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High-Resolution Melt Experiment

GETTING STARTED GUIDE

for use with:
QuantStudio™ 3 and 5 Real-Time PCR Systems
Applied Biosystems™ MeltDoctor™ HRM Reagents and Controls
Thermo Fisher Cloud
Applied Biosystems™ High-Resolution Melt Analysis Module

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About this guide

CAUTION! ABBREVIATED SAFETY ALERTS. Hazard symbols and hazard types specified in procedures may be abbreviated in this document. For the complete safety information, see the "Safety" appendix in this document.

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

About the HRM example experiments

For the purpose of explanation, this guide uses three example experiments to familiarize you with the process of setting up, performing, and analyzing high resolution melt experiments performed on the Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument. The example melt curve experiments are very basic and intended for instructional purposes only. The experiments employ MeltDoctor™ High-Resolution Melting (HRM) reagents and controls, which are available from Thermo Fisher Scientific. When designing and performing your own HRM experiments, adjust the reaction volumes and experiment parameters (controls, dyes, and PCR method) appropriate for your chosen materials and application.

Tips for running your own HRM experiment

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

1 HRM Experiment Overview

About HRM experiments

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

The Applied Biosystems™ qPCR Analysis Software can perform:

- **Mutation scanning experiments** – Screen DNA samples for new single-base changes, insertions/deletions, or other unknown mutations. The mutation scanning experiment product can be used for subsequent sequencing reactions.
- **Methylation studies** – Determine the percentage of methylated DNA in unknown samples.
- **Genotyping experiments** – Determine the genotype of a DNA sample.

HRM controls

For all types of experiments, the Applied Biosystems™ qPCR Analysis Software compares the melt curves of unknown samples against the melt curves of positive controls to identify groups of variants.

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

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

HRM experiment workflows

The following workflows illustrate the steps for performing a high-resolution melt curve experiment using MeltDoctor™ HRM Reagents on an Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument followed by analysis using the Applied Biosystems™ qPCR Analysis Software on the ThermoFisher Cloud.

Genotyping workflow

The following figure illustrates the general workflow for performing genotyping experiments using high resolution melt curve reagents and the QuantStudio™ 3 or 5 Instrument.

Calibrate your instrument

Prepare a MeltDoctor™ HRM calibration plate

▼

Perform the HRM calibration

▼

Design the HRM experiment

Design and order the primers

▼

Identify your controls

▼

Create the experiment, including targets, samples, controls, and run method

▼

Perform the experiment

Prepare the HRM reactions for the example experiment

▼

Load the plate and start the run

▼

Monitor the run

▼

Analyze the data

Create a project and add experiment data

▼

Review and (if necessary) edit the sample, target, task, and control assignments

▼

Review the results of the analysis and adjust the settings (if necessary)

▼

Publish the project data

Methylation study workflow

The following figure illustrates the general workflow for performing methylation study experiments using high resolution melt curve reagents and the QuantStudio™ 3 or 5 Instrument.

Calibrate your instrument

Prepare a MeltDoctor™ HRM calibration plate

▼

Perform the HRM calibration

▼

Design your HRM experiment

Design and order the primers

▼

Identify your controls and standards

▼

Create the experiment, including targets, samples, controls, standards, and run method

▼

Perform the experiment

Prepare the HRM reactions for the example experiment

▼

Load the plate and start the run

▼

Monitor the run

▼

Review Experiment Results and Adjust Parameters

Create a project and add experiment data

▼

(Optional) Add and define samples and targets

▼

Review/edit the sample, target, task, and control assignments

▼

Review the results of the analysis and adjust the settings (if necessary)

▼

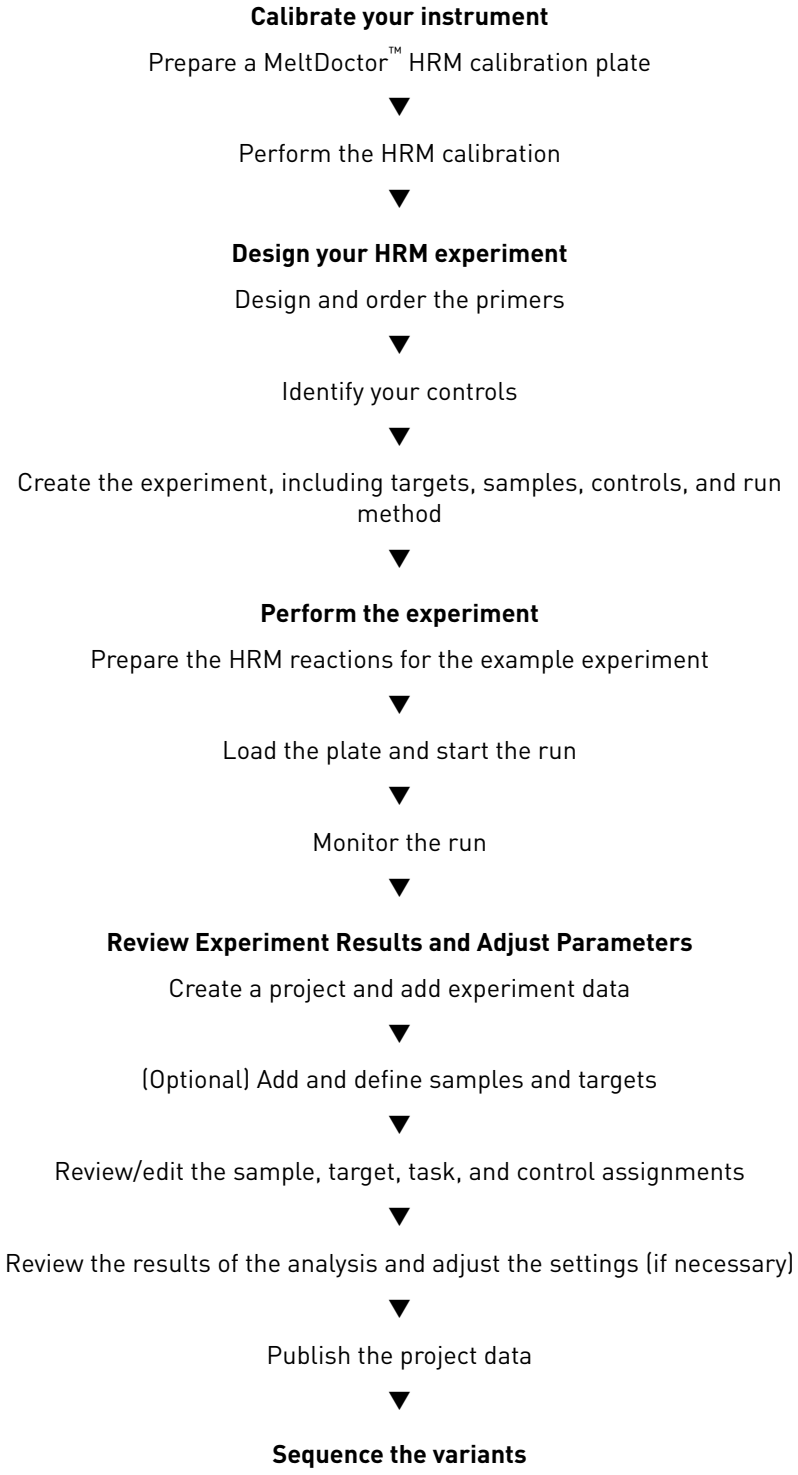
Publish the project data

▼

Sequence the variants

Mutation detection workflow

The following figure illustrates the general workflow for performing mutation-screening experiments using high resolution melt curve reagents and the QuantStudio™ 3 or 5 Instrument.



Materials and reagents

High-resolution melt (HRM) dyes

The melt profile of a PCR product is best obtained with high-resolution melt dyes (HRM dyes). HRM dyes are double-stranded 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.

Custom HRM dyes

This getting started guide describes procedures for calibrating your instrument and performing HRM experiments using the MeltDoctor™ HRM Dye. If you use a different HRM dye, prepare a custom HRM calibration plate and then calibrate your instrument for that dye (see your instrument user documentation for the calibration procedure). When the instrument is calibrated, follow the procedures provided, but replace the MeltDoctor™ HRM Dye with your own.

Note: See the *High-Resolution Melt Curve Getting Started Guide* for your QuantStudio™ 3 or 5 Instrument for instructions on performing a custom HRM dye calibration.

Note: Optimize your reactions for the HRM dye that you use, because each dye interacts uniquely with all other reaction components.

High-Resolution Melt (HRM) reagents and controls

The example experiments described in this document include the following Applied Biosystems™ MeltDoctor™ High-Resolution Melting (HRM) reagents and controls that can be used in your own experiments. For product details and ordering information, visit: **http://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-reagents/meltdoctor-hrm-reagents.html**

- **Applied Biosystems™ MeltDoctor™ HRM Master Mix** contains all PCR components (excluding template and primers) and has been formulated for optimal HRM performance across a wide range of genomic targets. It features a dNTP blend that includes dUTP, which minimizes carryover contamination by allowing amplicon degradation by uracil DNA glycosylase (UDG) in subsequent PCR reactions. The MeltDoctor™ HRM Master Mix does not require additional mixing prior to use, and was developed and optimized solely for HRM applications.
- **Applied Biosystems™ MeltDoctor™ HRM Positive Control Kit** provides nucleic acid controls that can be used to demonstrate and troubleshoot high resolution melt (HRM) analysis. The kit contains forward and reverse primers and three DNA templates representing the homozygous and heterozygous genotypes for a β-actin target gene (ACTB).
- **Applied Biosystems™ MeltDoctor™ HRM Calibration Standard** contains a DNA template and primers which, when mixed with PCR reagents and dye, can be used to prepare a plate for thermal and optical High Resolution Melt (HRM) calibration for the MeltDoctor™ HRM (High Resolution Melting) dye.
- **Applied Biosystems™ MeltDoctor™ HRM Calibration Plates** contain all the components required for dye and HRM calibration on the Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument. These plates provide the thermal and optical calibration required for accurate and reproducible discrimination between melt curves.

Required instruments and software

Compatible real-time qPCR instruments

This guide describes how to calibrate, set up, and perform high resolution melt experiments on the Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument. For instructions on performing HRM experiments on other real-time qPCR instruments manufactured by Thermo Fisher Scientific, refer to the high resolution melt documentation available from the Thermo Fisher Scientific website (**http://www.thermofisher.com/support**).

IMPORTANT! QuantStudio™ 3 or 5 Instrument firmware version 1.1 or later is required to perform HRM experiments.

About the Thermo Fisher Cloud and Applied Biosystems™ HRM Analysis Module

The high resolution melt curve data generated by QuantStudio™ 3 and 5 Real-Time PCR Systems is analyzed using the Applied Biosystems™ HRM Analysis Module available through the Thermo Fisher Cloud. The Thermo Fisher Cloud is a secure suite of cloud computing applications that provide storage and sharing of data generated on Applied Biosystems™ real-time PCR and capillary electrophoresis instruments. It seamlessly integrates data management and sharing (Thermo Fisher Cloud Storage), online analysis modules, and subscription-based access (Subscription Manager). Using the Thermo Fisher Cloud, you can store, access, analyze, and share results anytime and anywhere.

Note: Refer to **http://www.thermofisher.com/support** for answers to questions about Thermo Fisher Cloud security, including network access, data retention, and user authorization or authentication.

Note: As an alternative to using the Thermo Fisher Cloud or the Applied Biosystems™ HRM Analysis Module as described in this guide, you can use the Applied Biosystems™ High Resolution Melt Software v3.1 (Cat. No. A29881) to analyze your HRM experiments. The High Resolution Melt Software is a Windows™-compatible desktop application that performs high resolution melt (HRM) analysis of experiments generated by Applied Biosystems™ Real-Time PCR Systems, including the QuantStudio™ 3 and 5 Real-Time PCR Systems.

System requirements

Web Browser	Minimum version	Latest verified version	OS Compatibility
Microsoft™ Internet Explorer™	10.x	10.x	Windows™ 7.1+
Google™ Chrome™	25.x	42.x	Windows™ XP+ OSX 10.6+
Apple™ Safari™ (OSX)	7.x	8.x	6: OSX 10.7+ 7: OSX 10.9+
Mozilla™ Firefox™	16.x	36.x	Windows™ XP SP2+ OSX 10.6+

2 Calibrate your instrument

About the HRM calibration

You must calibrate your instrument for the HRM Dye before you can perform high resolution melting experiments. During the HRM calibration, the Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument amplifies the template in the HRM calibration consumable and performs a melt curve analysis of the PCR product. We recommend that you calibrate your instrument for each HRM dye that you are using or for each significant change in master mix composition.

IMPORTANT! If you are using a third-party HRM dye, perform a custom dye calibration for the HRM dye *before* you perform the HRM calibration. For more information on the custom dye calibration procedure, see the user documentation for your Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument.

Note: If you are using a third-party HRM dye:

- Prepare the calibration plate as described in this document, but adjust the HRM dye volumes according to the manufacturer's instructions.
- You might need to optimize your HRM reactions to account for differences in sensitivity resulting from the interaction between the dye and reaction components.

Prepare a pre-loaded HRM calibration plate

IMPORTANT! The region of interest, background, and uniformity calibrations must be current on your Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument before you can perform an HRM calibration. For more information on instrument calibration, see the user documentation for your instrument.

