curves differ for the variants: The green curves have a different shape, and the red and blue curves have a similar shape but different Tm.



Review the Difference Plot for outliers

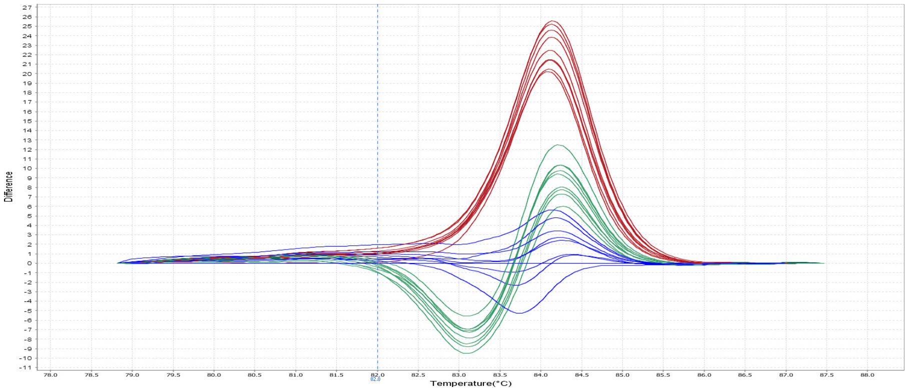
The Difference Plot displays the aligned data as the difference in fluorescence between the melt curve for a reference sample and the other melt curves. You can select a control or any well as a reference. After you select the reference, the software subtracts the reference curve from the other curves.

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

1. In the High Resolution Melt Plots screen, select the **Difference Plot** tab.
2. From the **Reference** dropdown menu (above the plot), select the wild type control as the reference, then review:
   - Variant groups – How many distinct clusters are displayed?
   - Outliers – How tight are the curves within each variant group?

Difference Plot example

In the example experiment, there are 3 distinct variant groups, 1 for each genotype. The wild type control (heterozygote) is selected as the reference (blue curves):



Review the software calls

The HRM Software automatically makes a call for each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. In the Results pane, click the **Well** column header to sort the results according to the well position.
2. For the positive controls, review:
    - Variant Call column – Do all of the positive control replicates have the correct call?
    - Silhouette Score column – Are the silhouette scores close to 1.0 (0.8 to 1.0)?

    Note: For wells with low silhouette scores (below 0.8), review the data.
3. For each replicate group, review:
    - Variant Call column – Do all replicates have the same call?
    - Silhouette Score column – Are the silhouette scores close to 1.0 (0.8 to 1.0)?

    Note: For wells with low silhouette scores (below 0.8), review the data.
4. To view the fluorescence data for certain wells, select the rows in the Results table.
5. In the Results pane, click the **Variant Call** column header to sort the results according to the variant call. For each variant call, review the samples that were assigned that call.

Omit outliers or change calls

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

For detailed instructions, see pages 34–35:

- Omit outliers from analysis
- Change calls made by the software
- Revert selected manual calls to the software Auto call
- Revert all manual calls for an assay

4

Perform an HRM Mutation Scanning Experiment

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

Perform an HRM Mutation Scanning Experiment

Design the HRM experiment (below)
↓
Prepare the HRM reactions (page 50)
↓
Amplify and melt the DNA (page 52)
↓
Review the high-resolution melting data (page 54)
↓
Sequence the variants (page 60)

Design the HRM experiment

Design and order the primers

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

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

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

2. If you want to use M13F and M13R primers in the sequencing reaction, add the appropriate M13 tail to the 5′ end of the primers:
   - M13F (add to the 5′ end of the forward primer):
     TGTAAAACGACGGCCAGT
   - M13R (add to the 5′ end of the reverse primer):
     CAGGAAACAGCTATGACC
3. Go to **www.appliedbiosystems.com**, then log into the Applied Biosystems Store if you have an account; register if you are a new user. For more instructions, see "Order custom primers" on page 92.

Plan to use controls

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

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

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

Prepare the HRM reactions

Required materials

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

Prepare the HRM reactions

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

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

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

Components	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL
Deionized water	7.6 µL	25.08 µL
Total reaction volume	**20.0 µL**	**66.00 µL**

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

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

Components	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL
Genomic DNA (20 ng/µL)	1.0 µL	3.30 µL
Deionized water	6.6 µL	21.78 µL
Total reaction volume	**20.0 µL**	**66.00 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume 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 each reaction replicate to 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.

Amplify and melt the DNA

After you have prepared the reaction plate, run the plate to amplify and melt the DNA and to generate HRM fluorescence data. This chapter contains brief instructions. For detailed instructions, see page 21.

Create and set up a new experiment file for the HRM run

Using the real-time PCR instrument software, open and set up the HRM experiment run file:

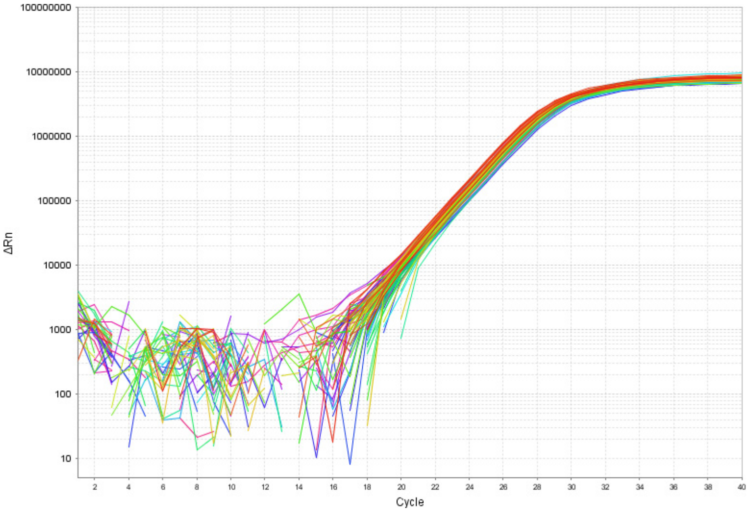
Setup	Setting
Experiment properties	• Experiment type: **Quantitation - Standard Curve** • Reagents: **Other**, then select the **Include Melt Curve** checkbox • Ramp speed: **Standard (~ 2 hours to complete a run)**
Target properties	• Reporter: **MeltDoctor** • Quencher: **None**
Plate layout	• Task for negative control wells: N • Passive Reference: **None**
Run method	• Reaction Volume Per Well: **20** µL • Ramp mode and rate (StepOne™ and StepOnePlus™ systems): Select **Continuous**, then set the ramp rate to 0.3% • Expert Mode (7500 systems): Select the checkbox • (7500 systems) Click **Select/View Filters**, then select only **Filter-1**

Run the plate

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	Denature	95 °C	10 sec
	Anneal	60 °C	1 min
	High resolution melting	95 °C	15 sec
	Anneal	60 °C	15 sec

Verify that the samples amplified and review the Tm peaks

1. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence



Note: Note which wells are outliers with C_T values that differ from replicates by more than 2. The outliers may produce erroneous HRM results.

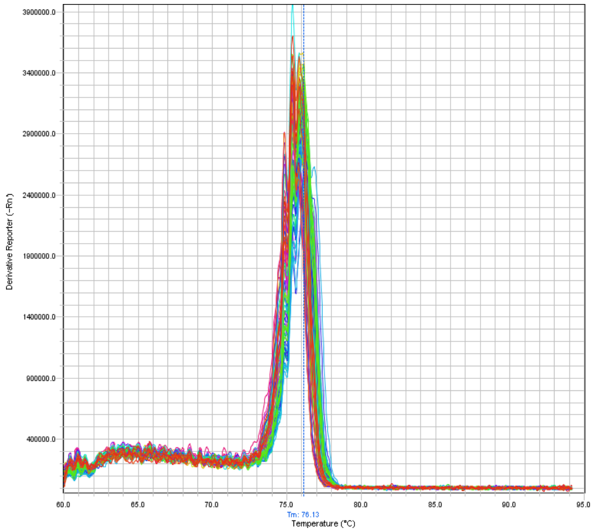
Note: If the Amplification Plot looks abnormal, refer to "Troubleshooting HRM Experiments" on page 79 to identify and resolve the problem.

2. Verify that the Melt Curve shows no unexpected Tm peaks:

   If the sequence you amplified contains more than 1 variant or a more complex mutation, you may see more than 1 Tm peak because of the large differences between the different sequence types.

   Note: Unexpected peaks at low temperatures may indicate contamination, primer dimers, or non-specific amplification.

   Note: The data may appear noisy because more data is collected during a high resolution melt curve than during a standard melt curve. The extra data are required for analysis with the HRM Software.



Review the high-resolution melting data

After you create, run, analyze, and save the *.eds experiment file on your real-time PCR system, use the High Resolution Melt Software v3.0 (HRM Software) to perform high resolution melting analysis of the data and screen the samples for mutations.

Example HRM experiments

Example HRM experiment files are located in *X*:\Applied Biosystems\HRM\Examples, where *X* is the drive where you installed the HRM Software.

