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

For detailed instructions and troubleshooting information, refer to the *HRM Experiments User Guide* (PN 4457847). You can download a PDF version from the Applied Biosystems website at **www.appliedbiosystems.com**.

Note: For safety and biohazard guidelines, refer to the "Safety" section in the *HRM Experiments User Guide* (PN 4457847). For every chemical, read the SDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1 Prepare the HRM reactions

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

2 Amplify and melt the DNA

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

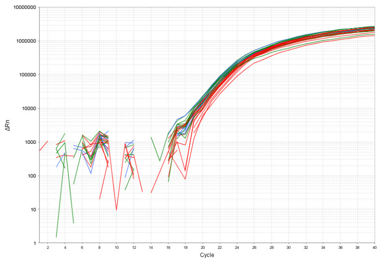
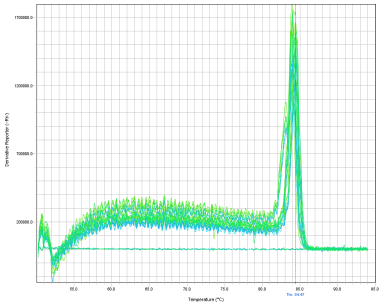
Setup	Setting
Experiment properties	• Experiment type: **Quantitation - Standard Curve** • Reagents: **Other**, then select the **Include Melt Curve** checkbox • Ramp speed: **Standard (~ 2 hours to complete a run)**
Target properties	• Reporter: **MeltDoctor** • Quencher: **None**
Plate layout	• Task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

2 Amplify and melt the DNA (continued)

b. Run the plate:

Stage	Step	Temp	Time
Holding	Enzyme activation	95 °C	10 min
Cycling (40 cycles)	Denature	95 °C	15 sec
	Anneal/extend	60 °C	1 min
Melt curve (for StepOne™ and StepOnePlus™ systems only: 0.3% ramp rate)	Denature	95 °C	10 sec
	Anneal	60 °C	1 min
	High resolution melt	95 °C	15 sec
	Anneal	60 °C	15 sec

c. Using the instrument system software, analyze the experiment file, verify that the samples amplified, review the peaks in the melt curve, then save the experiment file:

Plot	Example	Review the plot
Amplification Plot		Review the Amplification Plot for normal characteristics: • Fluorescence levels that exceed the threshold between cycles 8 and 35 • An exponential increase in fluorescence **Note:** Note which wells are outliers with C_T values that differ from replicates by more than 2.
Melt Curve		Verify that the Melt Curve shows no unexpected Tm peaks. If the sequence you amplified contains more than 1 SNP or a more complex mutation, you may see more than 1 Tm peak. **Note:** Unexpected peaks may indicate contamination, primer dimers, or non-specific amplification. **Note:** The data appear noisy because more data is collected during a high resolution melt curve than during a standard melt curve. The extra data are required for analysis with the HRM Software.

3 Review the high-resolution melt data

a. Using the HRM Software, open the *.eds experiment file from your Real-Time PCR System.

b. Make sure the HRM calibration file that is assigned to the HRM experiment is correct.

c. Add controls to the experiment on the Define screen, then assign the controls to wells on the Assign screen.

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

Note: For the Control Name, do not use the convention *variantN*, where *N* is any number (for example, *variant1*, *variant2*, and so on).

3 Review the high-resolution melt data (continued)

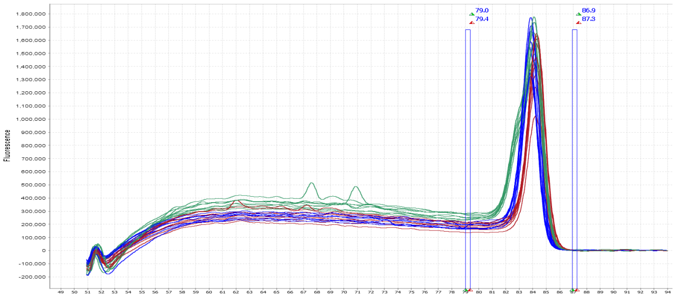
d. For each assay in the plate, select an assay, then specify the analysis settings:

- (Optional) **Pre- and post-melt regions** – Deselect the checkbox and manually enter temperatures to define the pre- and post-melt regions.
- **Number of variant groups**
  - If you defined and assigned controls, make sure the checkbox is selected so that the software will automatically determine the number of variant groups.
  - If you did not define and assign controls, deselect the checkbox and enter the number of variant groups to call for the selected assay.