IMPORTANT! Perform a background calibration immediately before you perform the HRM calibration.

Required materials

- Centrifuge with plate adaptor
- Powder-free gloves
- Safety goggles
- MeltDoctor™ HRM Calibration Plate, 0.1-mL ,96- or 384-well
  or
  MeltDoctor™ HRM Calibration Standard, 0.2-mL, 96-well

Calibration guidelines

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

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

Prepare the MeltDoctor™ HRM calibration plate

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

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

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

2. Remove the MeltDoctor™ HRM Calibration Plate from its packaging. Do not remove the optical film.

3. Vortex and centrifuge the MeltDoctor™ HRM Calibration Plate:
   a. Vortex the MeltDoctor™ HRM Calibration Plate for 5 seconds.

b. Centrifuge the MeltDoctor™ HRM Calibration Plate for 2 minutes at <1500 rpm.

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

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

Perform the HRM calibration

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

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

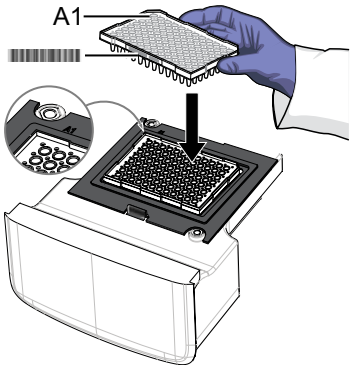
1. In the home screen, touch **Settings ▸ Maintenance and Service ▸ Calibrations ▸ Custom ▸ Custom Melt**.
2. Touch **PCR + Melt** or **Melt only** as appropriate for the dye that you are using.

   Note: Select **PCR + Melt** protocol if you are performing a calibration using MeltDoctor™ reagents.
3. Select or add a dye, then select a filter set (see filter-wavelength table in step 8 below).

   Note: Only the data points obtained by the selected filter set are used for calibration.

   Note: If using the SYBR™ Green dsDNA binding dye, the dye-DNA complex absorption maxima is 497 nm and the emission spectra maxima is 520 nm. If you are using a third-party HRM dye, refer to the manufacturer's documentation to determine the correct filter set for your dye.
4. (*Optional*) Touch **Reagents**, then enter reagent information.
5. Load the plate.
   a. Touch ⏏ to eject the instrument drawer.

b. Load the plate onto the plate adaptor so that:
   - Well A1 of the plate is in the top-left corner of the plate adapter.
   - The barcode faces the front of the instrument.



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

Note: (*For 96-well 0.2-mL blocks only*) Do not remove the black plate adapter before loading a plate or strip tubes. Strip tubes may fit loosely in the adapter, but when the drawer closes, the heated cover will apply the appropriate pressure to seat the tube strips securely in the adapter.

Note: The 384-well and 96-well Fast (0.1-mL) block configurations do not require a plate adapter.

c. Touch ⏏ to close the instrument drawer.

6. Touch **Start**.

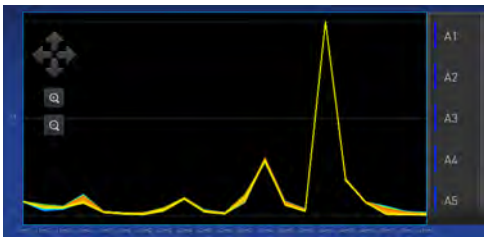
7. When the run is complete and the screen displays Calibration Complete, touch **View Results ▸ Details** to check the calibration status.

8. Review the plot. Passing calibration results show uniform signals with peaks aligned with the dye's wavelength.

Peak channel	Filter wavelength (nm)[1]	
	Excitation	Emission
x1-m1	470 ± 15	520 ± 15
x2-m2	520 ± 10	558 ± 12
x3-m3	550 ± 10	587 ± 10
x4-m4	580 ± 10	623 ± 14

Peak channel	Filter wavelength (nm)[1]	
	Excitation	Emission
x5-m5	640 ± 10	682 ± 14
x6-m6	662 ± 10	711 ± 12

[1] The central wavelengths are the optimized wavelengths.



Note: Example dye calibration plot.
The peaks for your dye may align with a different filter set.

9. Select an action depending on whether you determine the custom dye calibration passed or failed.

Calibration status	Action
Passed	• Touch **Accept Results** or **Reject Results**. **Note:** Accepting the results saves the calibration data to the instrument and overwrites existing data. • (*Optional*) Touch Transfer Eds to transfer the calibration data to a USB.
Failed	• Create another custom dye plate using the next dye concentration greater than the concentration determined in Determine the optimal dye concentration, then perform the calibration again. • Troubleshoot the failed calibration as explained in this guide.

10. Unload the plate.
    a. Touch ⏏ to eject the instrument drawer.
    b. Remove the plate.
    c. Touch ⏏ to close the instrument drawer.

⚠ **CAUTION! PHYSICAL INJURY HAZARD.** During instrument operation, the plate temperature can reach 100°C. Allow it to cool to room temperature before handling.

Note: If the instrument does not eject the plate, contact Support.

11. Discard the calibration plate.

You must add the custom dye to the desktop or cloud software dye libraries before creating, running, or analyzing experiments that use custom dyes. Refer to the *QuantStudio™ Design and Analysis desktop Software User Guide* (Pub. no. MAN0010408) or the *QuantStudio™ Design and Analysis cloud Software Help* (Pub. no. MAN0010414).

IMPORTANT! When specifying a custom dye (or creating a new dye), the name of the dye that you create must match the dye specified during the Experiment Setup (in the Design & Analysis Application Module).

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3 Prepare the HRM reactions and perform the experiment

Experiment workflow

The following workflows illustrate the general steps required to perform a high-resolution melt curve experiments using MeltDoctor™ HRM Reagents on an Applied Biosystems™ Real- Time PCR System.

Calibrate your instrument

▼

Design the HRM experiment

Design and order the primers

▼

Identify your controls

▼

(*Methylation study only*) Identify your standards

▼

Create the experiment on the QuantStudio™ 3 or 5 Instrument, including targets, samples, standards, and run method

▼

Perform the experiment

Prepare the HRM reactions for the example experiment

▼

Load the plate and start the run

▼

Monitor the run

▼

Analyze the data

▼

Sequence the variants

Required materials

- Microcentrifuge tubes
- Optical reaction plate appropriate for your real-time PCR instrument
- MicroAmp™ Optical Adhesive Film
- MeltDoctor™ HRM Master Mix
- Deionized water
- Pipettors and pipette tips
- Vortexer
- Centrifuge

- For each target sequence:
  - Forward and reverse primers (5 µM each)
  - DNA samples
- (*Methylation study only*) Methylated DNA standards
- (*Genotyping only*) Positive controls appropriate for your experiment

IMPORTANT! If you use HRM reagents from another manufacturer, you must calibrate your QuantStudio™ 3 or 5 Instrument using those dyes.

Note: For the purpose of explanation, the example experiment described in this guide uses the MeltDoctor™ HRM Positive Control Kit, which includes the following controls:

- MeltDoctor™ HRM Primer Mix (20X)
- MeltDoctor™ HRM Allele A DNA (20X)
- MeltDoctor™ HRM Allele G DNA (20X)
- MeltDoctor™ HRM Allele A/G DNA (20X)

Design primers

Primer design for genotyping

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

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

We recommend using these guidelines when designing primers:

Design attribute	Design guidelines
Amplicon	• Length is 60 to 250 basepairs (longer amplicons may require optimization) • Contains only one SNP • Does not contain a repeating element • Corresponds to a unique sequence within the sample
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 two G+C residues in the last five 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 four consecutive Gs. Also, make sure that the amplicon does not contain a repeating element.

Primer design for mutation detection

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 Thermo Fisher Scientific Store.

Design the primers so that they meet these guidelines:

Design attribute	Design guidelines
Amplicon	• Length is less than 250 basepairs • Does not contain a repeating element • Corresponds to a unique sequence within the sample
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 two G+C residues in the last five 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 four consecutive Gs. Also, make sure that the amplicon does not contain a repeating element.

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):

```
1 TGTAAAACGA CGGCCAGT
```

- M13R (add to the 5′ end of the reverse primer):

```
1 CAGGAAACAG CTATGACC
```

Primer design for methylation studies

Using Applied Biosystems™ 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 Thermo Fisher Scientific Store.

Design attribute	Design guidelines
Amplicon	• Length is less than 250 basepairs • Does not contain a repeating element • Corresponds to a unique sequence within the sample • 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)

Design attribute	Design guidelines
% GC content	30–80% GC content in each primer 3′ end No more than two G+C residues in the last five 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 four consecutive Gs. Also, make sure that the amplicon does not contain a repeating element.

Choose experiment controls

When designing your experiment, consider the use of controls appropriate for your application. The following table describes the recommended controls for high resolution melt curve experiments performed on the QuantStudio™ 3 or 5 Instrument.

Experiment type	Recommendation
Genotyping	Include controls for each target sequence in your genotyping experiment. • At least one negative control • At least one positive control to represent each expected variant Run 3-5 replicates of each control to improve your results. Running multiple positive controls allows you to more effectively define the natural spread or variation within different samples of the same sequence, or within replicates of the same genotype
Mutation detection	Include controls for each target sequence in your mutation detection 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.
Methylation study	Including controls for each target sequence in your methylation study: • At least one negative control • Methylated DNA standards that contain from 0% to 100% methylated DNA

Prepare the standards

If you are performing a methylation study, prepare the methylated DNA standards prior to plating the reactions.

Prepare the methylated DNA standards

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

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

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

Treat the samples and methylated DNA standards with bisulfite

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

We recommend that you use the Cells-to-CpG™ Bisulfite Conversion Kit (Cat. no. 4445555). For instructions, refer to the *Applied Biosystems™ Cells-to-CpG™ Bisulfite Conversion Kit Protocol* (Pub. no. 4448998).

Prepare the reactions

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

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

Components	Volume (µL)			
	384-well plate		96-well plate	
	1 reaction	3 replicates plus 10% excess	1 reaction	3 replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0	33.00	25.0	82.5
Primer 1 (5 µM)	1.2	3.96	3.0	9.9
Primer 2 (5 µM)	1.2	3.96	3.0	9.9
Deionized water	7.6	25.08	19.0	62.7
Total volume	**20.0**	**66.0**	**50.0**	**165.0**

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, standard, and unknown reactions in separate appropriately sized and labeled tubes:

Components	Volume (µL)			
	384-well plate		96-well plate	
	1 reaction	3 replicates plus 10% excess	1 reaction	3 replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0	33.00	25.0	82.5
Primer 1 (5 µM)	1.2	3.96	3.0	9.9
Primer 2 (5 µM)	1.2	3.96	3.0	9.9
Genomic DNA (20 ng/µL)	1.0	3.30	2.5	8.25
Deionized water	6.6	21.78	16.5	54.45
Total volume	**20.0**	**66.0**	**50.0**	**165.0**

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:

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

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

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

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

Perform the HRM experiment

Perform the HRM experiment

IMPORTANT! Before you perform high-resolution melt curve experiments on your Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument, you must perform an HRM calibration.

1. Use the QuantStudio™ Design and Analysis desktop Software to create a melt curve experiment template, then transfer the file to the QuantStudio™ 3 or 5 Instrument.

   For detailed instructions on creating and transferring an experiment template, refer to the user documentation for your Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument.

Experiment properties	Settings
Sample settings	Create a sample entry for each sample that you are evaluating, including separate positive controls for each expected variant.
Target settings	Create a target for each amplicon region and assign the dyes. **Note:** If you are running multiple assays on your plate, create a target for each target sequence that you are evaluating. The Applied Biosystems™ qPCR Analysis Software will separate the wells into different assays according to the target assigned to the well.
Control settings	Create and assign the controls used in the experiment (positive and negative). • Task for negative control wells: **NTC** • Passive Reference: **None**

Experiment properties	Settings
Run method	Program the experiment with the thermal cycling protocol for your instrument, including the following experiment properties: • Thermal Profile: Enter the time and temperature parameters for the PCR and subsequent melt curve, including both pre- and post-read data collection (if applicable). • Run Mode: **Standard** • Ramp Mode: – **Continuous** mode set to **0.025**°C/second *or* **No. of Data Points per Degree** mode set to ≥**10** data points. • Sample Volume (µL): **20** (384-well or 96-well Fast) or **50** (96-well standard) When in Continuous mode, the instrument uses a fixed ramp rate for the melt step and captures as many data point as possible within the time permitted. When in Data point per degree mode, the instrument adjusts the step and hold for the melt step to achieve the specified number of data points. IMPORTANT! If using Data points per degree mode, make sure to set the ramp mode to collect at least 10 data points per degree Celsius so that the instrument collects enough data for analysis.

The following table displays the thermal cycling method for performing PCR and melt curve using MeltDoctor™ reagents and controls.

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

2. Create an experiment from the template:
   a. In the home screen of the QuantStudio™ 3 or 5 Instrument, touch **Open Template**.
   b. *(Optional)* Touch an experiment category in the left column.
   c. Touch an experiment name.

3. Enter the experiment settings:

   Note: You can assign HRM experiments on your Applied Biosystems™ instrument before or after you prepare your HRM reactions.

   a. *(Optional)* Define the experiment properties, including Run File Name (.eds file name), Plate Barcode, Reagent Information, and Data Destination.