To follow the example HRM mutation scanning experiment used in this chapter, use the Mutation Scanning Example-HRM.eds file.

Note: The example files installed with the software are HRM experiment files and already have the appropriate HRM calibration files assigned to them.

Open and set up the HRM experiment

For more detailed instructions on how to open and set up an HRM experiment, see pages 26 through 30.

1. Open the *.eds experiment file that was run on your real-time PCR system.

   Note: If there is no default HRM calibration file selected for the instrument type, browse to and select the default HRM calibration file. To change the HRM calibration file for a selected experiment or for all subsequent HRM experiments, see page 95.

   IMPORTANT! Use the HRM calibration file name to make sure that the HRM calibration file that is assigned to the HRM experiment is correct. The HRM calibration file must be:

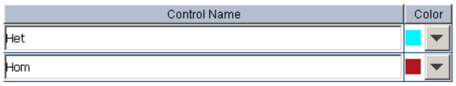
   - Run on the same instrument system (the same instrument, the same block type, and the same software version) as the run file
   - Run using the same reagents (HRM dye and master mix) as the reactions in your HRM experiment plate
   - Run using the same run conditions (the same ramp mode and ramp increment) as the run file.

   The HRM Software cannot distinguish between all calibration files. For example, it cannot distinguish between two different 7500 v2.0 instruments or between a StepOnePlus system and a StepOne system.

2. Set up and assign the controls:

   a. Add controls to the experiment in the Controls section of the Define screen.

   Controls in Mutation Scanning Example-HRM.eds:



Control Name	Color
Het	🟦 ▼
Hom	🟥 ▼

   Note: For control names, do not use the convention *variantN*, where *N* is any number (for example, *variant1*, *variant2*, and so on). The HRM Software uses the convention *variantN* when automatically assigning the variant calls.

   b. Assign controls to wells on the Assign screen.

   Note: You can assign only one control to a well. To change the control assigned to a well, remove the existing control assignment first, then make the new assignment.

3. For each assay in the plate, specify the analysis settings:

   a. View an analysis screen, click **Analysis Settings** (top right corner), then select an assay from the table of assays on the left.

b. Specify the settings for the pre-melt and post-melt regions:
   - Select the checkbox for the software to automatically set the pre- and post-melt regions for each well (default setting).
   - Deselect the checkbox to manually define the pre- and post-melt regions for all assay wells:
   - – **Pre-melt region** – Pre-melt stop temperature is next to the start of the melt transition region and pre-melt start temperature is 0.5 to 1 °C lower than the pre-melt stop temperature.
   - – **Post-melt region** – Post-melt start temperature next to the end of the melt transition region and post-melt stop temperature 0.5 to 1 °C higher than the pre-melt stop temperature.

c. For the number of variant groups, make sure the checkbox is selected so that the software will automatically determine the number of variant groups to call based on the melt curve differences for the selected assay (default setting).

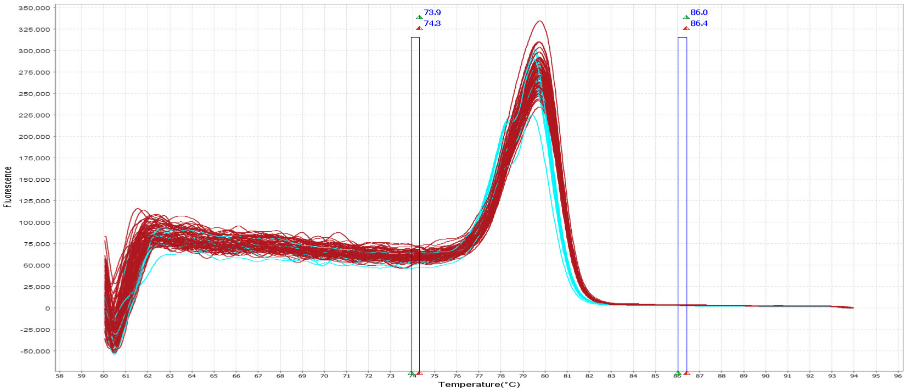
IMPORTANT! If you assigned controls, you need to select the checkbox for the software to automatically set the number of variant groups. The algorithms that assign variant calls according to an entered number of variant groups are incompatible with the algorithms that assign variant calls according to the controls that are assigned. If you assign controls and enter the number of variant groups, you will get unpredictable results.

d. Click **Apply Analysis Settings** to close the analysis settings and reanalyze the experiment.

e. After the reanalysis is complete, save the experiment.

Note: When you save the HRM experiment the first time, "-HRM" is added to the file name to indicate that the experiment run file is associated with an HRM calibration file and contains HRM data. You cannot open an HRM experiment file using the instrument software. The original experiment run file is maintained. Keep the "-HRM" in the HRM experiment file name to distinguish between the *.eds files.

4. (Optional) View the Derivative Melt Curves, set the pre- and post-melt regions as close as possible to the melting transition region, as in the example below, click **Analyze**, then save the changes.



About the melting profiles

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

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

Review the populations in the Aligned Melt Curves plot

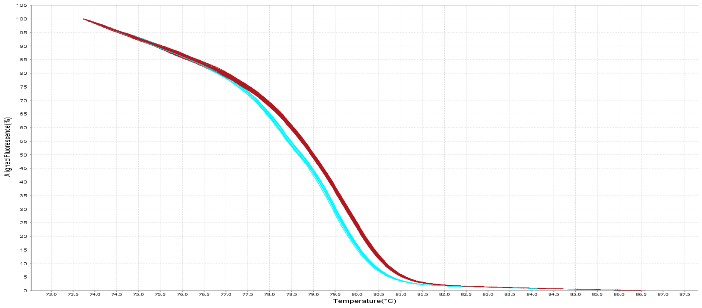
The Aligned Melt Curves plot displays the melt curves as % melt (0–100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions.

1. In the navigation pane, select to view the **High Resolution Melt Plots** screen, then select the **Aligned Melt Curves** tab.

2. Review:
   - Wild type controls – Do the melt curves for the wild type controls cluster well? Are there any outliers?
   - Possible mutations – Are there any samples with melt curves that are different from the wild type melt curves?

Aligned Melt Curves example

In the example experiment, there is 1 distinct variant group for the wild type (Hom) samples. The samples with curves that vary from the wild type form another distinct variant group and may contain mutations.



Review the Difference Plot for outliers

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

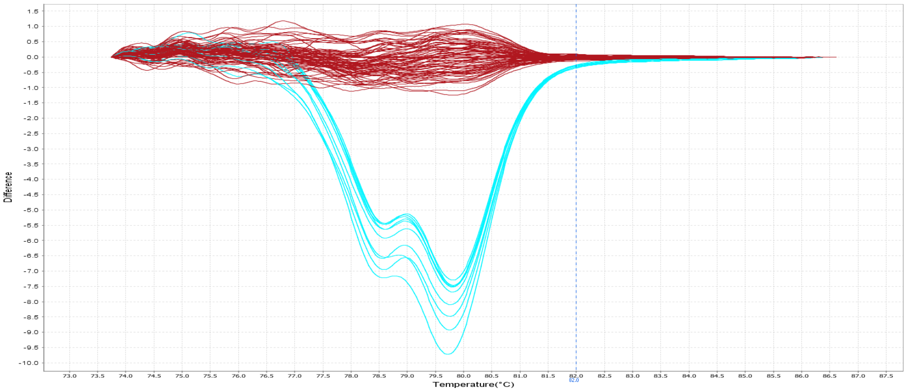
The Difference Plot allows you to more easily see small differences between curves and identify outliers.

1. In the High Resolution Melt Plots screen, select the **Difference Plot** tab.
2. From the **Reference** dropdown menu (above the plot), select a control or any well as the reference, then review:
   - Variant groups – How many distinct clusters are displayed?
   - Outliers – How tight are the curves within each variant group?

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

Difference Plot example

In the example experiment, there are 2 distinct variant groups. The wild type control (homozygote) is selected as the reference (red curves):



Review the software calls

The HRM Software automatically makes a call for each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. In the Results pane, click the **Well** column header to sort the results according to the well position.
2. For each replicate group, review:
   - Variant Call column – Do all replicates have the same call?
   - Silhouette Score column – Are the silhouette scores close to 1.0 (0.8 to 1.0)?

   Note: For wells with low silhouette scores (below 0.8), review the data.
3. To view the fluorescence data for certain wells, select the rows in the Results table.
4. In the Results pane, click the **Variant Call** column header to sort the results according to the variant call. Scan the results for samples that were not assigned the same call as the wild type control.

Omit outliers or change calls

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

For detailed instructions, see pages 34–35:

- Omit outliers from analysis
- Change calls made by the software
- Revert selected manual calls to the software Auto call
- Revert all manual calls for an assay

Sequence the variants

After you identify the variants in the HRM software, dilute or purify the PCR product from the HRM reactions, then sequence the variants.