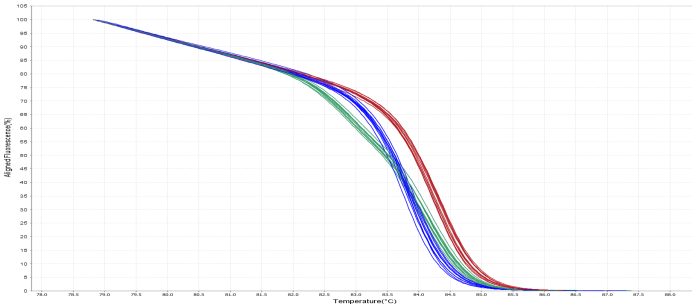
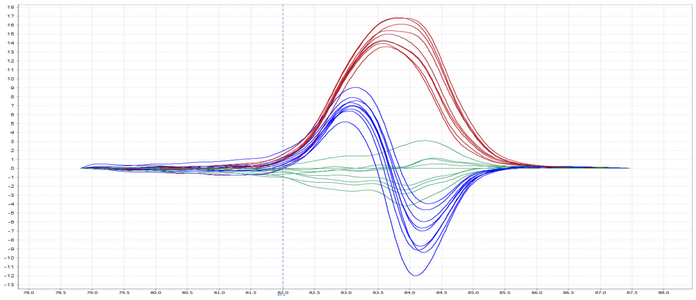
e. Click **Apply Analysis Settings** to close the analysis settings and reanalyze the experiment.

f. Save the HRM experiment file.

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



h. Review the plots:

Plot	Example	Review the plot
Aligned Melt Curves		• How many different variant groups are displayed? Does this number correspond to the number of variant groups you were expecting? • Are there any curves within a variant group that do not cluster tightly with the other samples in that group?
Difference Plot		Select a control or any well as the reference, then review: Select one of the wild type controls as the reference, then review: • Variant groups – How many distinct clusters are displayed? • Outliers – How tight are the curves within each variant group? **Note:** Try selecting different wells as the reference to find the optimal display of the groups.

3 Review the high-resolution melt data (continued)

i. Review the software calls, then omit outliers or change calls as necessary:

Sample type	Review the software calls
Positive controls	• Variant Call column – Do all of the positive control replicates have the correct call? • Silhouette Score column – Are the silhouette scores close to 1.0 (0.8 to 1.0)?
Replicate groups	• Variant Call column – Do all replicates have the same call? • Silhouette Score column – Are the silhouette scores close to 1.0 (0.8 to 1.0)?
All samples	Sort the results according to the variant call. For each variant call, review the samples that were assigned that call.

Note: For wells with low silhouette scores (below 0.8), review the data.

Note: Remember to click **Analyze** to reanalyze the data after you omit outliers or change calls.

Ordering information

Item	Source
MeltDoctor™ HRM Calibration Plate, Fast 96-Well	Applied Biosystems PN 4425618
MeltDoctor™ HRM Calibration Standard (20X), 1 mL	Applied Biosystems PN 4425562
MeltDoctor™ HRM Master Mix: • 5 mL bottle • 5 × 5 mL bottle • 10 × 5 mL bottle	Applied Biosystems • PN 4415440 • PN 4415452 • PN 4415450
MeltDoctor™ HRM Positive Control Kit	Applied Biosystems PN 4410126
MeltDoctor™ HRM Reagent Kit	Applied Biosystems PN 4425557
High Resolution Melt Software v3.0: • 1 license • 10 licenses	Applied Biosystems • PN 4461357 • PN 4461456

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