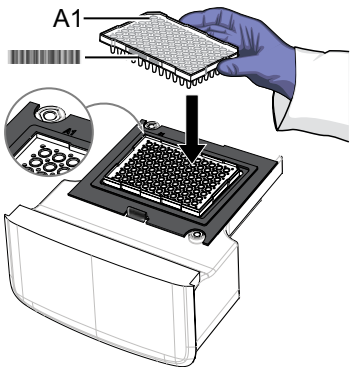
   b. *(Optional)* Edit the run method. Add, remove, or edit a step, stage, melt curve, or data collection location. Also, adjust the cover temperature, sample volume, or number of cycles as needed.

   c. *(Optional)* Assign sample names to the plate wells and view Well ID, Targets, or Dyes.

4. Load the plate.

   a. Touch ⏏ to eject the instrument drawer.

   b. Load the plate onto the plate adaptor so that:
      - Well A1 of the plate is in the top-left corner of the plate adapter.
      - The barcode faces the front of the instrument.



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

   Note: (*For 96-well 0.2-mL blocks only*) Do not remove the black plate adapter before loading a plate or strip tubes. Strip tubes may fit loosely in the adapter, but when the drawer closes, the heated cover will apply the appropriate pressure to seat the tube strips securely in the adapter.

   Note: The 384-well and 96-well Fast (0.1-mL) block configurations do not require a plate adapter.

   c. Touch ⏏ to close the instrument drawer.

5. Touch **Start Run**.

Confirm amplification and check the run data

Note: See the Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument user documentation for instructions on viewing amplification data during the real-time PCR run.

1. During or immediately following the real-time run, review the amplification data for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence

   If the Amplification Plot contains abnormalities, see the Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument user documentation for identify and resolve the problem.

2. Review the amplification data for potential outlier wells with C_T values that differ from replicates by more than two. Record the location of the outlying wells on the plate for possible omission during the analysis.

   Note: The outliers can produce erroneous HRM results.

Unload the instrument and transfer the data

When the run is complete:

1. Unload the plate.
   a. Touch ⏏ to eject the instrument drawer.
   b. Remove the plate.
   c. Touch ⏏ to close the instrument drawer.

   ⚠ **CAUTION! PHYSICAL INJURY HAZARD.** During instrument operation, the plate temperature can reach 100°C. Allow it to cool to room temperature before handling.

   Note: If the instrument does not eject the plate, contact Support.

2. Transfer the data to the Thermo Fisher Cloud for analysis:
   a. In the home screen, touch **Transfer File**.
   b. Touch **Cloud**, **USB**, or **Network** to select the data destination.
   c. Navigate to and select your folder destination.
   d. Touch **OK**, then touch **Transfer**.

4 Analyze an HRM genotyping experiment

HRM genotyping analysis workflow

The following workflow illustrates the general steps required to perform a high-resolution melt curve genotyping experiment using MeltDoctor™ HRM Reagents on an Applied Biosystems™ Real- Time PCR System followed by analysis using the Applied Biosystems™ qPCR Analysis Software.

Calibrate your instrument

▼

Design the HRM experiment

▼

Perform the experiment

▼

Analyze the data

Create a project and add experiment data

▼

Review and (if necessary) edit the sample, target, task, and control assignments

▼

Review the results of the analysis and adjust the settings (if necessary)

▼

Publish the project data

Configure your analysis project

After you create your HRM project using the Thermo Fisher Cloud and populate it with experiments, use the Applied Biosystems™ qPCR Analysis Software HRM Module to correct any experiment settings prior to the analysis.

Note: As an alternative to using the Thermo Fisher Cloud or the Applied Biosystems™ HRM Analysis Module as described in this guide, you can use the Applied Biosystems™ High Resolution Melt Software v3.1 (Cat. No. A29881) to analyze your HRM experiments. The High Resolution Melt Software is a Windows™-compatible desktop application that performs high resolution melt (HRM) analysis of experiments generated by Applied Biosystems™ Real-Time PCR Systems, including the QuantStudio™ 3 and 5 Real-Time PCR Systems.

Log into the Thermo Fisher Cloud

Analysis of the HRM experiment data is performed using the Applied Biosystems™ qPCR Analysis Software HRM Module, which is available on the Thermo Fisher Cloud. The Thermo Fisher Cloud is a cloud storage and applications environment that provides scientists with a secure location to store, analyze, and share data. If connected to the internet, you can upload your HRM experiment data directly from your Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument or upload the experiment files from anywhere you have web access.

Note: For more information on the Thermo Fisher Cloud, go to **https://www.thermofisher.com/cloud**.

To log into the Thermo Fisher Cloud:

- Using an internet browser, go to **https://apps.thermofisher.com** , then log into the Thermo Fisher Cloud using your user account and password.

  If you do not have a Thermo Fisher Cloud user account, click **Create an account**, then follow the on-screen instructions to create an account for your projects. The Applied Biosystems™ qPCR Analysis Software HRM Module is available from within the Thermo Fisher Cloud.

Create a project and add experiment data

1. Click (Manage Projects) to view the Dashboard.
2. Create the project:
   a. Click **New Project**.
   b. In the Create Project dialog box, enter a name for the project, select the folder within which you want to place the project, then click **OK**.

      Note: The project name cannot exceed 50 characters and cannot include any of the following characters: / \ < > * ? " | : ; & % $ @ ^ ( ) !

3. From the Manage Data screen, add any additional experiment data to the project.

To import experiment data stored on...	Action
Your computer	1. Click **Import from local**. 2. From the Open dialog box, select one or more experiment files (.sds or .eds), then click **Open**. **Note:** Ctrl- or Shift-click to select multiple files. Wait for the Applied Biosystems™ Software to upload the selected data. 3. Click **Close** prompted that the import is complete.
Thermo Fisher Cloud	1. Click **Import from Thermo Fisher Cloud**. 2. Select one or more experiment files (.sds or .eds) from the table, then click **Add**. 3. When you are done adding files to the queue, click **OK**. 4. Click **Close** prompted that the import is complete.

4. Repeat step 3 until your project contains all of the desired experiment data.

5. Click the appropriate analysis module on the left side of the screen to begin the analysis.

Manage samples and targets

The Applied Biosystems™ qPCR Analysis Software populates the Overview screen with the samples and targets present in the experiments added to the project. If necessary, you can add, edit, or remove the samples and targets as needed before the analysis.

- **Create** a new sample or target:
  a. From the Samples or Targets table in the Overview screen, click **Actions** ▸ **Add**.
  b. In the New Sample/Target dialog box, enter a name for the new sample or target (up to 256 characters), then edit the properties of the new sample/target.
  c. Click **OK**.
- **Update** an existing sample or target by editing the entry directly in the table.

  Note: Alternately, select a sample or target from the table, then select **Actions** ▸ **Update**.
- **Delete** a sample or target:
  a. From the Samples or Targets table in the Overview screen, select the sample or target of interest, then click **Actions** ▸ **Delete**.
  b. In the confirmation dialog box, click **OK** to delete the sample or target.

Manage HRM controls

The Applied Biosystems™ qPCR Analysis Software populates the Overview screen with the controls present in the experiments added to the project.

If necessary, you can add, edit, or remove controls as needed before the analysis:

- **Create** a new control:
  a. From the Controls table in the Overview screen, click **Actions** ▸ **Add**.
  b. In the New Control dialog box, enter a name for the new control (up to 256 characters), then edit the properties of the new control.
  c. Click **OK**.
- **Update** an existing control by editing the entry directly in the table.

  Note: Alternately, select a control from the table, then select **Actions** ▸ **Update**.
- **Delete** a control:
  a. From the Controls table in the Overview screen, select the control of interest, then click **Actions** ▸ **Delete**.
  b. In the confirmation dialog box, click **OK** to delete the control.

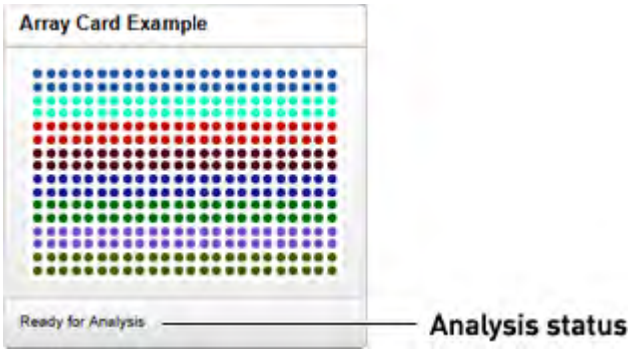
Review and edit the plate setups

After configuring your project with all necessary samples and targets, use the Plate Setup screen to review the experiments for problems that can prevent the analysis of the project. The Applied Biosystems™ qPCR Analysis Software displays plate configuration errors that can prohibit analysis in the margin beneath each image of the related experiment. Before you can analyze your project, you must use the Plate Setup screen to address them.

To review the plate setup information for your project:

1. Select **Plate Setup** to display Plate Setup screen.
2. From the Plate Setup screen, review the experiment records for errors.
3. If errors are present, click the experiment record of interest and address the problem that is preventing the analysis of the file.

   Note: The software displays plate configuration problems that will prevent analysis of an experiment beneath the image of the related plate.



Apply samples and targets

If the sample or target assignments of one or more of your experiments contain errors or are missing, you can use the Applied Biosystems™ qPCR Analysis Software to correct the problem prior to analysis.

Note: When reviewing a plate layout, click **Actions ▸ Clear Well Setup** to remove the well information (sample, task, and target assignments) from the selected wells in the plate grid.

1. From the Plate Setup screen, select the experiment that you want to modify.
2. (Optional) From the Edit Plate screen, click **View**, then select **Target** and **Sample** to color the plate setup according to the element that you intend to modify.
3. Select the wells of the plate layout to which you want to apply the target or sample.
4. When the wells are selected, click the appropriate field to the right of the plate grid, then select the appropriate item from the list.

   Note: If you have not yet created a sample or target, enter the name in the appropriate field and press **Enter** to create the new sample or target.
5. Once you are finished making changes to the plate layout, click **Analyze** to reanalyze your project.

Specify and assign tasks

If the task assignments of one or more of your experiments contain errors or are missing, you can use the Applied Biosystems™ qPCR Analysis Software to correct the problem prior to analysis.

Note: When reviewing a plate layout, click **Actions ▸ Clear Well Setup** to remove the well information (sample, task, and target assignments) from the selected wells in the plate grid.

1. From the Plate Setup screen, select the experiment record that you want to modify.
2. From the Edit Plate screen, click **View**, then select **Task** to color the plate setup according to task assignment.
3. Select the wells of the plate layout to which you want to apply a task.

4. When the wells are selected, click the **Task** menu, then select the appropriate task from the list.

   Available tasks include:

   - **Unknown** – The task for wells that contain a sample with unknown genotype, variant content, or percentage methylation.
   - **NTC** – The task for wells that contain water or buffer instead of sample (no template controls). No amplification of the target should occur in negative control wells.
   - **Positive Controls** - Wells that contain one of the following:
     - A template known to generate a specific genotype call for one or both alleles (**Heterozygote** or **Homozygote**).
     - A wild type control (**Wild Type**).
     - Methylated DNA standards that contain from 0% to 100% methylated DNA (add a custom control label).

   Note: The Task/Control dropdown list displays the controls present in the Controls pane of the Overview screen. See "Manage HRM controls" on page 34 for information on adding and editing controls.

5. Repeat steps 3 and 4 as needed.

6. Once you have completed making changes to the plate layout, click **Analyze** to reanalyze your project.

Review HRM genotyping data

The Applied Biosystems™ qPCR Analysis Software High Resolution Melt module supports the analysis of HRM genotyping experiments, where melt curve technique is used to determine the genotype of unknown DNA samples. During the analysis, the software compares the melt curves of the unknown samples against those generated from a set of DNA standards (positive controls) that represent the genotypes for the target sequence (Allele 1 homozygous, Allele 2 homozygous, and Allele 1/Allele 2 heterozygous). Based on the comparison, the software assigns calls to the unknown samples according to the fit of the unknown melt curves to those of the controls.

In general, review of genotyping results occurs in the following steps:

1. Define the analysis settings, then analyze the project.
2. Perform an initial review of the High Resolution Melt Plots, the Plate Layout, and the Well Table to evaluate the genotype calls made by the Applied Biosystems™ Software.
3. Perform a thorough review of the QC Summary to evaluate the samples that triggered QC flags. Review the raw data and amplification data for the samples that exhibit abnormal amplification.
4. If necessary, adjust the analysis settings or modify the calls manually.

After evaluating the results, publish the analyzed data.

Configure the analysis settings

When a project is created, the Applied Biosystems™ qPCR Analysis Software processes the project data using the default analysis settings of the experiments added to the project. If desired, you can modify the analysis settings from the Quality Control & Results screen (for example, manual versus automatic thresholding or stringent versus relaxed quality thresholds).

1. From the Quality Control & Results screen, select an experiment of interest.
2. From the Review Result screen, click **Analysis Settings**.
3. From the Edit Analysis Setting dialog box, modify the analysis settings as desired.