Dilute the PCR product

1. After the PCR amplification, spin the HRM reaction plate at 100 × g for 1 minute.
2. Perform DNA quantitation of the PCR products for the selected variants, then dilute to 0.5–1.5 ng/µL with water.
3. Use the dilution ratio to determine whether you need to purify the PCR product before performing the sequencing reactions:

How much did you dilute the PCR product?	Next step
<1:20	Purify the PCR product using ExoSAP-IT® (next procedure) before performing the sequencing reactions.
>1:20	Perform the sequencing reactions using the diluted DNA (page 61).

Purify the PCR product

If you diluted the PCR product less than 1:20, purify the PCR product using ExoSAP-IT®.

1. Combine the diluted PCR product and ExoSAP-IT in a clean MicroAmp® Fast Optical Reaction Plate:

Component	Volume
Diluted PCR product	10 µL
ExoSAP-IT®	2 µL
Total reaction volume	**12 µL**

2. Mix the reactions well by pipetting up and down with a multichannel pipettor, then seal the plate with MicroAmp® Clear Adhesive Film.
3. Spin the plate at 1600 × g for 30 seconds.
4. Load the plate in the thermal cycler, cover the plate with a MicroAmp® Optical Film Compression Pad, then run the reactions in a thermal cycler:
   - Reaction volume: 12 µL
   - Thermal profile:

Stage	Temp	Time
1	37 °C	30 min
2	80 °C	15 min
3	4 °C	∞

5. After the run is complete, spin the plate at 100 × g for 1 minute.

Perform the sequencing reactions

Perform fast cycle sequencing with modifications to the protocol for the BigDye® Terminator v1.1 Cycle Sequencing Kit. If your PCR products contain an M13 tail from the primers you used in the HRM amplification reactions, use the M13F and M13R primers for the forward and reverse primers.

1. On ice, prepare 8 µL of Sequencing Master Mix for each sample:

Component	Volume
BigDye® Terminator v1.1	2 µL
Forward primer or reverse primer (3.2 pmol each)	1 µL
Deionized water	4 µL
BigDye® Terminator v1.1, v3.1 5X Sequencing Buffer	1 µL
Total volume per reaction	**8 µL**

Note: Include 5–10% excess volume in the master mix to compensate for pipetting error.

2. Transfer 8 µL of Sequencing Master Mix to wells of a 96-well reaction plate.

3. Add 2 µL of diluted DNA to the appropriate wells of the reaction plate, then pipet up and down to mix.

4. Seal the plate with MicroAmp® Clear Adhesive Film, then spin briefly.

5. Run the reactions in a Veriti™ 96-Well Fast Thermal Cycler:
   - Reaction volume: 10 µL
   - Thermal profile:

Stage	Step	Temp	Time
Holding	Denaturation	96 °C	1 min
Cycle sequencing (25 cycles)	Denaturation	96 °C	10 sec
	Annealing	50 °C	3 sec
	Extension	60 °C	75 sec
Holding	Holding	4 °C	∞

Note: Use a rapid thermal ramp (1 °C/second) for each new temperature.

6. After the run is complete, spin the plate briefly.

Purify the sequencing reaction

Use the BigDye XTerminator® Purification Kit to remove unincorporated BigDye® terminators. For more instructions on the purification or on transferring the plate to the DNA Analyzer, refer to the *BigDye XTerminator® Purification Kit Protocol* (PN 4374408).

1. Spin the reaction plate briefly, then add BigDye XTerminator reagents:
    a. Add 45 µL of SAM™ Solution to each reaction.
    b. Vortex the BigDye XTerminator® Solution thoroughly, then use a wide-bore pipette tip to add 10 µL to each reaction.
2. Seal the plate with MicroAmp® Clear Adhesive Film, then verify that each well is sealed.
3. Vortex the plate for 30 minutes.
4. Spin the plate at 1000 × g for 2 minutes.

Run the sequencing reaction products

Run the sequencing reaction products on an Applied Biosystems 3500/3500*xl* DNA Analyzer, 3730/3730*xl* DNA Analyzer, 3130/3130*xl* Genetic Analyzer, or 3100/3100-*Avant*™ Genetic Analyzer. For more instructions, refer to the user guide for your instrument.

Item	Applied Biosystems 3500/3500*xl* DNA Analyzer with 3500 Data Collection Software v1.0	Applied Biosystems 3730/3730*xl* DNA Analyzers with Data Collection Software v1.1, v2.0, or v3.0	Applied Biosystems 3130/3130*xl* DNA Analyzer with Data Collection Software v2.0	ABI PRISM® 3100/3100-*Avant*™ Genetic Analyzer with Data Collection Software v2.0
Polymer	POP-7™ polymer	POP-7™ polymer	POP-6™ polymer	POP-6™ polymer
Array	50 cm	36 cm	36 cm	36 cm
Run file	RapidSeq_BDX_50_POP7	BDX_RapidSeq36_POP7	BDX_RapidSeq36_POP6	BDX_RapidSeq36_POP6
Mobility file	Kb_3500_POP6_BDV1	KB_3730_POP7_BDTv1.mob	Kb_3130_POP6_BDV1.mob	Kb_3100_POP6_BDV1.mob
Basecaller	KB	KB	KB	KB

5

Perform HRM Methylation Studies

Perform HRM methylation studies to determine the percentage of methylated DNA in unknown samples.

Perform HRM Methylation Studies

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Design the HRM experiment

Design and order the primers

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

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

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

2. Go to **www.appliedbiosystems.com**, then log into the Applied Biosystems Store if you have an account; register if you are a new user. For more instructions, see "Order custom primers" on page 92.

Plan to use controls

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

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

Optimize the HRM reactions

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

1. Prepare the HRM reactions: Test different reaction conditions.

2. Amplify and melt the DNA: Review the C_T values to quantify the efficiency of the PCR reaction.

3. Review the HRM data: Review the specificity of the PCR reaction and the melting behavior of the PCR fragments.

4. Perform electrophoresis of the PCR products on high-percentage agarose gels: Verify the size of the amplicon and review the specificity of the PCR reaction.

Prepare the HRM reactions

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

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

Prepare the methylated DNA standards

Use the Cells-to-CpG™ Methylated and Unmethylated gDNA Control Kit, and mix different ratios of 100% methylated and 0% methylated DNA. For example:

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

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

Treat the samples and methylated DNA standards with bisulfite

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

We recommend that you use one of the Cells-to-CpG™ Bisulfite Conversion Kits. For instructions, refer to the *Cells-to-CpG™ Bisulfite Conversion Kit (2x96) Protocol* (PN 4449006) or the *Cells-to-CpG™ Bisulfite Conversion Kit (50) Protocol* (PN 4448998).

Required materials

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

Prepare the HRM reactions

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

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

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

Components	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL
Deionized water	7.6 µL	25.08 µL
Total reaction volume	**20.0 µL**	**66.00 µL**

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

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

Components	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL
Genomic DNA (20 ng/µL)	1.0 µL	3.30 µL
Deionized water	6.6 µL	21.78 µL
Total reaction volume	**20.0 µL**	**66.00 µL**

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

3. Vortex the reactions to mix, then spin the tubes briefly.

4. Prepare a reaction plate appropriate for your instrument:

   a. Pipet each reaction replicate to the appropriate wells of the optical reaction plate.

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

c. Confirm that the liquid is at the bottom of the wells in the reaction plate.

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

Amplify and melt the DNA

After you have prepared the reaction plate, run the plate to amplify and melt the DNA and to generate HRM fluorescence data. This chapter contains brief instructions. For detailed instructions, see page 21.

Create and set up a new experiment file for the HRM run

Using the real-time PCR instrument software, open and set up the HRM experiment run file:

Setup	Setting
Experiment properties	• Experiment type: **Quantitation - Standard Curve** • Reagents: **Other**, then select the **Include Melt Curve** checkbox • Ramp speed: **Standard (~ 2 hours to complete a run)**
Target properties	• Reporter: **MeltDoctor** • Quencher: **None**
Plate layout	• Task for negative control wells: N • Passive Reference: **None**
Run method	• Reaction Volume Per Well: **20** µL • Ramp mode and rate (StepOne™ and StepOnePlus™ systems): Select **Continuous**, then set the ramp rate to 0.3% • Expert Mode (7500 systems): Select the checkbox • (7500 systems) Click **Select/View Filters**, then select only **Filter-1**

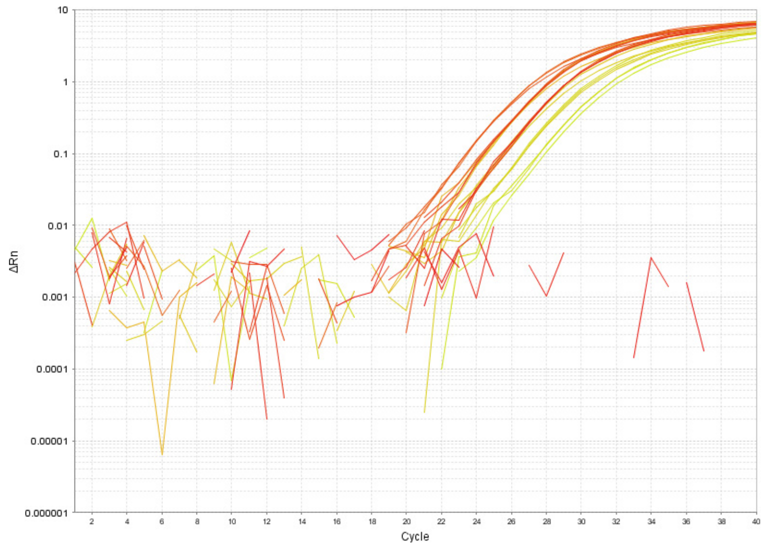
Run the plate

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	Denature	95 °C	10 sec
	Anneal	60 °C	1 min
	High resolution melting	95 °C	15 sec
	Anneal	60 °C	15 sec

Note: For methylation experiments, adjust the annealing temperature during the amplification to increase or decrease the extent of the PCR bias.

Verify that the samples amplified and review the Tm peaks

1. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence



Note: Note which wells are outliers with C_T values that differ from replicates by more than 2. The outliers may produce erroneous HRM results.

Note: If the Amplification Plot looks abnormal, refer to "Troubleshooting HRM Experiments" on page 79 to identify and resolve the problem.

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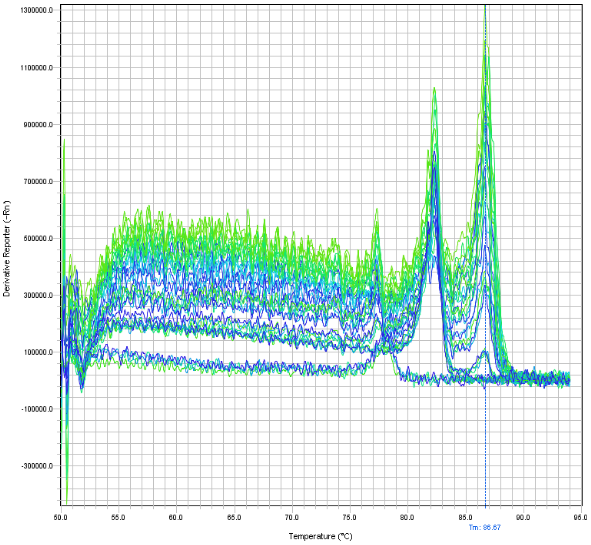
2. Verify that the Melt Curve shows no unexpected Tm peaks:

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

   Note: Unexpected peaks at low temperatures may indicate contamination, primer dimers, or non-specific amplification.

   Note: The data may appear noisy because more data is collected during a high resolution melt curve than during a standard melt curve. The extra data are required for analysis with the HRM Software.



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

Review the high-resolution melting data

After you create, run, analyze, and save the *.eds experiment file on your real-time PCR system, use the High Resolution Melt Software v3.0 (HRM Software) to perform high resolution melting analysis of the data and review the methylation data.

Example HRM experiments

Example HRM experiment files are located in *X*:\Applied Biosystems\HRM\Examples, where *X* is the drive where you installed the HRM Software.

To follow the example HRM methylation study used in this chapter, use the Low Percent Methylation Titration-HRM.eds file.

Note: The example files installed with the software are HRM experiment files and already have the appropriate HRM calibration files assigned to them.

Open and set up the HRM experiment

For more detailed instructions on how to open and set up an HRM experiment, see pages 26 through 30.

1. Open the *.eds experiment file that was run on your real-time PCR system.

   Note: If there is no default HRM calibration file selected for the instrument type, browse to and select the default HRM calibration file. To change the HRM calibration file for a selected experiment or for all subsequent HRM experiments, see page 95.

   IMPORTANT! Use the HRM calibration file name to make sure that the HRM calibration file that is assigned to the HRM experiment is correct. The HRM calibration file must be:

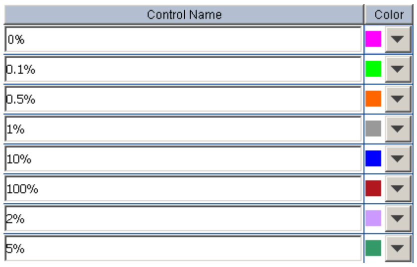
   - Run on the same instrument system (the same instrument, the same block type, and the same software version) as the run file
   - Run using the same reagents (HRM dye and master mix) as the reactions in your HRM experiment plate
   - Run using the same run conditions (the same ramp mode and ramp increment) as the run file.

   The HRM Software cannot distinguish between all calibration files. For example, it cannot distinguish between two different 7500 v2.0 instruments or between a StepOnePlus system and a StepOne system.

2. Set up and assign the controls:

   a. Add controls to the experiment in the Controls section of the Define screen.

   Controls in Low Percent Methylation Titration-HRM.eds:



Control Name	Color
0%	🟪
0.1%	🟩
0.5%	🟧
1%	◼
10%	🟦
100%	🟥
2%	🟪
5%	🟩

   Note: Add a control for each known methylation percentage.

   Note: For control names, do not use the convention *variantN*, where *N* is any number (for example, *variant1*, *variant2*, and so on). The HRM Software uses the convention *variantN* when automatically assigning the variant calls.

   b. Assign controls to wells on the Assign screen.

   Note: You can assign only one control to a well. To change the control assigned to a well, remove the existing control assignment first, then make the new assignment.

3. For each assay in the plate, specify the analysis settings:

   a. View an analysis screen, click **Analysis Settings** (top right corner), then select an assay from the table of assays on the left.

   b. Specify the settings for the pre-melt and post-melt regions:

      - Select the checkbox for the software to automatically set the pre- and post-melt regions for each well (default setting).
      - Deselect the checkbox to manually define the pre- and post-melt regions for all assay wells:
      - **Pre-melt region** – Pre-melt stop temperature is next to the start of the melt transition region and pre-melt start temperature is 0.5 to 1 °C lower than the pre-melt stop temperature.
      - **Post-melt region** – Post-melt start temperature next to the end of the melt transition region and post-melt stop temperature 0.5 to 1 °C higher than the pre-melt stop temperature.

c. For the number of variant groups, make sure the checkbox is selected so that the software will automatically determine the number of variant groups to call based on the melt curve differences for the selected assay (default setting).

IMPORTANT! If you assigned controls, you need to select the checkbox for the software to automatically set the number of variant groups. The algorithms that assign variant calls according to an entered number of variant groups are incompatible with the algorithms that assign variant calls according to the controls that are assigned. If you assign controls and enter the number of variant groups, you will get unpredictable results.

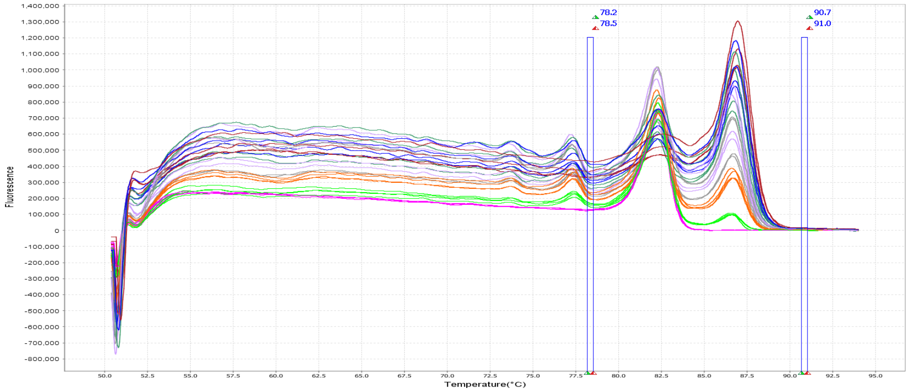
---

d. Click **Apply Analysis Settings** to close the analysis settings and reanalyze the experiment.

e. After the reanalysis is complete, save the experiment.

Note: When you save the HRM experiment the first time, "-HRM" is added to the file name to indicate that the experiment run file is associated with an HRM calibration file and contains HRM data. You cannot open an HRM experiment file using the instrument software. The original experiment run file is maintained. Keep the "-HRM" in the HRM experiment file name to distinguish between the *.eds files.

4. (Optional) View the Derivative Melt Curves, set the pre- and post-melt regions as close as possible to the melting transition region, as in the example below, click **Analyze**, then save the changes.



About the melting profiles

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

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

Review the populations in the Aligned Melt Curves plot

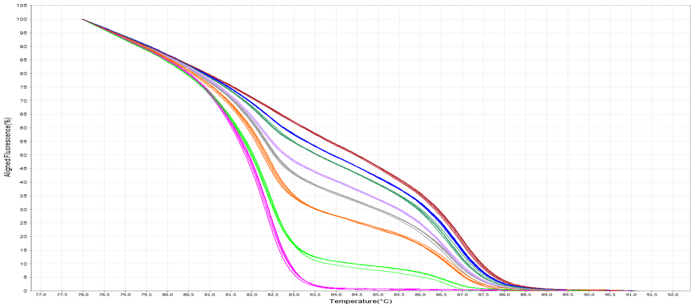
The Aligned Melt Curves plot displays the melt curves as % melt (0–100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions.