Group	Settings
Target Settings	Select the method (automatic or manual) that the Applied Biosystems™ Software will use to compute the pre- and post-melt ranges, grouping, and variant removal: • Define whether the pre- and post-melt range for each target will be computed automatically or manually: – **Auto Set Melt Range** – If you are using automatic settings, select the checkbox to have the software calculate the pre- and post- melt ranges for the specific target. – **Pre-Melt Start/Stop** and **Post-Melt Start/Stop** – If you are using manual settings, enter the manual pre- and post-range values for the appropriate targets. • Define whether the genotype groups for each target will be determined automatically or manually: – **Auto Determine # of Groups** – If you are using automatic settings, select the checkbox to have the software calculate the number of groups for the specific target. – **Number of Groups** – If you are using manual settings, enter the number of groups for the appropriate targets. • **Remove all Manual Variants on reanalysis** – Select to have the software omit from the analysis all wells that have been manually been labeled as a variant.
Flag Settings	Specify the quality measures that the Applied Biosystems™ Software will compute during the analysis. 1. In the Use column, select the check boxes for flags you want to apply during analysis. 2. If an attribute, condition, and value are listed for a flag, you can specify the setting for applying the flag. For example, with the default setting for the no amplification flag (NOAMP), wells are flagged if the amplification algorithm result is less than 0.1. **Note:** If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting. 3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag. Rejected wells are not considered for data analysis.

4. When done modifying the analysis settings, click **Finish**.

Review the quality data

After the Applied Biosystems™ qPCR Analysis Software processes your project, you can use the Quality Control & Results screen to review the quality data generated by the analysis. The software provides a variety of options to review the quality data; however, the strategy that you employ will depend on the type of experiment you are performing and the samples/targets that you are evaluating. The following procedure describes a general approach to data review and provides an overview of the software features.

1. If you have not already done so, click **Analyze** to analyze your project.
2. Click **Quality Control & Results** to view the Quality Control & Results screen.
3. Review the experiment data for quality flags generated during the analysis.

   Note: The Applied Biosystems™ Software displays summaries of the quality data in the margin beneath each experiment. You can view the identity of the triggered flags by mousing over an experiment of interest.

   Alternatively, to quickly find the experiments in your project that generated quality flags, click (Plate View), then click the Flags column heading to sort the experiments that generated quality flags to the top of the table. To examine the data that triggered the flag, click the corresponding link in the Name column.

   In response to the presence of quality flags, consider the following resolutions:

   - Change the quality settings in the analysis settings:
     - Adjust the sensitivity of the quality flags so that more wells or fewer wells are flagged.
     - Deactivate the quality flags that triggered by the data.
   - Omit individual wells from the analysis.
4. If flags or irregularities are present, or you would like to review the melt curve data for a specific experiment, click the experiment of interest.
5. When viewing an experiment, display the Well Table:

Tool	Use this tool to...
Mouse/cursor	Select wells. To select: • An individual well, select the well in the Well table. • More than one well at a time, press the **Ctrl** key or **Shift** key when you select the wells in the Well table. When you select wells in the Well table, the corresponding data points are selected in the amplification plot.
Actions menu	**Omit/Un-Omit** well from the analysis. After you omit or un-omit a well, click **Analyze** to reanalyze the project. For omitted wells, the software: • Does not display data in the Well table (data are either Unknown or empty/blank). • Does not include the omitted wells in the analysis.
or	Expand or collapse the Well table by clicking the appropriate symbol in the vertical bar between the plot and table.

6. Review the data in the Well Table data.

Column	Description
Well	The location of the well on the reaction plate. For example, P18 indicates that the sample is found in row P, column 18.
Omit	The omission status of the related well.
Target	The ID (a unique name or number) of the nucleic acid sequence targeted by the assay added to the well.
Sample	The ID (a unique name or number) of the sample.
Task/Control	The task assigned to the well, where the task is the function that a sample performs on the plate.
Variant	The call for the sample in well. Can be assigned by software (Auto) or manually.
Method	The method used to apply the call to the well: Auto(matic) if calculated by the Applied Biosystems™ qPCR Analysis Software or Manual if user-applied.
Silhouette Score	The *modified* silhouette score calculated for the well, which measures distinguishably of the associated melt curve relative to the other curves in the assay (Rousseeuw, 1987.). For each melt curve, the software calculates a *modified* silhouette score, which ranges from 0 to 100. A score closer to 100 indicates that a melt curve is more similar to curves assigned the same variant call than to curves called differently. Lower silhouette scores indicate that a melt curve is less similar to curves assigned the same variant call. **IMPORTANT!** The modified silhouette score differs from the standard silhouette score (Lovmar, *et. al.,* 2005.) in that the software assigns the score to each identified cluster instead of to each data point in the cluster. In addition, the modified value ranges from 0 to 100.
Tm1/Tm2/Tm3	The 1st, 2nd, and 3rd calculated melt temperature (Tm) for the well (if present). **Note:** Blank table cells indicate that the software calculated no Tm for the well at the indicated position.
Amp Status	The amplification status for the well: amplification, no amplification, reviewed, and undetermined.
Amp Score	The amplification score calculated for the well.
Cq Conf	The Cq confidence score calculated for the well.
Ct	The C_T calculated for the related well.
Ct Mean	The arithmetic mean generated from the C_Ts calculated for the technical replicates of the well.
Ct SD	The standard deviation generated from the C_Ts calculated for the technical replicates of the well.
Flags	The number of flags generated for the well.
Quality data	The quality flags generated by the associated well.

7. When ready, click ‹ to return to the thumbnails view.

Review the controls and replicate populations for outliers

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

1. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.

2. In the Quality Control & Results pane, select **Plot ▸ Aligned Melt** to review the Aligned Melt Curve Plot.

3. If using positive controls, confirm the calls for the positive controls:
   a. From the well table, select the wells containing a positive control to highlight the corresponding melt curve in the Aligned Melt Curves Plot.
   b. Confirm that the color of the line corresponds to the correct genotype.
   c. Repeat steps a and b for the wells containing the other positive controls.

4. Screen the negative controls to ensure that samples failed to amplify:
   a. From the well table, select the wells containing a negative control to highlight the corresponding melt curve in the Aligned Melt Curves Plot.
   b. Confirm that the selected wells in the well table are negative controls, and not unknown samples.

   Samples that grouped with the negative controls may:
   - Contain no DNA
   - Contain PCR inhibitors
   - Be homozygous for a sequence deletion

5. Confirm the results of the samples that did not group tightly or are grouped with negative controls by retesting them.

6. If you select to run replicate reactions, carefully review your data set for curves that do not align tightly with the other samples in the group (outliers) to ensure the accuracy of the genotype calls. If outliers are present, confirm the results of the associated samples by retesting them.

7. Look for how many different variant groups (different colors) are displayed. If you see more than you were expecting, you may have sample contamination or may need to modify the analysis settings.

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

Review and adjust the pre- and post-melt regions

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

About the pre- and post-melt regions

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

- **Pre-melt region** – The pair of lines to the left of the peak indicate the pre-melt Start and Stop temperatures when every amplicon is double-stranded. Fluorescence data from the pre-melt region corresponds to 100% fluorescence in the Aligned Melt Curves Plot.
- **Active melt region** – The data peak indicates the active melt region of the plot. Data from the active melt region are used to plot the Aligned Melt Curves Plot.
- **Post-melt region** – The set of lines to the right of the peak indicate the post-melt Start and Stop temperatures when every amplicon is single-stranded. Fluorescence data from the post-melt region correspond to 0% fluorescence in the Aligned Melt Curves Plot.

Review and adjust the pre- and post-melt regions

1. Display the Derivative Plot for the experiment of interest:
   a. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Derivative Melt** to view the Derivative Melt Plot.
2. Select **Target ▸ *<assay>*** to view the melt data for the desired assay.
3. In the Derivative Melt plot, set the pre-melt region:
   a. Click and drag the Pre-melt Stop temperature line (red arrow on the left) adjacent to the start of the melt transition region.
   b. Click and drag the Pre-melt Start temperature line (green arrow on the left) approximately 0.2°C to 0.5°C from the Pre-melt Stop temperature line.

   Note: The Pre-melt region should be within a flat area where there are no large spikes or slopes present in the fluorescence levels.
4. Set the post-melt region:
   a. Click and drag the Post-melt Start temperature line (black arrow on the right) adjacent to the end of the melt transition region.
   b. Click and drag the Post-melt Stop temperature line (yellow arrow on the right) approximately 0.2°C to 0.5°C from the Post-melt Start temperature line.

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

5. Click **Analyze**.

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

Confirm the genotype calls

The Applied Biosystems™ qPCR Analysis Software calls 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 Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
2. In the Quality Control & Results pane, select **Plot ▸ Difference** to review the Difference Melt Curve Plot.
3. From the View By drop-down list, select **Well Table** to review the genotyping calls generated by the software.
4. In the well table, click the **Well** column header to sort the results according to the well position.
5. For the positive controls, review:
   - **Variant Call column** – Do all of the positive control replicates have the correct call?
   - **Cq Confidence column** – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?

   Note: If any of the controls are outliers, omit them from the HRM analysis, then reanalyze.
6. For each replicate group, review:
   - **Variant Call column** – Do all replicates have the same call?
   - **Cq Confidence column** – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?
7. Select the rows in the Results table to view the corresponding fluorescence data in the Difference Melt Plot.
8. In the Results pane, click the **Variant** column header to sort the results according to the variant call. For each variant call, review the samples that are assigned that call.

Review the Multicomponent plot

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

Review the Multicomponent Plot for:

- MeltDoctor™ HRM dye or your custom reporter dye
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

1. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
2. In the Quality Control & Results tab, select all wells in the plate grid.
3. Select **Plot ▸ Multicomponent** to view the Multicomponent Plot.
4. Select **Target ▸ *<assay>*** to view the data for the desired assay.
5. Select one unknown well in the plate layout to display the corresponding data in the Multicomponent Plot.

   Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.
6. Click (View Options), then select **Color By ▸ Dye**.

   If the Legend is not displayed, also select **Show Legend**.
7. Confirm that the dye signals in the Multicomponent Plot increase throughout the PCR, indicating normal amplification.
8. In the Well Table or Plate Grid, select all negative control wells, then confirm that they did not amplify. If amplification has taken place, the negative controls may be contaminated.

When reviewing the Multicomponent Plot, look for:

- **Reporter dye** – The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- **Any irregularities in the signal** – There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative control wells** – There should not be any amplification in the negative control wells.

Review the Amplification Plot

If you collected real-time data for your experiment, review the amplification data to further understand the flags triggered by the experiment data. The Amplification Plot screen displays amplification of all samples in the selected wells. Use the amplification plots to confirm the results of the experiment:

- **ΔRn vs Cycle** – This plot displays ΔRn as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle** – This plot displays Rn as a function of cycle number. You can use this plot to identify and examine irregular amplification.
- **C_T vs Well** – This plot displays C_T as a function of well position. You can use this plot to locate outlying amplification (outliers)

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

1. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
2. In the Quality Control & Results tab, select all wells in the plate grid.
3. Select **Plot ▸ Amplification** to view the Multicomponent Plot.
4. Select **Target ▸ *<assay>*** to view the data for the desired assay.
5. In the Amplification Plot, click (View Options), then select:
   - **Plot Type ▸ ΔRn vs Cycle**
   - **Color By ▸ Sample**
   - **Graph Type ▸ Log**

   If the Legend is not displayed, also select **Show Legend**.
6. Verify that the threshold is set correctly.
7. Repeat above steps for all targets.
8. In the Amplification Plot, click (View Options), then select:
   - **Plot Type ▸ C_T vs Well**
   - **Color By ▸ Sample**
9. Confirm that the populations of technical replicates have achieved similar amplification.

When you analyze the Amplification Plot, look for:

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

Perform manual calls

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

1. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
2. In the Quality Control & Results pane, select the experiment of interest.
3. In the Review Result screen, select one or more wells within a Melt Curve Plot, Plate Layout, or Well Table.
4. Click **Actions**, then select **Manual Call**.
5. From the Manual Call dialog box, you can assign the sample to:
   - **An existing variant call** - Click **Select Existing**, select the appropriate call from the Group drop-down menu, then click **OK**.
   - **A new variant call** - Click **Create New**, enter a name for the new call in the Group field, select a color, then click **OK**.

   In the Plate Layout tab, the upper right corner of the sample well is marked with a red triangle.
   In the Well Table tab, in the Method column, Manual appears next to the selected sample.
6. Repeat the steps above to assign more manual calls.
7. Click **Analyze** to reanalyze the data using the manual calls.

Omit wells from the analysis

To omit the data from one or more wells that you do not want included in the analysis:

- Select one or more wells in a plot or table, then click **Actions** ▸ **Omit**. After the wells are omitted, click **Analyze** to reanalyze the project without the omitted well(s).

5 Analyze an HRM mutation detection experiment

HRM mutation detection analysis workflow

The following workflow illustrates the general steps required to perform a high-resolution melt curve mutation detection experiment using MeltDoctor™ HRM Reagents on an Applied Biosystems™ Real- Time PCR System followed by analysis using the Applied Biosystems™ qPCR Analysis Software.

Calibrate your instrument

▼

Design your HRM experiment

▼

Perform the experiment

▼

Analyze the data

Create a project and add experiment data

▼

(Optional) Add and define samples and targets

▼

Review/edit the sample, target, task, and control assignments

▼

Review the results of the analysis and adjust the settings (if necessary)

▼

Publish the project data

▼

Sequence the variants

Configure your analysis project

After you create your HRM project using the Thermo Fisher Cloud and populate it with experiments, use the Applied Biosystems™ qPCR Analysis Software HRM Module to correct any experiment settings prior to the analysis.