1. In the navigation pane, select to view the **High Resolution Melt Plots** screen, then select the **Aligned Melt Curves** tab.

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

Aligned Melt Curves example

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



Review the Difference Plot for outliers

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

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

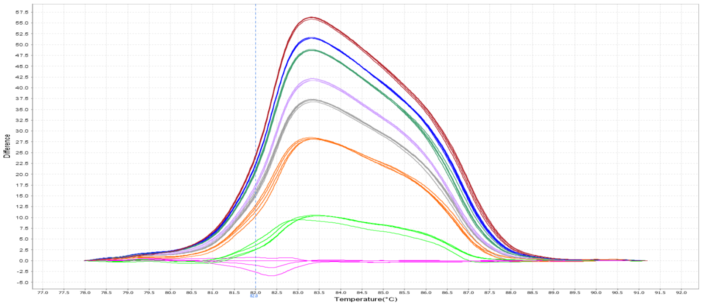
1. In the High Resolution Melt Plots screen, select the **Difference Plot** tab.

2. From the **Reference** dropdown menu (above the plot), select a control or any well as the reference, then review:
    - Variant groups – How many distinct clusters are displayed?
    - Outliers – How tight are the curves within each variant group?

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

Difference Plot example

In the example experiment, there are 8 distinct variant groups, 1 for each methylation standard. The 0% methylation standard is selected as the reference (pink curves):



Review the software calls

The HRM Software automatically makes a call for each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. In the Results pane, click the **Well** column header to sort the results according to the well position.
2. For the methylation standard controls, review:
   - Variant Call column – Do all of the methylation standard controls have the correct call?
   - Silhouette Score column – Are the silhouette scores close to 1.0 (0.8 to 1.0)?

   Note: For wells with low silhouette scores (below 0.8), review the data.
3. To view the fluorescence data for certain wells, select the rows in the Results table.

Omit outliers or change calls

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

For detailed instructions, see pages 34–35:

- Omit outliers from analysis
- Change calls made by the software
- Revert selected manual calls to the software Auto call
- Revert all manual calls for an assay

Sequence the variants

After you identify the samples that contain methylated C residues in the amplified region, dilute or purify the PCR product from the HRM reactions, then sequence the variants. Because the samples were treated with bisulfite before the HRM reactions, the U residues in the sequencing results correspond to methylated C residues.

Dilute the PCR product

1. After the PCR amplification, spin the HRM reaction plate at 100 × g for 1 minute.
2. Perform DNA quantitation of the PCR products for the selected variants, then dilute to 0.5–1.5 ng/µL with water.
3. Use the dilution ratio to determine whether you need to purify the PCR product before performing the sequencing reactions:

How much did you dilute the PCR product?	Next step
<1:20	Purify the PCR product using ExoSAP-IT® (next procedure) before performing the sequencing reactions.
>1:20	Perform the sequencing reactions using the diluted DNA (page 76).

Purify the PCR product

If you diluted the PCR product less than 1:20, purify the PCR product using ExoSAP-IT®.

1. Combine the diluted PCR product and ExoSAP-IT in a clean MicroAmp® Fast Optical Reaction Plate:

Component	Volume
Diluted PCR product	10 µL
ExoSAP-IT®	2 µL
Total reaction volume	**12 µL**

2. Mix the reactions well by pipetting up and down with a multichannel pipettor, then seal the plate with MicroAmp® Clear Adhesive Film.
3. Spin the plate at 1600 × g for 30 seconds.

4. Load the plate in the thermal cycler, cover the plate with a MicroAmp® Optical Film Compression Pad, then run the reactions in a thermal cycler:
   - Reaction volume: 12 µL
   - Thermal profile:

Stage	Temp	Time
1	37 °C	30 min
2	80 °C	15 min
3	4 °C	∞

5. After the run is complete, spin the plate at 100 × g for 1 minute.

Perform the sequencing reactions

Perform fast cycle sequencing with modifications to the protocol for the BigDye® Terminator v1.1 Cycle Sequencing Kit. If your PCR products contain an M13 tail from the primers you used in the HRM amplification reactions, use the M13F and M13R primers for the forward and reverse primers.

1. On ice, prepare 8 µL of Sequencing Master Mix for each sample:

Component	Volume
BigDye® Terminator v1.1	2 µL
Forward primer or reverse primer (3.2 pmol each)	1 µL
Deionized water	4 µL
BigDye® Terminator v1.1, v3.1 5X Sequencing Buffer	1 µL
Total volume per reaction	**8 µL**

Note: Include 5–10% excess volume in the master mix to compensate for pipetting error.

2. Transfer 8 µL of Sequencing Master Mix to wells of a 96-well reaction plate.

3. Add 2 µL of diluted DNA to the appropriate wells of the reaction plate, then pipet up and down to mix.

4. Seal the plate with MicroAmp® Clear Adhesive Film, then spin briefly.

5. Run the reactions in a Veriti™ 96-Well Fast Thermal Cycler:
    - Reaction volume: 10 µL
    - Thermal profile:

Stage	Step	Temp	Time
Holding	Denaturation	96 °C	1 min
Cycle sequencing (25 cycles)	Denaturation	96 °C	10 sec
	Annealing	50 °C	3 sec
	Extension	60 °C	75 sec
Holding	Holding	4 °C	∞

Note: Use a rapid thermal ramp (1 °C/second) for each new temperature.

6. After the run is complete, spin the plate briefly.

Purify the sequencing reaction

Use the BigDye XTerminator® Purification Kit to remove unincorporated BigDye® terminators. For more instructions on the purification or on transferring the plate to the DNA Analyzer, refer to the *BigDye XTerminator® Purification Kit Protocol* (PN 4374408).

1. Spin the reaction plate briefly, then add BigDye XTerminator reagents:
    a. Add 45 µL of SAM™ Solution to each reaction.
    b. Vortex the BigDye XTerminator® Solution thoroughly, then use a wide-bore pipette tip to add 10 µL to each reaction.
2. Seal the plate with MicroAmp® Clear Adhesive Film, then verify that each well is sealed.
3. Vortex the plate for 30 minutes.
4. Spin the plate at 1000 × g for 2 minutes.

Run the sequencing reaction products

Run the sequencing reaction products on an Applied Biosystems 3500/3500*xl* DNA Analyzer, 3730/3730*xl* DNA Analyzer, 3130/3130*xl* Genetic Analyzer, or 3100/3100-*Avant*™ Genetic Analyzer. For more instructions, refer to the user guide for your instrument.

Item	Applied Biosystems 3500/3500*xl* DNA Analyzer with 3500 Data Collection Software v1.0	Applied Biosystems 3130/3130*xl* DNA Analyzer with Data Collection Software v2.0	ABI PRISM® 3100/3100-*Avant*™ Genetic Analyzer with Data Collection Software v2.0
Polymer	POP-6™ polymer	POP-4™ polymer	POP-4™ polymer
Array	50 cm	36 cm	36 cm
Run file	StsSeq_BDX_50_POP6	BDX_RapidSeq36_POP4	BDX_RapidSeq36_POP4
Mobility file	Kb_3500_POP6_BDV1	Kb_3130_POP4_BDV1.mob	Kb_3100_POP4_BDV1.mob
Basecaller	KB	KB	KB

6

Troubleshooting HRM Experiments

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

Observation	Page
Abnormal amplification plots	
Late amplification: C_T value >30 for a majority of samples	80
Some late amplification: C_T value >30 for some samples	80
PCR inhibition: Amplification curve with low slope and C_T values higher than expected	81
Nonspecific amplification: Decreased PCR efficiency and multiple amplicons	82
Abnormal HRM curves	
Replicates are widely spread: Sample replicates show a wide spread in HRM curves	83
Multiple melt regions: Complex melt curves with multiple melting regions	83
More than three different variant calls (HRM genotyping experiments only)	84
Messy HRM curves: Diagonal wavy curves below heterozygous clusters	84

For more guidance on troubleshooting, refer to:

- Applied Biosystems Real-Time PCR Troubleshooting Tool: **www.appliedbiosystems.com/troubleshoot**
- *Applied Biosystems 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.

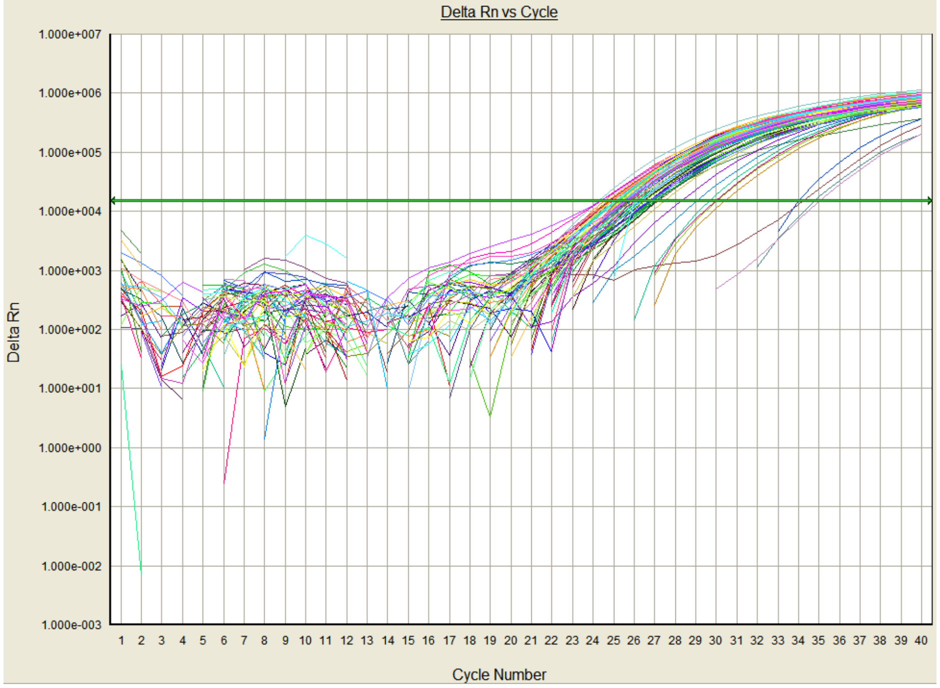
Some late amplification: C_T value >30 for some samples

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 81 to troubleshoot PCR inhibition in your HRM reactions.

PCR inhibition: Amplification curve with low slope and C_T 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 Reagents (see page 93 for reaction component volumes). 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 Reagents (see page 93 for reaction component volumes). You can add up to 0.5 µM of each primer to each reaction.
Amplicon is greater than 200 bp.	Increase the extension times during the amplification reaction.
Primers are amplifying multiple targets.	Perform a BLAST search to ensure primer specificity. If the primers are not specific, design new primers.
	Reduce the number of amplification cycles.

Nonspecific amplification: Decreased PCR efficiency and multiple amplicons

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

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

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

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

Possible causes	Recommended action
Population spread	Use multiple controls for HRM analysis to help you define the population spread.
Incorrect salt concentration.	Perform a $MgCl_2$ titration to find the optimal salt concentration for each reaction. **Note:** For some nucleic acid extraction kits, the elution buffer contains high salt concentrations.
DNA starting concentrations vary widely between samples.	Make sure that the starting DNA concentrations are similar for the samples that you are testing.
Low PCR efficiencies.	Ensure efficient PCR.

Multiple melt regions: Complex melt curves with multiple melting regions

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

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

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

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

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

Messy HRM curves: Diagonal wavy curves below heterozygous clusters

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

Possible cause	Recommended action
Negative controls and unamplified samples are included in the HRM analysis.	• Assign negative controls to the experiment in the instrument software before you open them with the HRM Software. or • Omit negative controls and unamplified samples from the HRM analysis. Refer to the *High Resolution Melt Software Help System*.

Ordering Information

Materials and equipment for HRM calibration and HRM experiments

MeltDoctor™ HRM reagents

Item	Applied Biosystems Part Number
MeltDoctor™ HRM Calibration Plate, Fast 96-Well	4425618
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), 500 µL	4410126
MeltDoctor™ HRM Reagents: • AmpliTaq Gold® 360 DNA Polymerase • AmpliTaq Gold® 360 Buffer • 360 GC Enhancer • GeneAmp® dNTP Blend • MeltDoctor™ HRM Dye (20X)	4425557

Equipment and software

Item	Source
StepOne™ Real-Time PCR System: • StepOne™ Real-Time PCR System: • StepOne™ Real-Time PCR System with Tower • StepOne™ Real-Time PCR System with Laptop	Applied Biosystems • PN 4376357 • PN 4376374 • PN 4376373
StepOnePlus™ Real-Time PCR System: • StepOnePlus™ Real-Time PCR System • StepOnePlus™ Real-Time PCR System with Tower • StepOnePlus™ Real-Time PCR System with Laptop	Applied Biosystems • PN 4376600 • PN 4376599 • PN 4376598
7500 Fast Real-Time PCR System: • 7500 Fast Real-Time PCR System with Notebook Computer • 7500 Fast Real-Time PCR System with Tower Computer	Applied Biosystems • PN 4351106 • PN 4351107

Item	Source
High Resolution Melt Software v3.0: • 1 license • 10 licenses	Applied Biosystems • PN 4461357 • PN 4461456
Primer Express® Software v3.0 or later	Applied Biosystems
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® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL • MicroAmp® Fast Optical 48-Well Reaction Plate	Applied Biosystems • PN 4346906 and 4366932 • PN 4375816
MicroAmp® Optical Adhesive Film: • 25 films • 100 films	Applied Biosystems • PN 4360954 • PN 4311971
MicroAmp® 48-Well Optical Adhesive Film: • 25 films • 100 films	Applied Biosystems • PN 4375928 • PN 4375323
Microcentrifuge tubes	MLS
Pipettors and pipette tips	MLS

Materials for bisulfite conversion

Item	Source
Cells-to-CpG™ Bisulfite Conversion Kit (2x96)	Applied Biosystems PN 4445554
Cells-to-CpG™ Bisulfite Conversion Kit (50)	Applied Biosystems PN 4445555
Cells-to-CpG™ Bisulfite Conversion and Quantitation Control Kit	Applied Biosystems PN 4445553
Cells-to-CpG™ Methylated and Unmethylated gDNA Control Kit	Applied Biosystems PN 4445552

Materials and equipment for sequencing variants after HRM analysis

Equipment

Item	Source
Applied Biosystems DNA sequencer: • Applied Biosystems 3500/3500*xl* DNA Analyzer • Applied Biosystems 3730/3730*xl* DNA Analyzer • Applied Biosystems 3130/3130*xl* DNA Analyzer • Applied Biosystems 3100/3100-*Avant* DNA Analyzer	Applied Biosystems
Veriti® 96-Well Fast Thermal Cycler	Applied Biosystems
Centrifuge with plate adapters	MLS
Microcentrifuge	MLS
Vortexer	MLS

Supplies

Item	Source
MicroAmp® Clear Adhesive Film, 100 films	Applied Biosystems PN 4306311
MicroAmp® Optical Film Compression Pad	Applied Biosystems
Wide-bore (>1 mm) pipette tips: • Wide-Orifice Tips • Clear Wide Bore Tips	• Rainin Instrument LLC • Axygen Scientific Inc.
Microcentrifuge tubes	MLS
Pipettors and pipette tips	MLS

Reagents

For the SDS of any chemical not distributed by Life Technologies, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Item	Source
BigDye® Terminator v1.1 Cycle Sequencing Kit, 100 reactions	Applied Biosystems PN 4337450
BigDye XTerminator® Purification Kit, 2 mL (~100 20-µL reactions)	Applied Biosystems PN 4376486
M13 forward and reverse sequencing primers: • M13 Forward (–20), 2 µg • M13 Reverse, 2 µg **Note:** Use only if the HRM PCR product contains the M13 sequences.	Invitrogen • PN N520-02 • PN N530-02
UltraPure™ DNase/RNase-Free Distilled Water, 500 mL	Invitrogen PN 10977-015
ExoSAP-IT®, 100 reactions	USB Corporation PN 78200
Deionized Water	MLS

B Supplemental Information and Procedures

This appendix contains supplemental information and procedures for preparing and running HRM reactions and for using the High Resolution Melt Software v3.0.

- About HRM dyes 89
- Calibrate your instrument using other HRM dyes 89
- Prepare your own HRM calibration plate 90
- Prepare the DNA templates 91
- Order custom primers 92
- Optimizing the reaction conditions 93
- Computer requirements 94
- Change the HRM calibration file 95
- Guidelines for experiments with multiple HRM assays 96
- Publishing the data 96

About HRM dyes

The melting profile of a PCR product is best obtained with high-resolution melting 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 (Kent *et al.*, 2007).

Calibrate your instrument using other HRM dyes

For each HRM dye that you plan to use in HRM experiments, you need to first calibrate your instrument for that HRM dye. If you choose to use a dye other than the MeltDoctor™ HRM Dye, follow this workflow to calibrate your instrument:

1. Perform a background calibration. Follow the instructions on page 10.
   a. Prepare the background calibration plate.
   b. Run the background calibration plate.
   c. Review the background calibration results.

2. Amplify the DNA in the HRM calibration plate.
   a. Prepare the calibration plate – Prepare your own HRM calibration plate using the MeltDoctor™ HRM Calibration Standard with your dye and your own master mix.
   b. Run your HRM calibration plate to amplify the DNA.
   c. Verify that the HRM calibration samples amplified – Use the instructions on page 13 as guidelines.
3. Calibrate the instrument to use your HRM dye.
   a. Run the HRM calibration plate for the custom dye calibration – Use the instructions on page 13 as guidelines.
   b. Review the custom dye calibration results – Use the instructions on page 14 as guidelines.
4. Perform a melt curve to generate the HRM calibration file.
   a. Run the HRM calibration plate for the HRM calibration.
   b. Verify that the Melt Curve contains only one Tm peak – Use the instructions on page 16 as guidelines.