Note: As an alternative to using the Thermo Fisher Cloud or the Applied Biosystems™ HRM Analysis Module as described in this guide, you can use the Applied Biosystems™ High Resolution Melt Software v3.1 (Cat. No. A29881) to analyze your HRM experiments. The High Resolution Melt Software is a Windows™-compatible desktop application that performs high resolution melt (HRM) analysis of experiments generated by Applied Biosystems™ Real-Time PCR Systems, including the QuantStudio™ 3 and 5 Real-Time PCR Systems.

Log into the Thermo Fisher Cloud

Analysis of the HRM experiment data is performed using the Applied Biosystems™ qPCR Analysis Software HRM Module, which is available on the Thermo Fisher Cloud. The Thermo Fisher Cloud is a cloud storage and applications environment that provides scientists with a secure location to store, analyze, and share data. If connected to the internet, you can upload your HRM experiment data directly from your Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument or upload the experiment files from anywhere you have web access.

Note: For more information on the Thermo Fisher Cloud, go to **https://www.thermofisher.com/cloud**.

To log into the Thermo Fisher Cloud:

- Using an internet browser, go to **https://apps.thermofisher.com** , then log into the Thermo Fisher Cloud using your user account and password.

  If you do not have a Thermo Fisher Cloud user account, click **Create an account**, then follow the on-screen instructions to create an account for your projects. The Applied Biosystems™ qPCR Analysis Software HRM Module is available from within the Thermo Fisher Cloud.

Create a project and add experiment data

1. Click (Manage Projects) to view the Dashboard.
2. Create the project:
   a. Click **New Project**.
   b. In the Create Project dialog box, enter a name for the project, select the folder within which you want to place the project, then click **OK**.

      Note: The project name cannot exceed 50 characters and cannot include any of the following characters: / \ < > * ? " | : ; & % $ @ ^ ( ) !

3. From the Manage Data screen, add any additional experiment data to the project.

To import experiment data stored on...	Action
Your computer	1. Click **Import from local**. 2. From the Open dialog box, select one or more experiment files (.sds or .eds), then click **Open**. **Note:** Ctrl- or Shift-click to select multiple files. Wait for the Applied Biosystems™ Software to upload the selected data. 3. Click **Close** prompted that the import is complete.
Thermo Fisher Cloud	1. Click **Import from Thermo Fisher Cloud**. 2. Select one or more experiment files (.sds or .eds) from the table, then click **Add**. 3. When you are done adding files to the queue, click **OK**. 4. Click **Close** prompted that the import is complete.

4. Repeat step 3 until your project contains all of the desired experiment data.

5. Click the appropriate analysis module on the left side of the screen to begin the analysis.

Manage samples and targets

The Applied Biosystems™ qPCR Analysis Software populates the Overview screen with the samples and targets present in the experiments added to the project. If necessary, you can add, edit, or remove the samples and targets as needed before the analysis.

- **Create** a new sample or target:
  a. From the Samples or Targets table in the Overview screen, click **Actions** ▸ **Add**.
  b. In the New Sample/Target dialog box, enter a name for the new sample or target (up to 256 characters), then edit the properties of the new sample/target.
  c. Click **OK**.
- **Update** an existing sample or target by editing the entry directly in the table.
  Note: Alternately, select a sample or target from the table, then select **Actions** ▸ **Update**.
- **Delete** a sample or target:
  a. From the Samples or Targets table in the Overview screen, select the sample or target of interest, then click **Actions** ▸ **Delete**.
  b. In the confirmation dialog box, click **OK** to delete the sample or target.

Manage HRM controls

The Applied Biosystems™ qPCR Analysis Software populates the Overview screen with the controls present in the experiments added to the project.

If necessary, you can add, edit, or remove controls as needed before the analysis:

- **Create** a new control:
  a. From the Controls table in the Overview screen, click **Actions** ▸ **Add**.
  b. In the New Control dialog box, enter a name for the new control (up to 256 characters), then edit the properties of the new control.
  c. Click **OK**.
- **Update** an existing control by editing the entry directly in the table.

  Note: Alternately, select a control from the table, then select **Actions** ▸ **Update**.
- **Delete** a control:
  a. From the Controls table in the Overview screen, select the control of interest, then click **Actions** ▸ **Delete**.
  b. In the confirmation dialog box, click **OK** to delete the control.

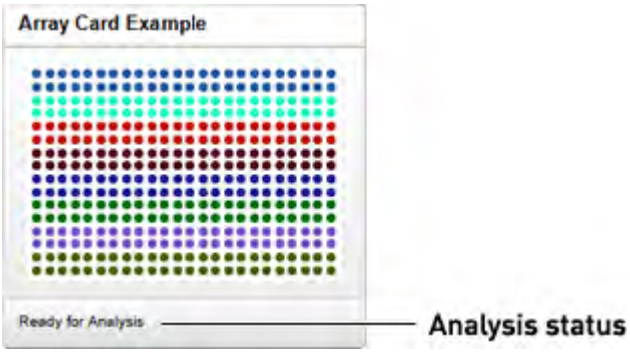
Review and edit the plate setups

After configuring your project with all necessary samples and targets, use the Plate Setup screen to review the experiments for problems that can prevent the analysis of the project. The Applied Biosystems™ qPCR Analysis Software displays plate configuration errors that can prohibit analysis in the margin beneath each image of the related experiment. Before you can analyze your project, you must use the Plate Setup screen to address them.

To review the plate setup information for your project:

1. Select **Plate Setup** to display Plate Setup screen.
2. From the Plate Setup screen, review the experiment records for errors.
3. If errors are present, click the experiment record of interest and address the problem that is preventing the analysis of the file.

   Note: The software displays plate configuration problems that will prevent analysis of an experiment beneath the image of the related plate.



Apply samples and targets

If the sample or target assignments of one or more of your experiments contain errors or are missing, you can use the Applied Biosystems™ qPCR Analysis Software to correct the problem prior to analysis.

Note: When reviewing a plate layout, click **Actions ▸ Clear Well Setup** to remove the well information (sample, task, and target assignments) from the selected wells in the plate grid.

1. From the Plate Setup screen, select the experiment that you want to modify.
2. (Optional) From the Edit Plate screen, click **View**, then select **Target** and **Sample** to color the plate setup according to the element that you intend to modify.
3. Select the wells of the plate layout to which you want to apply the target or sample.
4. When the wells are selected, click the appropriate field to the right of the plate grid, then select the appropriate item from the list.

   Note: If you have not yet created a sample or target, enter the name in the appropriate field and press **Enter** to create the new sample or target.
5. Once you are finished making changes to the plate layout, click **Analyze** to reanalyze your project.

Specify and assign tasks

If the task assignments of one or more of your experiments contain errors or are missing, you can use the Applied Biosystems™ qPCR Analysis Software to correct the problem prior to analysis.

Note: When reviewing a plate layout, click **Actions ▸ Clear Well Setup** to remove the well information (sample, task, and target assignments) from the selected wells in the plate grid.

1. From the Plate Setup screen, select the experiment record that you want to modify.
2. From the Edit Plate screen, click **View**, then select **Task** to color the plate setup according to task assignment.
3. Select the wells of the plate layout to which you want to apply a task.

4. When the wells are selected, click the **Task** menu, then select the appropriate task from the list.

   Available tasks include:

   - **Unknown** – The task for wells that contain a sample with unknown genotype, variant content, or percentage methylation.
   - **NTC** – The task for wells that contain water or buffer instead of sample (no template controls). No amplification of the target should occur in negative control wells.
   - **Positive Controls** - Wells that contain one of the following:
     - A template known to generate a specific genotype call for one or both alleles (**Heterozygote** or **Homozygote**).
     - A wild type control (**Wild Type**).
     - Methylated DNA standards that contain from 0% to 100% methylated DNA (add a custom control label).

   Note: The Task/Control dropdown list displays the controls present in the Controls pane of the Overview screen. See "Manage HRM controls" on page 34 for information on adding and editing controls.

5. Repeat steps 3 and 4 as needed.

6. Once you have completed making changes to the plate layout, click **Analyze** to reanalyze your project.

Review HRM mutation detection data

The Applied Biosystems™ qPCR Analysis Software High Resolution Melt module supports the analysis of HRM mutation detection experiments, where melt curve technique is used to screen DNA samples for new single-base changes, insertions/deletions, or other unknown mutations. During the analysis, the software compares the melt curves of unknown samples to those generated from one or more DNA samples that contain wild type sequences (positive controls). Based on the comparison, the software assigns calls to the unknown samples according to the fit of the unknown melt curves to those of the controls. For the unknowns, possible calls are either "wild type" if the sample matches a control or "variant X" if the sample does not.

In general, review of genotyping results occurs in the following steps:

1. Define the analysis settings, then analyze the project.
2. Perform an initial review of the High Resolution Melt Plots, the Plate Layout, and the Well Table to evaluate the calls made by the Applied Biosystems™ Software.
3. Perform a thorough review of the QC Summary to evaluate the samples that triggered QC flags. Review the raw data and amplification data for the samples that exhibit abnormal amplification.
4. If necessary, adjust the analysis settings or modify the calls manually.

After evaluating the results, publish the analyzed data.

Configure the analysis settings

When a project is created, the Applied Biosystems™ qPCR Analysis Software processes the project data using the default analysis settings of the experiments added to the project. If desired, you can modify the analysis settings from the Quality Control & Results screen (for example, manual versus automatic thresholding or stringent versus relaxed quality thresholds).

1. From the Quality Control & Results screen, select an experiment of interest.
2. From the Review Result screen, click **Analysis Settings**.
3. From the Edit Analysis Setting dialog box, modify the analysis settings as desired.

Group	Settings
Target Settings	Select the method (automatic or manual) that the Applied Biosystems™ Software will use to compute the pre- and post-melt ranges, grouping, and variant removal: • Define whether the pre- and post-melt range for each target will be computed automatically or manually: – **Auto Set Melt Range** – If you are using automatic settings, select the checkbox to have the software calculate the pre- and post- melt ranges for the specific target. – **Pre-Melt Start/Stop** and **Post-Melt Start/Stop** – If you are using manual settings, enter the manual pre- and post-range values for the appropriate targets. • Define whether the genotype groups for each target will be determined automatically or manually: – **Auto Determine # of Groups** – If you are using automatic settings, select the checkbox to have the software calculate the number of groups for the specific target. – **Number of Groups** – If you are using manual settings, enter the number of groups for the appropriate targets. • **Remove all Manual Variants on reanalysis** – Select to have the software omit from the analysis all wells that have been manually been labeled as a variant.
Flag Settings	Specify the quality measures that the Applied Biosystems™ Software will compute during the analysis. 1. In the Use column, select the check boxes for flags you want to apply during analysis. 2. If an attribute, condition, and value are listed for a flag, you can specify the setting for applying the flag. For example, with the default setting for the no amplification flag (NOAMP), wells are flagged if the amplification algorithm result is less than 0.1. **Note:** If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting. 3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag. Rejected wells are not considered for data analysis.

4. When done modifying the analysis settings, click **Finish**.

Review the quality data

After the Applied Biosystems™ qPCR Analysis Software processes your project, you can use the Quality Control & Results screen to review the quality data generated by the analysis. The software provides a variety of options to review the quality data; however, the strategy that you employ will depend on the type of experiment you are performing and the samples/targets that you are evaluating. The following procedure describes a general approach to data review and provides an overview of the software features.

1. If you have not already done so, click **Analyze** to analyze your project.
2. Click **Quality Control & Results** to view the Quality Control & Results screen.
3. Review the experiment data for quality flags generated during the analysis.

   Note: The Applied Biosystems™ Software displays summaries of the quality data in the margin beneath each experiment. You can view the identity of the triggered flags by mousing over an experiment of interest.

   Alternatively, to quickly find the experiments in your project that generated quality flags, click (Plate View), then click the Flags column heading to sort the experiments that generated quality flags to the top of the table. To examine the data that triggered the flag, click the corresponding link in the Name column.

   In response to the presence of quality flags, consider the following resolutions:

   - Change the quality settings in the analysis settings:
     - Adjust the sensitivity of the quality flags so that more wells or fewer wells are flagged.
     - Deactivate the quality flags that triggered by the data.
   - Omit individual wells from the analysis.
4. If flags or irregularities are present, or you would like to review the melt curve data for a specific experiment, click the experiment of interest.
5. When viewing an experiment, display the Well Table:

Tool	Use this tool to...
Mouse/cursor	Select wells. To select: • An individual well, select the well in the Well table. • More than one well at a time, press the **Ctrl** key or **Shift** key when you select the wells in the Well table. When you select wells in the Well table, the corresponding data points are selected in the amplification plot.
Actions menu	**Omit/Un-Omit** well from the analysis. After you omit or un-omit a well, click **Analyze** to reanalyze the project. For omitted wells, the software: • Does not display data in the Well table (data are either Unknown or empty/blank). • Does not include the omitted wells in the analysis.
or	Expand or collapse the Well table by clicking the appropriate symbol in the vertical bar between the plot and table.

6. Review the data in the Well Table data.

Column	Description
Well	The location of the well on the reaction plate. For example, P18 indicates that the sample is found in row P, column 18.
Omit	The omission status of the related well.
Target	The ID (a unique name or number) of the nucleic acid sequence targeted by the assay added to the well.
Sample	The ID (a unique name or number) of the sample.
Task/Control	The task assigned to the well, where the task is the function that a sample performs on the plate.
Variant	The call for the sample in well. Can be assigned by software (Auto) or manually.
Method	The method used to apply the call to the well: Auto(matic) if calculated by the Applied Biosystems™ qPCR Analysis Software or Manual if user-applied.
Silhouette Score	The *modified* silhouette score calculated for the well, which measures distinguishably of the associated melt curve relative to the other curves in the assay (Rousseeuw, 1987.). For each melt curve, the software calculates a *modified* silhouette score, which ranges from 0 to 100. A score closer to 100 indicates that a melt curve is more similar to curves assigned the same variant call than to curves called differently. Lower silhouette scores indicate that a melt curve is less similar to curves assigned the same variant call. **IMPORTANT!** The modified silhouette score differs from the standard silhouette score (Lovmar, *et. al.*, 2005.) in that the software assigns the score to each identified cluster instead of to each data point in the cluster. In addition, the modified value ranges from 0 to 100.
Tm1/Tm2/Tm3	The 1st, 2nd, and 3rd calculated melt temperature (Tm) for the well (if present). **Note:** Blank table cells indicate that the software calculated no Tm for the well at the indicated position.
Amp Status	The amplification status for the well: amplification, no amplification, reviewed, and undetermined.
Amp Score	The amplification score calculated for the well.
Cq Conf	The Cq confidence score calculated for the well.
Ct	The C_T calculated for the related well.
Ct Mean	The arithmetic mean generated from the C_Ts calculated for the technical replicates of the well.
Ct SD	The standard deviation generated from the C_Ts calculated for the technical replicates of the well.
Flags	The number of flags generated for the well.
Quality data	The quality flags generated by the associated well.

7. When ready, click ❮ to return to the thumbnails view.

Review the controls and replicate populations for outliers

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

1. Display the Aligned Melt Plot for the experiment of interest:
   a. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Aligned Melt** to view the Aligned Melt plot.
2. If using Wild type controls, confirm the calls for the positive controls:
   a. From the well table, select the wells containing a positive control to highlight the corresponding melt curve in the Aligned Melt Curves Plot.
   b. Confirm that the wild type controls cluster well and review the population for outliers.
   c. Repeat steps a and b for the wells containing the other positive controls.
3. Screen the negative controls to ensure that samples failed to amplify:
   a. From the well table, select the wells containing a negative control to highlight the corresponding melt curve in the Aligned Melt Curves Plot.
   b. Confirm that the selected wells in the well table are negative controls, and not unknown samples.

   Samples that grouped with the negative controls may:
   - Contain no DNA
   - Contain PCR inhibitors
   - Be homozygous for a sequence deletion
4. If you run replicate reactions, carefully review your data set for curves that do not align tightly with the other samples in the group (outliers). If outliers are present, confirm the results of the associated samples by retesting them.
5. Review the data:
   - **Possible mutations** – Review the curves for samples with melt curves that are different from the wild type melt curves.
   - **Unexpected peaks** – Confirm that the Derivative Melt Curve shows no unexpected Tm peaks. If the sequence you amplified contains more than one variant or a more complex mutation, you may see more than one Tm peak. Unexpected peaks can indicate contamination, primer dimers, or non-specific amplification.

Review and adjust the pre- and post-melt regions

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

About the pre- and post-melt regions

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

- **Pre-melt region** – The pair of lines to the left of the peak indicate the pre-melt Start and Stop temperatures when every amplicon is double-stranded. Fluorescence data from the pre-melt region corresponds to 100% fluorescence in the Aligned Melt Curves Plot.
- **Active melt region** – The data peak indicates the active melt region of the plot. Data from the active melt region are used to plot the Aligned Melt Curves Plot.
- **Post-melt region** – The set of lines to the right of the peak indicate the post-melt Start and Stop temperatures when every amplicon is single-stranded. Fluorescence data from the post-melt region correspond to 0% fluorescence in the Aligned Melt Curves Plot.

Review and adjust the pre- and post-melt regions

1. Display the Derivative Plot for the experiment of interest:
   a. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Derivative Melt** to view the Derivative Melt Plot.
2. Select **Target ▸ *<assay>*** to view the melt data for the desired assay.
3. In the Derivative Melt plot, set the pre-melt region:
   a. Click and drag the Pre-melt Stop temperature line (red arrow on the left) adjacent to the start of the melt transition region.
   b. Click and drag the Pre-melt Start temperature line (green arrow on the left) approximately 0.2°C to 0.5°C from the Pre-melt Stop temperature line.

   Note: The Pre-melt region should be within a flat area where there are no large spikes or slopes present in the fluorescence levels.
4. Set the post-melt region:
   a. Click and drag the Post-melt Start temperature line (black arrow on the right) adjacent to the end of the melt transition region.
   b. Click and drag the Post-melt Stop temperature line (yellow arrow on the right) approximately 0.2°C to 0.5°C from the Post-melt Start temperature line.

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

5. Click **Analyze**.

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

Review the populations in the Aligned Melt Plot

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

1. Display the Aligned Melt Plot for the experiment of interest:
   a. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Aligned Melt** to view the Aligned Melt plot.
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?
3. Repeat the steps for the remaining experiments in your project.

Review the Difference Melt Curves 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. Display the Difference Plot for the experiment of interest:
   a. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Difference Melt** to view the Difference Melt Plot.
2. From the Reference drop-down menu, select a control or any well as the reference, then review:
   - **Variant clusters** – How many distinct clusters are displayed?
   - **Outliers** – How tight are the curves within each variant cluster?

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

Confirm the software calls

The Applied Biosystems™ qPCR Analysis Software calls 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. Display the Aligned Plot for the experiment of interest:
   a. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Aligned Melt** to view the Aligned Melt Plot.
2. In the Well Table, select **Well Table** from the View By drop-down list to review the calls generated by the software.
3. In the well table, click the **Well** column header to sort the results according to the well position.
4. For each replicate group, review:
   - **Variant Call column** – Do all replicates have the same call?
   - **Confidence column** – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?
5. Select the rows in the Results table to view the corresponding fluorescence data in the Aligned Melt Plot.
6. In the well table, click the **Variant** 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.

Perform manual calls

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

1. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
2. In the Quality Control & Results pane, select the experiment of interest.
3. In the Review Result screen, select one or more wells within a Melt Curve Plot, Plate Layout, or Well Table.
4. Click **Actions**, then select **Manual Call**.

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

   In the Plate Layout tab, the upper right corner of the sample well is marked with a red triangle.
   In the Well Table tab, in the Method column, Manual appears next to the selected sample.
6. Repeat the steps above to assign more manual calls.
7. Click **Analyze** to reanalyze the data using the manual calls.

Omit wells from the analysis

To omit the data from one or more wells that you do not want included in the analysis:

- Select one or more wells in a plot or table, then click **Actions** ▸ **Omit**. After the wells are omitted, click **Analyze** to reanalyze the project without the omitted well(s).

(*Optional*) Sequence the variants

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

6 Analyze an HRM methylation study

HRM methylation study analysis workflow

The following workflow illustrates the general steps required to perform a high-resolution melt curve methylation experiment using MeltDoctor™ HRM Reagents on an Applied Biosystems™ Real- Time PCR System followed by analysis using the Applied Biosystems™ qPCR Analysis Software.

Calibrate your instrument

▼

Design your HRM experiment

▼

Perform the experiment

▼

Analyze the data

Create a project and add experiment data

▼

(Optional) Add and define samples and targets

▼

Review/edit the sample, target, task, and control assignments

▼

Review the results of the analysis and adjust the settings (if necessary)

▼

Publish the project data

▼

Sequence the variants

Configure your analysis project

After you create your HRM project using the Thermo Fisher Cloud and populate it with experiments, use the Applied Biosystems™ qPCR Analysis Software HRM Module to correct any experiment settings prior to the analysis.

Note: As an alternative to using the Thermo Fisher Cloud or the Applied Biosystems™ HRM Analysis Module as described in this guide, you can use the Applied Biosystems™ High Resolution Melt Software v3.1 (Cat. No. A29881) to analyze your HRM experiments. The High Resolution Melt Software is a Windows™-compatible desktop application that performs high resolution melt (HRM) analysis of experiments generated by Applied Biosystems™ Real-Time PCR Systems, including the QuantStudio™ 3 and 5 Real-Time PCR Systems.

Log into the Thermo Fisher Cloud

Analysis of the HRM experiment data is performed using the Applied Biosystems™ qPCR Analysis Software HRM Module, which is available on the Thermo Fisher Cloud. The Thermo Fisher Cloud is a cloud storage and applications environment that provides scientists with a secure location to store, analyze, and share data. If connected to the internet, you can upload your HRM experiment data directly from your Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument or upload the experiment files from anywhere you have web access.

Note: For more information on the Thermo Fisher Cloud, go to **https://www.thermofisher.com/cloud**.

To log into the Thermo Fisher Cloud:

- Using an internet browser, go to **https://apps.thermofisher.com** , then log into the Thermo Fisher Cloud using your user account and password.

  If you do not have a Thermo Fisher Cloud user account, click **Create an account**, then follow the on-screen instructions to create an account for your projects. The Applied Biosystems™ qPCR Analysis Software HRM Module is available from within the Thermo Fisher Cloud.

Create a project and add experiment data

1. Click (Manage Projects) to view the Dashboard.
2. Create the project:
   a. Click **New Project**.
   b. In the Create Project dialog box, enter a name for the project, select the folder within which you want to place the project, then click **OK**.

      Note: The project name cannot exceed 50 characters and cannot include any of the following characters: / \ < > * ? " | : ; & % $ @ ^ ( ) !

3. From the Manage Data screen, add any additional experiment data to the project.

To import experiment data stored on...	Action
Your computer	1. Click **Import from local**. 2. From the Open dialog box, select one or more experiment files (.sds or .eds), then click **Open**. **Note:** Ctrl- or Shift-click to select multiple files. Wait for the Applied Biosystems™ Software to upload the selected data. 3. Click **Close** prompted that the import is complete.
Thermo Fisher Cloud	1. Click **Import from Thermo Fisher Cloud**. 2. Select one or more experiment files (.sds or .eds) from the table, then click **Add**. 3. When you are done adding files to the queue, click **OK**. 4. Click **Close** prompted that the import is complete.

4. Repeat step 3 until your project contains all of the desired experiment data.

5. Click the appropriate analysis module on the left side of the screen to begin the analysis.

Manage samples and targets

The Applied Biosystems™ qPCR Analysis Software populates the Overview screen with the samples and targets present in the experiments added to the project. If necessary, you can add, edit, or remove the samples and targets as needed before the analysis.

- **Create** a new sample or target:
  a. From the Samples or Targets table in the Overview screen, click **Actions ▸ Add**.
  b. In the New Sample/Target dialog box, enter a name for the new sample or target (up to 256 characters), then edit the properties of the new sample/target.
  c. Click **OK**.
- **Update** an existing sample or target by editing the entry directly in the table.
  Note: Alternately, select a sample or target from the table, then select **Actions ▸ Update**.
- **Delete** a sample or target:
  a. From the Samples or Targets table in the Overview screen, select the sample or target of interest, then click **Actions ▸ Delete**.
  b. In the confirmation dialog box, click **OK** to delete the sample or target.

Manage HRM controls

The Applied Biosystems™ qPCR Analysis Software populates the Overview screen with the controls present in the experiments added to the project.

If necessary, you can add, edit, or remove controls as needed before the analysis:

- **Create** a new control:
  a. From the Controls table in the Overview screen, click **Actions** ▸ **Add**.
  b. In the New Control dialog box, enter a name for the new control (up to 256 characters), then edit the properties of the new control.
  c. Click **OK**.
- **Update** an existing control by editing the entry directly in the table.

  Note: Alternately, select a control from the table, then select **Actions** ▸ **Update**.
- **Delete** a control:
  a. From the Controls table in the Overview screen, select the control of interest, then click **Actions** ▸ **Delete**.
  b. In the confirmation dialog box, click **OK** to delete the control.

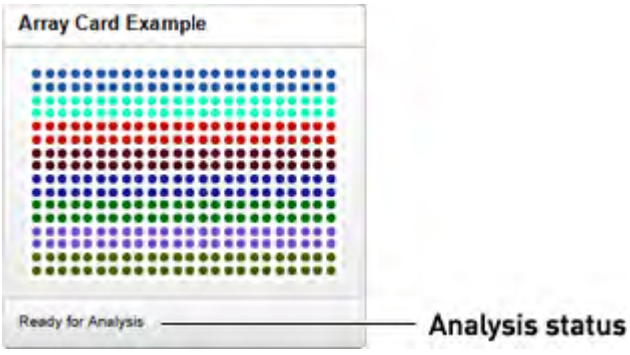
Review and edit the plate setups

After configuring your project with all necessary samples and targets, use the Plate Setup screen to review the experiments for problems that can prevent the analysis of the project. The Applied Biosystems™ qPCR Analysis Software displays plate configuration errors that can prohibit analysis in the margin beneath each image of the related experiment. Before you can analyze your project, you must use the Plate Setup screen to address them.

To review the plate setup information for your project:

1. Select **Plate Setup** to display Plate Setup screen.
2. From the Plate Setup screen, review the experiment records for errors.
3. If errors are present, click the experiment record of interest and address the problem that is preventing the analysis of the file.