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

Prepare your own HRM calibration plate

This procedure is for preparing your own 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 amplification run, 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 Reagents 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.

If you are using a different HRM dye, prepare your HRM calibration plate using the MeltDoctor™ HRM Calibration Standard with your dye and your own master mix.

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

Component	**Volume (µL)**		
	1 reaction	**55 reactions**	**110 reactions**
MeltDoctor™ HRM Master Mix	10	550	1100
MeltDoctor™ HRM Calibration Standard (20×)	1	55	110
Deionized water	9	495	990
Total volume	**20**	**1100**	**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.

Prepare the DNA templates

1. Purify all the DNA samples in an HRM experiment using the same method. Watch out for salt carryover because it will subtly change the thermodynamics of the DNA melting transition.

2. Perform agarose gel electrophoresis and spectrophotometry to make sure the DNA template is intact and is not contaminated with other DNAs, RNAs, proteins, or organic chemicals. Proteins and organic chemicals may inhibit the PCR amplification, and contaminating DNAs and RNAs may result in sub-optimal PCR performance or increased change of non-specific amplification.

3. Determine the quantity of DNA using spectrophotometry. If too little DNA template is added to the reaction, the fluorescence signal may not be sufficient for successful HRM analysis. If too much DNA template is added to the reaction, the PCR may be inhibited.

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

Order custom primers

1. Go to **www.appliedbiosystems.com**, then log into the Applied Biosystems Store if you have an account; register if you are a new user.
2. Below the Custom Primers & Probes heading, click **Custom Unlabeled Primers**, then click **Sequence Detection Primers**.
3. In the Ordering Information tab, select the check box next to the quantity of primers to order, then click **Configure**.
4. Follow the instructions on the web page to configure the primers:
   a. Select purification and formulation options.
   b. Enter or upload the primer names and sequences.
   c. Review the oligos to order.

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

Optimizing the reaction conditions

If you want to optimize the reaction conditions, use the MeltDoctor™ HRM Reagents. See Table 1 for the recommended reaction component volumes.

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

Table 1 Recommended reaction component volumes using the MeltDoctor™ HRM Reagents

Components	Volume for one 10-µL reaction	Volume for one 20-µL reaction	Final concentration range
AmpliTaq Gold® 360 Buffer, 10✕	1 µL	2 µL	1✕
25 mM Magnesium Chloride	0.6 to 0.9 µL	1.2 to 1.8 µL	1.5 to 3.5 mM
GeneAmp® dNTP Blend, 10 mM	0.1 to 0.3 µL	0.2 to 0.6 µL	100 to 300 µM each
Primer 1 (5 µM)	0.4 to 1.0 µL	0.8 to 2.0 µL	0.2 to 0.5 µM
Primer 2 (5 µM)	0.4 to 1.0 µL	0.8 to 2.0 µL	0.2 to 0.5 µM
MeltDoctor™ HRM Dye (20✕)	0.5 µL	1.0 µL	1✕
AmpliTaq Gold® 360 DNA Polymerase (5 U/µL)	0.1 to 0.3 µL	0.2 to 0.6 µL	0.05 to 0.15 U/µL
Human gDNA (20 ng/µL)	0.5 µL	1 µL	10 pg/µL to 10 ng/µL
Deionized water	As needed	As needed	–
Total volume	**10 µL**	**20 µL**	–

Computer requirements

The hardware and software requirements for running HRM Software are listed in the table below.

Component	Recommended Requirements	Minimum Requirements‡
Computer	• Intel® Core™ 2 Duo processor or compatible processor, 2.4 GHz • 4 GB of RAM • One hard drive with 60 GB available§ • 20/48X IDE CD-ROM# • USB v2.0 • Ethernet network interface adapter (10BASE-T) • UL listed • CE marked • FCC labeled	• Intel® Pentium® 4 processor or compatible processor, 1.2 GHz • 1 GB of RAM • One hard drive with 10 GB available • 20/48X IDE CD-ROM# • USB v1.1 • Ethernet network interface adapter (10BASE-T) • UL listed • CE marked • FCC labeled
Monitor	• 1280 × 1024 pixel resolution for full screen display • 16-inch color monitor • 32-bit color • UL listed	• 1280 × 1024 pixel resolution for full screen display • 16-inch color monitor • 32-bit color • UL listed
Operating System	Microsoft® Windows® XP Operating System, Service Pack 3 or greater	Microsoft® Windows® XP Operating System, Service Pack 2 or greater

‡ The Minimum Requirements column lists the lowest specifications that permit the installer to install the software. The minimum requirements may not provide optimal performance. Life Technologies does not guarantee support of an installation in this environment.
§ For optimal performance of the software, partition the hard drives on your computer.
# Not necessary if downloading the software from www.appliedbiosystems.com.

Change the HRM calibration file

IMPORTANT! The HRM calibration file must be:

- Run on the same instrument as the run file
- Run in the same reaction plate type as the run file (96-well Fast or 48-well Fast)
- Run with the same software version as the run file
- Run using the same run conditions (the same ramp mode and ramp increment) as the run file

---

View the HRM calibration file

Open the Experiment Properties screen to view the name of the calibration file that is assigned to the HRM experiment file.

Change (overwrite) the HRM calibration file

You can change (overwrite) the HRM calibration file just for the HRM experiment that you are viewing.

1. In the menu bar, select **Analysis** ▸ **Change Calibration File**.
2. Browse to and select the appropriate HRM calibration file (*.eds file), then click **OK**.

Change the default HRM calibration file

This option changes the default HRM calibration file for *all subsequent new* HRM experiments run on a particular instrument.

1. From the menu bar, select **Analysis** ▸ **Select Default Calibration File**.
2. Select the appropriate instrument from the dropdown menu, then browse to and select the appropriate HRM calibration file (*.eds file), then click **OK**.

Note: If you change the default calibration file, it changes the calibration file only for subsequent new HRM experiments for that instrument type. Changing the default calibration file does not change the calibration file for previously analyzed experiments.

Guidelines for experiments with multiple HRM assays

In the HRM Software, an assay is defined as a specific combination of target and dye. You can have multiple assays in the same plate, and the HRM Software analyzes each assay separately, using distinct analysis settings.

If your reaction plate contains multiple HRM assays, make sure that you:

- Assign a different target for each HRM assay in the instrument software before you open the file with the HRM Software. The HRM Software analyzes data for each target separately.
- Select the assay from the Assay dropdown menu before you review the results or make any changes to the HRM experiment file.

Publishing the data

You can publish the data from the HRM Software in several ways:

Icon	Icon location	Action
	Toolbar	Export data.
	Toolbar	Create slides.
🖨 Print Report...	Toolbar	Print a report.
NA	NA	Copy and paste data from the well table to a text or spreadsheet application.
	Plot	Copy and paste the plot to a text, spreadsheet, or slide.
🖨	Plot	Print a plot.
	Plot	Save a plot as an image file.

For information on performing these procedures, click ? **or press F1** in the HRM Software to access the Help system.

C

Software Warranty Information

Computer configuration

Applied Biosystems supplies or recommends certain configurations of computer hardware, software, and peripherals for use with its instrumentation.
Applied Biosystems reserves the right to decline support for or impose extra charges for supporting nonstandard computer configurations or components that have not been supplied or recommended by Applied Biosystems. Applied Biosystems also reserves the right to require that computer hardware and software be restored to the standard configuration prior to providing service or technical support. For systems that have built-in computers or processing units, installing unauthorized hardware or software may void the Warranty or Service Plan.

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Limited warranty

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Warranty period commencement date

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Warranty claims

Warranty claims must be made within the applicable warranty period.

Warranty exceptions

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D

Safety

This appendix covers:

- General chemical safety 101
- SDSs 101
- Biological hazard safety 102

General chemical safety

Chemical hazard warning

⚠ **WARNING! CHEMICAL HAZARD.** Before handling any chemicals, refer to the Safety Data Sheet (SDS) provided by the manufacturer, and observe all relevant precautions.

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About SDSs" on page 101.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

SDSs

About SDSs

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

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

Obtaining SDSs

The SDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain SDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **SDS**.
2. In the Keyword Search field, enter the chemical name, product name, SDS part number, or other information that appears in the SDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

Note: For the SDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Biological hazard safety

General biohazard

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

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **www.cdc.gov/biosafety/publications/index.htm**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

www.cdc.gov

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Documentation and Support

Related documentation

HRM documentation

The following documents about HRM experiments are available:

- *High Resolution Melt Software Help* – Installed with and accessible from within the HRM Software, the Help system describes the software and provides procedures for common tasks.
- Quick reference cards provide abbreviated procedures for performing HRM experiments using MeltDoctor™ HRM Reagents and HRM Software:
  - *HRM Genotyping Quick Reference Card* (PN 4457857)
  - *HRM Mutation Scanning Quick Reference Card* (PN 4457855)
  - *HRM Methylation Studies Quick Reference Card* (PN 4457856)
- *A Guide to High Resolution Melting (HRM) Analysis* (Stock number O-081740) – This document provides background information on HRM analysis to help you perform robust HRM experiments.