   Note: The software displays plate configuration problems that will prevent analysis of an experiment beneath the image of the related plate.



Apply samples and targets

If the sample or target assignments of one or more of your experiments contain errors or are missing, you can use the Applied Biosystems™ qPCR Analysis Software to correct the problem prior to analysis.

Note: When reviewing a plate layout, click **Actions ▸ Clear Well Setup** to remove the well information (sample, task, and target assignments) from the selected wells in the plate grid.

1. From the Plate Setup screen, select the experiment that you want to modify.
2. (Optional) From the Edit Plate screen, click **View**, then select **Target** and **Sample** to color the plate setup according to the element that you intend to modify.
3. Select the wells of the plate layout to which you want to apply the target or sample.
4. When the wells are selected, click the appropriate field to the right of the plate grid, then select the appropriate item from the list.

   Note: If you have not yet created a sample or target, enter the name in the appropriate field and press **Enter** to create the new sample or target.
5. Once you are finished making changes to the plate layout, click **Analyze** to reanalyze your project.

Specify and assign tasks

If the task assignments of one or more of your experiments contain errors or are missing, you can use the Applied Biosystems™ qPCR Analysis Software to correct the problem prior to analysis.

Note: When reviewing a plate layout, click **Actions ▸ Clear Well Setup** to remove the well information (sample, task, and target assignments) from the selected wells in the plate grid.

1. From the Plate Setup screen, select the experiment record that you want to modify.
2. From the Edit Plate screen, click **View**, then select **Task** to color the plate setup according to task assignment.
3. Select the wells of the plate layout to which you want to apply a task.

4. When the wells are selected, click the **Task** menu, then select the appropriate task from the list.

   Available tasks include:

   - **Unknown** – The task for wells that contain a sample with unknown genotype, variant content, or percentage methylation.
   - **NTC** – The task for wells that contain water or buffer instead of sample (no template controls). No amplification of the target should occur in negative control wells.
   - **Positive Controls** - Wells that contain one of the following:
     - A template known to generate a specific genotype call for one or both alleles (**Heterozygote** or **Homozygote**).
     - A wild type control (**Wild Type**).
     - Methylated DNA standards that contain from 0% to 100% methylated DNA (add a custom control label).

   Note: The Task/Control dropdown list displays the controls present in the Controls pane of the Overview screen. See "Manage HRM controls" on page 34 for information on adding and editing controls.

5. Repeat steps 3 and 4 as needed.

6. Once you have completed making changes to the plate layout, click **Analyze** to reanalyze your project.

Review HRM methylation data

The Applied Biosystems™ qPCR Analysis Software High Resolution Melt module supports the analysis of HRM methylation experiments, where melt curve technique is used to determine the percentage of methylated DNA in unknown samples. During the analysis, the software compares the melt curves of the unknown samples against those generated from a set of methylated DNA standards (positive controls). Based on the comparison, the software assigns calls to the unknown samples according to the fit of the unknown melt curves to those of the standard ladder.

In general, review of HRM methylation results occurs in the following steps:

1. Define the analysis settings, then analyze the project.
2. Perform an initial review of the High Resolution Melt Plots, the Plate Layout, and the Well Table to evaluate the calls made by the Applied Biosystems™ Software.
3. Perform a thorough review of the QC Summary to evaluate the samples that triggered QC flags. Review the raw data and amplification data for the samples that exhibit abnormal amplification.
4. If necessary, adjust the analysis settings or modify the calls manually.

After evaluating the results, publish the analyzed data.

Configure the analysis settings

When a project is created, the Applied Biosystems™ qPCR Analysis Software processes the project data using the default analysis settings of the experiments added to the project. If desired, you can modify the analysis settings from the Quality Control & Results screen (for example, manual versus automatic thresholding or stringent versus relaxed quality thresholds).

1. From the Quality Control & Results screen, select an experiment of interest.
2. From the Review Result screen, click **Analysis Settings**.
3. From the Edit Analysis Setting dialog box, modify the analysis settings as desired.

Group	Settings
Target Settings	Select the method (automatic or manual) that the Applied Biosystems™ Software will use to compute the pre- and post-melt ranges, grouping, and variant removal: • Define whether the pre- and post-melt range for each target will be computed automatically or manually: – **Auto Set Melt Range** – If you are using automatic settings, select the checkbox to have the software calculate the pre- and post- melt ranges for the specific target. – **Pre-Melt Start/Stop** and **Post-Melt Start/Stop** – If you are using manual settings, enter the manual pre- and post-range values for the appropriate targets. • Define whether the genotype groups for each target will be determined automatically or manually: – **Auto Determine # of Groups** – If you are using automatic settings, select the checkbox to have the software calculate the number of groups for the specific target. – **Number of Groups** – If you are using manual settings, enter the number of groups for the appropriate targets. • **Remove all Manual Variants on reanalysis** – Select to have the software omit from the analysis all wells that have been manually been labeled as a variant.
Flag Settings	Specify the quality measures that the Applied Biosystems™ Software will compute during the analysis. 1. In the Use column, select the check boxes for flags you want to apply during analysis. 2. If an attribute, condition, and value are listed for a flag, you can specify the setting for applying the flag. For example, with the default setting for the no amplification flag (NOAMP), wells are flagged if the amplification algorithm result is less than 0.1. **Note:** If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting. 3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag. Rejected wells are not considered for data analysis.

4. When done modifying the analysis settings, click **Finish**.

Review the quality data

After the Applied Biosystems™ qPCR Analysis Software processes your project, you can use the Quality Control & Results screen to review the quality data generated by the analysis. The software provides a variety of options to review the quality data; however, the strategy that you employ will depend on the type of experiment you are performing and the samples/targets that you are evaluating. The following procedure describes a general approach to data review and provides an overview of the software features.

1. If you have not already done so, click **Analyze** to analyze your project.
2. Click **Quality Control & Results** to view the Quality Control & Results screen.
3. Review the experiment data for quality flags generated during the analysis.

   Note: The Applied Biosystems™ Software displays summaries of the quality data in the margin beneath each experiment. You can view the identity of the triggered flags by mousing over an experiment of interest.

   Alternatively, to quickly find the experiments in your project that generated quality flags, click (Plate View), then click the Flags column heading to sort the experiments that generated quality flags to the top of the table. To examine the data that triggered the flag, click the corresponding link in the Name column.

   In response to the presence of quality flags, consider the following resolutions:

   - Change the quality settings in the analysis settings:
     - Adjust the sensitivity of the quality flags so that more wells or fewer wells are flagged.
     - Deactivate the quality flags that triggered by the data.
   - Omit individual wells from the analysis.
4. If flags or irregularities are present, or you would like to review the melt curve data for a specific experiment, click the experiment of interest.
5. When viewing an experiment, display the Well Table:

Tool	Use this tool to...
Mouse/cursor	Select wells. To select: • An individual well, select the well in the Well table. • More than one well at a time, press the **Ctrl** key or **Shift** key when you select the wells in the Well table. When you select wells in the Well table, the corresponding data points are selected in the amplification plot.
Actions menu	**Omit/Un-Omit** well from the analysis. After you omit or un-omit a well, click **Analyze** to reanalyze the project. For omitted wells, the software: • Does not display data in the Well table (data are either Unknown or empty/blank). • Does not include the omitted wells in the analysis.
or	Expand or collapse the Well table by clicking the appropriate symbol in the vertical bar between the plot and table.

6. Review the data in the Well Table data.

Column	Description
Well	The location of the well on the reaction plate. For example, P18 indicates that the sample is found in row P, column 18.
Omit	The omission status of the related well.
Target	The ID (a unique name or number) of the nucleic acid sequence targeted by the assay added to the well.
Sample	The ID (a unique name or number) of the sample.
Task/Control	The task assigned to the well, where the task is the function that a sample performs on the plate.
Variant	The call for the sample in well. Can be assigned by software (Auto) or manually.
Method	The method used to apply the call to the well: Auto(matic) if calculated by the Applied Biosystems™ qPCR Analysis Software or Manual if user-applied.
Silhouette Score	The *modified* silhouette score calculated for the well, which measures distinguishably of the associated melt curve relative to the other curves in the assay (Rousseeuw, 1987.). For each melt curve, the software calculates a *modified* silhouette score, which ranges from 0 to 100. A score closer to 100 indicates that a melt curve is more similar to curves assigned the same variant call than to curves called differently. Lower silhouette scores indicate that a melt curve is less similar to curves assigned the same variant call. **IMPORTANT!** The modified silhouette score differs from the standard silhouette score (Lovmar, *et. al.*, 2005.) in that the software assigns the score to each identified cluster instead of to each data point in the cluster. In addition, the modified value ranges from 0 to 100.
Tm1/Tm2/Tm3	The 1st, 2nd, and 3rd calculated melt temperature (Tm) for the well (if present). **Note:** Blank table cells indicate that the software calculated no Tm for the well at the indicated position.
Amp Status	The amplification status for the well: amplification, no amplification, reviewed, and undetermined.
Amp Score	The amplification score calculated for the well.
Cq Conf	The Cq confidence score calculated for the well.
Ct	The C_T calculated for the related well.
Ct Mean	The arithmetic mean generated from the C_Ts calculated for the technical replicates of the well.
Ct SD	The standard deviation generated from the C_Ts calculated for the technical replicates of the well.
Flags	The number of flags generated for the well.
Quality data	The quality flags generated by the associated well.

7. When ready, click ❮ to return to the thumbnails view.

Review the controls and replicate populations for outliers

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

1. Display the Aligned Plot for the experiment of interest:
   a. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Aligned Melt** to view the Aligned Melt Plot.
2. Screen the negative controls to ensure that samples failed to amplify:
   a. From the well table, select the wells containing a negative control to highlight the corresponding melt curve in the Aligned Melt Curves Plot.
   b. Confirm that the selected wells in the well table are negative controls, and not unknown samples.

   Samples that grouped with the negative controls may:
   - Contain no DNA
   - Contain PCR inhibitors
   - Be homozygous for a sequence deletion
3. If you run replicate reactions, carefully review your data set for curves that do not align tightly with the other samples in the group (outliers). If outliers are present, confirm the results of the associated samples by retesting them.
4. Review the data:
   - **Methylated DNA standards** – Do the melt curves for the methylated DNA standards cluster well? Are there any outliers?
   - **Define methylation range for unknowns** – Which methylated standard melt curves are above and below the melt curves for the unknowns? For example, if the melt curve for an unknown sample lies between the melt curves for the 5% and 10% methylated standards, the unknown sample contains between 5% and 10% methylated nucleotides.

Review and adjust the pre- and post-melt regions

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

About the pre- and post-melt regions

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

- **Pre-melt region** – The pair of lines to the left of the peak indicate the pre-melt Start and Stop temperatures when every amplicon is double-stranded. Fluorescence data from the pre-melt region corresponds to 100% fluorescence in the Aligned Melt Curves Plot.
- **Active melt region** – The data peak indicates the active melt region of the plot. Data from the active melt region are used to plot the Aligned Melt Curves Plot.
- **Post-melt region** – The set of lines to the right of the peak indicate the post-melt Start and Stop temperatures when every amplicon is single-stranded. Fluorescence data from the post-melt region correspond to 0% fluorescence in the Aligned Melt Curves Plot.

Review and adjust the pre- and post-melt regions

1. Display the Derivative Plot for the experiment of interest:
   a. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Derivative Melt** to view the Derivative Melt Plot.
2. Select **Target ▸ *<assay>*** to view the melt data for the desired assay.
3. In the Derivative Melt plot, set the pre-melt region:
   a. Click and drag the Pre-melt Stop temperature line (red arrow on the left) adjacent to the start of the melt transition region.
   b. Click and drag the Pre-melt Start temperature line (green arrow on the left) approximately 0.2°C to 0.5°C from the Pre-melt Stop temperature line.

   Note: The Pre-melt region should be within a flat area where there are no large spikes or slopes present in the fluorescence levels.
4. Set the post-melt region:
   a. Click and drag the Post-melt Start temperature line (black arrow on the right) adjacent to the end of the melt transition region.
   b. Click and drag the Post-melt Stop temperature line (yellow arrow on the right) approximately 0.2°C to 0.5°C from the Post-melt Start temperature line.

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

5. Click **Analyze**.

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

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 any control or well as a reference to subtract the selected curve from the others.

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

1. Display the Difference Plot for the experiment of interest:
   a. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Difference Melt** to view the Difference Melt Plot.
2. From the **Reference** drop-down menu, select a control or any well as the reference, then review:
   - **Variant clusters** – How many distinct clusters are displayed?
   - **Outliers** – How tight are the curves within each variant cluster?
3. Repeat step 3 to review the data using the other reference samples to find the optimal display of the clusters.

Confirm the software calls

The Applied Biosystems™ qPCR Analysis Software calls 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. Display the Difference Plot for the experiment of interest:
   a. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Difference Melt** to view the Difference Melt Plot.
2. From the View By drop-down list, select **Well Table** to review the calls generated by the software.
3. In the well table, click the **Well** column header to sort the results according to the well position.

4. For the methylation standard controls, review:
   - **Variant Call column** – Do all of the methylation standard controls have the correct call?
   - **Confidence column** – Are there any outliers within the replicate group? Do the values for the replicate group differ from the confidence values for the other replicate groups in the plate?

   Note: If any of the controls are outliers, omit them from the HRM analysis, then reanalyze.

5. Select the rows in the Results table to view the corresponding fluorescence data in the Difference Melt Plot.

Perform manual calls

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

1. In the Applied Biosystems™ qPCR Analysis Software, select the **Quality Control & Results** tab.
2. In the Quality Control & Results pane, select the experiment of interest.
3. In the Review Result screen, select one or more wells within a Melt Curve Plot, Plate Layout, or Well Table.
4. Click **Actions**, then select **Manual Call**.
5. From the Manual Call dialog box, you can assign the sample to:
   - **An existing variant call** - Click **Select Existing**, select the appropriate call from the Group drop-down menu, then click **OK**.
   - **A new variant call** - Click **Create New**, enter a name for the new call in the Group field, select a color, then click **OK**.

   In the Plate Layout tab, the upper right corner of the sample well is marked with a red triangle.
   In the Well Table tab, in the Method column, Manual appears next to the selected sample.
6. Repeat the steps above to assign more manual calls.
7. Click **Analyze** to reanalyze the data using the manual calls.

Omit wells from the analysis

To omit the data from one or more wells that you do not want included in the analysis:

- Select one or more wells in a plot or table, then click **Actions ▸ Omit**. After the wells are omitted, click **Analyze** to reanalyze the project without the omitted well(s).

(*Optional*) Sequence the variants

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

A

Troubleshooting

Troubleshoot the HRM calibration

The following table contains common problems encountered during an HRM calibration. For more detailed information, contact Thermo Fisher Scientific support.

Observation	Possible cause	Recommended action
Instrument does not eject the plate	The adhesive cover may have adhered the plate to the heated cover within the instrument.	Unload the instrument.
Instrument malfunction	Multiple possible causes	Contact a local Thermo Fisher Scientific Field Services office.

Troubleshoot an HRM experiment

The following table contains solutions to common problems encountered when performing high-resolution melt experiments. For more detailed information, contact Thermo Fisher Scientific support.

Observation	Possible cause	Recommended action
Late amplification: Ct 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.	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.
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.	Negative controls and unamplified samples are included in the HRM analysis.	Omit negative controls and unamplified samples from the HRM analysis.

Observation	Possible cause	Recommended action
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.	The amplicon contains more than 1 SNP.	Sequence the PCR product to confirm whether the amplicon contains more than one SNP. If the sequencing reveals more SNPs, redesign the primers so that the amplicon contains only one SNP.
	The amplicon is too long.	Redesign the primers to reduce the amplicon size.
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.	The amplicon contains more than one SNP (genotyping experiments only).	Sequence the PCR product to confirm whether the amplicon contains more than one SNP. If the sequencing reveals more SNPs, redesign the primers so that the amplicon contains only one SNP.
	The amplicon is too long.	Redesign the primers to reduce the amplicon size.
Nonspecific amplification: Decreased PCR efficiency and multiple amplicons Decreased PCR efficiency and multiple amplicons may affect the melting behavior of the true target amplicons.	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.
PCR inhibition: Amplification curve with low slope and Ct values higher than expected The amplification curve has a low slope and the amplification reaction may not reach the plateau phase. HRM resolution may be affected by the lower increase in fluorescence.	DNA sample contains contaminants that inhibit PCR.	Dilute the samples 1:10 or 1:100, then repeat the HRM reactions.
	Incorrect salt concentration.	Perform a $MgCl_2$ titration to find the optimal salt concentration for each reaction.
	Reaction does not contain sufficient enzyme.	Optimize the reaction using the MeltDoctor™ HRM Reagent Kit. You can add up to 0.15 U/µL AmpliTaq Gold™ 360 DNA Polymerase to each reaction.
	Reaction does not contain sufficient primer.	Optimize the reaction using the MeltDoctor™ HRM Reagent Kit. You can add up to 0.5 µM of each primer to each reaction.
	Amplicon is greater than 200 bp.	Increase the extension times during the amplification reaction.
	Primers are amplifying multiple targets.	Perform a BLAST search to ensure primer specificity. If the primers are not specific, design new primers.
		Reduce the number of amplification cycles.

Observation	Possible cause	Recommended action
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.	Population spread	Use multiple controls for HRM analysis to help you define the population spread.
	Incorrect salt concentration.	Perform a $MgCl_2$ titration to find the optimal salt concentration for each reaction.
	DNA starting concentrations vary widely between samples.	Make sure that the starting DNA concentrations are similar for the samples that you are testing.
	Low PCR efficiencies.	Ensure efficient PCR.
Some late amplification: Ct value >30 for some samples Sample outliers with Ct 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.	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 "PCR inhibition: Amplification curve with low slope and Ct values higher than expected" on page 75 to troubleshoot PCR inhibition in your HRM reactions.

B

Supplemental procedures

Prepare the HRM calibration plate

IMPORTANT! Before you can perform an HRM calibration, confirm that the region of interest, background, and uniformity calibrations are current. For more information on instrument calibration, see the user documentation for your Applied Biosystems™ QuantStudio™ 3 or 5 Real-Time PCR Instrument.

IMPORTANT! Perform a background calibration immediately before the HRM calibration.

Required materials

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

Prepare a custom HRM calibration plate

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

Component	Volume (µL)					
	384-well plate		Fast 96-well plate		Standard 96-well plate	
	1 rxn.	425 rxns.	1 rxn.	110 rxns.	1 rxn.	110 rxns.
AmpliTaq Gold™ 360 Master Mix	10	4250	10	1100	25.0	2750
Custom dye (20×) (typical dye concentration 0.1 µM	1	425	1	110	2.5	275
MeltDoctor™ HRM Calibration Standard (20×)	1	425	1	110	2.5	275
Deionized water	8	3400	8	880	22.5	2200
Total volume	**20**	**8500**	**20**	**2200**	**50**	**5500**

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:

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

IMPORTANT! Accurate pipetting is required for proper calibration.

5. Seal the reaction plate with optical adhesive film, then briefly centrifuge the reaction plate to force the contents to the bottom of the wells.

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

IMPORTANT! Empty wells can cause the calibration to fail.

Prepare a 96-well 0.2-mL HRM calibration plate

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

IMPORTANT! Use the HRM calibration plate immediately after preparing it.

IMPORTANT! You must perform the custom dye calibration and HRM calibration on the same day that the HRM calibration plate is prepared.

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

Required materials

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

Prepare the 96-well 0.2-mL custom HRM calibration plate

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

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

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

   IMPORTANT! Accurate pipetting is required for proper calibration.

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

   IMPORTANT! Empty wells may cause the calibration to fail.

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

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

Prepare the DNA templates

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

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

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

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

Optimizing the reaction conditions

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

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

Table 1 Recommended component volumes for using the MeltDoctor™ HRM Reagent Kit

Components	Volume for one 20-µL reaction	Final concentration	Acceptable concentration range
AmpliTaq Gold™ 360 Buffer, 10×	2.0 µL	1×	1×
25 mM Magnesium Chloride	1.6 µL	2mM	1.5 to 3.5mM
GeneAmp™ dNTP Blend, 10mM	0.4 µL	200 µM each	100 to 300 µM each
Primer 1 (5 µM)	1.2 µL	0.3 µM	0.2 to 0.5 µM
Primer 2 (5 µM)	1.2 µL	0.3 µM	0.2 to 0.5 µM
MeltDoctor™ HRM Dye (20X)	1.0 µL	1X	0.5X to 2X
AmpliTaq Gold™ 360 DNA Polymerase (5 U/µL)	0.4 µL	0.1 U/µL	0.05 to 0.15 U/µL
Human gDNA (20 ng/µL)	1.0 µL	1 ng/µL	10 pg/µL to 10 ng/µL
Deionized water	**11.2 µL**		
Total volume	**20.0 µL**		

Documentation and support

Obtaining information from the Help system

The Applied Biosystems™ qPCR Analysis Software Help has a Help system that describes how to use each feature of the user interface. Access the Help system by doing one of the following:

- Click in the toolbar of the Applied Biosystems™ qPCR Analysis Software Help window.
- Select **Help ▸ Contents and Index**.
- Press **F1**.

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

- Reviewing the table of contents
- Searching for a specific topic
- Searching an alphabetized index

You can also access PDF versions of all documents in the Applied Biosystems™ qPCR Analysis Help document set from the Help system.

Customer and technical support

Visit **thermofisher.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
  - Product FAQs
  - Software, patches, and updates
- Order and web support
- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

Glossary

amplification efficiency (EFF%)	Calculation of the efficiency of the PCR amplification in a standard curve experiment. EFF% is calculated using the slope of the regression line in the standard curve. A slope close to -3.32 indicates optimal, 100% PCR amplification efficiency. To use amplification efficiency in a gene expression project: • On the instrument where you collected the comparative C_T ($\Delta\Delta C_T$) data that will be used in the project, run a standard curve experiment to determine the efficiency. • In the Applied Biosystems™ qPCR Analysis Software, enter the amplification efficiency in the Efficiency table in the Relative Quantification Settings tab in the Analysis Settings dialog box.
amplification plot	Display of data collected during the cycling stage of PCR amplification. The amplification plot can be viewed as: • Baseline-corrected normalized reporter (ΔRn) vs. cycle • Normalized reporter (Rn) vs. cycle
assays	A PCR reaction mix that contains primers to amplify a target and a reagent to detect the amplified target.
automatic baseline	An analysis setting for the Baseline Threshold algorithm in which the software identifies the start and end cycles for the baseline in the amplification plot.
automatic threshold	An analysis setting for the Baseline Threshold algorithm in which the software calculates the baseline start and end cycles and the threshold in the amplification plot. The software uses the baseline and threshold to calculate the threshold cycle (C_q).
baseline	In the amplification plot, the baseline is a cycle-to-cycle range that defines background fluorescence. This range can be set manually on a target-by-target basis, or automatically, where the software sets the baseline for each individual well.
Baseline Threshold algorithm	Expression estimation algorithm (C_q) which subtracts a baseline component and sets a fluorescent threshold in the exponential region for quantification.
baseline-corrected normalized reporter (ΔRn)	In experiments that contain data from real-time PCR, the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. In the ΔRn vs Cycle amplification plot, ΔRn is calculated at each cycle as: ΔRn (cycle) = Rn (cycle) - Rn (baseline), where Rn = normalized reporter

cycle threshold	See threshold cycle (C_T).
cycling stage	See threshold cycle (C_T).
C_T	See threshold cycle (C_T).
C_T algorithm	See Baseline Threshold algorithm.
flag	A quality control (QC) indicator which, when applied by the software to a well during analysis, indicates a possible issue with that reaction. A summary of the flags identified in the project is displayed in the Flag Summary screen.
negative control (NC)	See no template control (NTC).
no template control (NTC)	In the software, the task for targets in wells that contain water or buffer instead of sample. No amplification should occur in negative control wells. Also called 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 in to the analysis; no information is permanently discarded.
outlier	A data point that deviates significantly from the values of an associated group (for example, the other technical replicates for a sample).
plate grid (plate layout)	An illustration of the grid of wells and assigned content in the reaction plate, array card, or OpenArray™ plate. The number of rows and columns in the grid depends on the plate or card that you use. In the software, you can use the plate grid to view well assignments and results. The plate grid can be printed, included in a report, exported, and saved as a slide for a presentation.
projects	The Applied Biosystems™ qPCR Analysis Software organizes the analysis of experiment data by project, which represents the association of the raw data, all experimental setup information, and any associated settings used to perform the analysis. Once created, projects can be shared with other users and transferred to/from the repository. **Note:** Projects to not contain the data from experiments uploaded to the repository; they link the data for analysis without affecting the original data files.
quencher	A molecule attached to the 3' end of TaqMan™ probes to prevent the reporter from emitting fluorescence signal while the probe is intact. With TaqMan™ probes, a nonfluorescent quencher-minor groove binder (NFQ-MGB) can be used as the quencher.
replicates	Identical reactions containing identical components and volumes.

reporter	A fluorescent dye used to detect amplification. With TaqMan™ reagents, the reporter dye is attached to the 5' end. With SYBR™ Green reagents, the reporter dye is SYBR™ Green dye.
Rn	See *normalized reporter (Rn)*.
ROX dye	A dye used as the passive reference.
run method	The reaction volume and the thermal profile (thermal cycling parameters) for the instrument run.
sample	The biological tissue or specimen that you are testing for a target gene.
task	In the software, the type of reaction performed in the well for the target.
technical replicates	Reactions that contain identical components and volumes, and that evaluate the same sample; important for evaluating precision.
thermal profile	The part of the run method that specifies the temperature, time, ramp, number of cycles, and data collection points for all steps and stages of the instrument run.
threshold	In amplification plots, the threshold is the level of fluorescence above the baseline and within the exponential amplification region. For the Baseline Threshold algorithm, the threshold can be determined automatically (see *automatic threshold*), or it can be set manually (see *manual threshold*).
threshold cycle (C_T)	The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.
unknown	In the software, the task for the target in wells that contain the sample being tested.

References

Lovmar, L., Ahlford, A., Jonsson M., and Syvänen A. 2005. *Silhouette scores for assessment of SNP genotype clusters.* BMC Genomics, 6: 35.

Rousseeuw, P. 1987. *Silhouettes: A graphical aid to the interpretation and validation of cluster analysis.* Journal of Computational and Applied Mathematics. 20: 53–65.