Access the HRM Help system

To access the *High Resolution Melt Software Help System* from within the HRM Software, click ? in the software window, select **Help ▸ High Resolution Melt Software Help**, or press **F1**.

7500 Fast, StepOne™, and StepOnePlus™ System documentation

The following related documents are available from Applied Biosystems:

Instrument and software	Document	Part number
7500 Fast System and 7500 Software v2.0 or later	*Applied Biosystems 7500/7500 Fast Real-Time PCR System Maintenance Guide*	4387777
	Applied Biosystems 7500 Software v2.0 Help	NA
StepOne™ and StepOnePlus™ Systems and StepOne Software v2.2 or later	*Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Installation, Networking, and Maintenance Guide*	4376782
	Applied Biosystems StepOne™ Software Help	NA

How to obtain support

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

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, SDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

Glossary

aligned melt curves plot	In HRM experiments, a plot of the aligned fluorescence (0–100%) over temperature (°C). The melt curves have been aligned to the same fluorescence level using the pre- and post-melt regions set in the HRM Settings tab.
amplicon	A segment of DNA amplified during PCR.
Analysis Settings library	In the software, a collection of analysis settings to use in experiments. You can save settings and reuse them. You cannot edit or import settings into the library.
control	In HRM experiments, a sample of known genotype or melt-curve shape. When you assign controls, you can: • Assign multiple controls for each cluster to more effectively define the natural spread or variation that is present within different samples of the same sequence, or replicates of the same genotype. • Assign as many controls as appropriate for the type of HRM experiment that you are performing.
Control Library	In the software, an editable collection of controls to use in HRM experiments. The controls in the library contain the control name and control color and, optionally, comments about the control.
derivative melt curve plot	The default view of the melting curve. It plots the negative derivative data (-Rn′) vs. temperature (°C) for high resolution melt curve experiments.
difference plot	In HRM experiments, a plot of the aligned data as the difference in fluorescence between the reference curve and the other melt (dissociation) curves. After you select the reference curve, the software subtracts the reference curve from the other curves.
dissociation curve	See melt curve.
Dye Library	In the software, a collection of dyes to use in experiments. Custom dyes can be added to the library, but system dyes cannot be removed.
experiment file	An electronic record that contains all information about a particular plate, including metadata (name, barcode, comments), plate setup (well contents, assay definitions), run method (thermal cycling profile), run results, analysis settings, analysis results, and other plate-specific data. Experiment files have the suffixes *.eds (experiment document single), *.edt (template), and *.edm (multiple.)
export	A software feature that allows you to export experiment setup files, experiment results, instrument information, and analysis results to spreadsheet, presentation, or text files. You can edit the default location of the exported file.

forward primer	Oligonucleotide that flanks the 5′ end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.
heterozygote	Samples having both allele 1 and allele 2.
high resolution melting (HRM) experiment	An experiment that generates and analyzes high-resolution melt curves from an HRM reaction.
homozygote	Samples having only allele 1 or only allele 2.
HRM calibration	Type of calibration in which the instrument is calibrated to accommodate MeltDoctor™ HRM Dye or custom dyes for analysis of HRM experiments.
import	A software feature that allows you to import plate setup information before performing an HRM analysis. You can also import information into some libraries in the system.
melt curve	A plot of data collected during the melt curve stage. Peaks in the melt curve can indicate the melting temperature (Tm) of the target, or they can identify nonspecific PCR amplification. In the software, you can view the melt curve as normalized reporter (Rn) vs. temperature or as derivative reporter (-Rn′) vs. temperature. In a high resolution melting experiment, you can view the melt curve as fluorescence vs. temperature. Also called dissociation curve.
melt curve characteristics	The melt curve shape and the difference in melting temperature (Tm) values.
melting temperature (Tm)	The temperature at which 50% of the DNA is double-stranded and 50% of the DNA is dissociated into single-stranded DNA. The Tm is displayed in the melt curve.
melting transition region	In melt curve experiments, the region before and after the melting temperature (Tm).
negative control (NC)	In the software, the task for targets in wells that contain water or buffer instead of sample. No amplification of the target should occur in negative control wells. Negative controls are needed to control as amplification controls, but they are not appropriate for HRM analysis. HRM Software automatically omits wells from the HRM analysis if the wells contain a negative control task. Previously called no template control (NTC).
no template control (NTC)	See negative control (NC).
omit well	An action that you perform before reanalysis to omit one or more wells from analysis. Because no algorithms are applied to omitted wells, omitted wells contain no results. You can add wells back to the analysis; no information is permanently discarded.
outlier	A measurement that deviates significantly from the measurement of the other replicates for that same sample.

passive reference	A dye that produces fluorescence signal independent of PCR amplification, and that is added to each reaction at a constant concentration. Because the passive reference signal should be consistent across all wells, it is used to normalize the reporter dye signal to account for non-PCR related fluorescence fluctuations caused by minor well-to-well differences in volume. Normalization to the passive reference signal generally results in data with noticeably high precision among technical replicates.
plate layout	An illustration of the grid of wells and assigned content in the reaction plate. The number of rows and columns in the grid depends on the sample block that you use. In the software, you can use the plate layout as a selection tool to assign well contents, to view well assignments, and to view results. The plate layout can be printed, included in a report, exported, and saved as a slide for a presentation.
plate setup file	A file (*.txt, *.csv, *.xml, *.sds, or *.sdt) that contains setup information such as the well number, sample name, sample color, target name, dyes, and other reaction plate contents.
positive control	A DNA sample with a known variant call.
primer mix	PCR reaction component that contains the forward primer and reverse primer designed to amplify the target.
Real-time PCR Data Markup Language (RDML)	A reporting format that is compliant with the Minimum Information for Publication for Quantitative Real Time Experiments (MIQE) guidelines (**http://www.rdml.org**).
reference	In an HRM experiment, the melt curve selected by a user in the difference plot to use as a basis for comparison. The software displays the aligned data as the difference in fluorescence between the reference curve and the other melt curves.
reject well	An action that the software performs during analysis to remove one or more wells from further analysis if a specific flag is applied to the well. Rejected wells contain results calculated up to the point of rejection.
replicate group	A user-defined biological grouping. A replicate group may be a set of identical reactions in an experiment.
replicates	Total number of identical reactions containing identical components and volumes.
reporter	A fluorescent dye used to detect amplification. With MeltDoctor™ HRM Reagents, the reporter dye is MeltDoctor™ HRM Dye, a DNA-binding dye.
reverse primer	An oligonucleotide that flanks the 3′ end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.
sample	The template that you are testing for a target gene.
sample definition file	A tab-delimited file (*.txt or *.csv) that contains the following setup information: well number, sample name, and custom sample properties.

Sample Library	In the software, an editable collection of samples to use in experiments. The samples in the library contain the sample name and sample color, and, optionally, comments about the sample.
silhouette score	A measure of the distinguishability of a melt curve relative to other melt curves in the assay (Rousseeuw, 1987). For each melt curve, the HRM Software assigns a modified silhouette score, a value that ranges from 0 to 1. Note that for standard silhouette scores (Lovmar, *et al.*, 2005), the score is assigned to each identified cluster instead of to each data point in the cluster and the value ranges from -1 to 1. In the HRM Software, you can find the score in the Silhouette Score column of the Well Table on an Analysis screen. Silhouette scores close to 1.0 (0.8 to 1.0) are desirable and indicate that a melt curve is more similar to melt curves that were assigned the same variant call than to melt curves that were assigned different variant calls. Lower silhouette scores indicate that the melt curve is less similar to melt curves that were assigned the same variant call.
target	The nucleic acid sequence to amplify and detect.
target color	In the software, a color assigned to a target to identify the target in the plate layout and analysis plots.
Target Library	In the software, an editable collection of targets to use in experiments. Targets in the library contain the target name, reporter, quencher, and target color. The targets in the library may also contain comments about the target.
task	In the software, the type of reaction performed in the well for the target. Available tasks: • Unknown • Negative Control
template	In the software, the type of nucleic acid to add to the PCR reaction.
thermal profile	Part of the run method that specifies the temperature, time, ramp, and data collection points for all steps and stages of the instrument run.
Tm	See melting temperature (Tm).
unknown	In the software, the task for the target in wells that contain the sample being tested. In HRM experiments, the task for the target in wells that contain a sample with an unknown melt curve profile.
variant	In an HRM experiment, a sample (or group of samples) with a unique melt curve (that is, the melt curve is different from the melt curves of other samples or controls used in the experiment). The software determines melt curve differences by the melting temperature (Tm) and the shape of the melt curve.

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