

Perform an HRM Genotyping Experiment

This quick reference card provides brief procedures for performing an HRM genotyping experiment using MeltDoctor™ HRM Master Mix and Applied Biosystems High Resolution Melting Software. See the *Applied Biosystems High Resolution Melting Getting Started Guide* for details and for procedures using the MeltDoctor™ HRM Reagents Kit.

Note: For safety and biohazard guidelines, refer to the “Safety” section in the *Applied Biosystems High Resolution Melting Getting Started Guide* (PN 4393102). For every chemical, read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1 Prepare the HRM reactions

Components	Volume for one reaction	
	20- μ L reactions (384-well Fast reaction plate or 96-well Fast reaction plate)	50- μ L reactions (96-well standard reaction plate)
MeltDoctor™ HRM Master Mix	10.0 μ L	25.0 μ L
Primer 1 (5 μ M)	1.2 μ L	3.0 μ L
Primer 2 (5 μ M)	1.2 μ L	3.0 μ L
Genomic DNA (20 ng/ μ L)	1.0 μ L	2.5 μ L
Deionized water	6.6 μ L	16.5 μ L
Total reaction volume	20 μL	50 μL

IMPORTANT! Applied Biosystems recommends performing at least 3 technical replicates of each reaction. Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.

2 Amplify and melt the DNA

Note: If you are performing your experiment on the 7900HT Fast instrument using a 96-well reaction plate, spin the plate after you amplify the DNA, and perform the melt curve in a separate run.

- a. Create and set up the HRM run file:

Run file setting	Applied Biosystems 7900HT Fast Real-Time PCR System with SDS Software v2.3 or later	Applied Biosystems 7500 Fast Real-Time PCR System with 7500 Software v2.0 or later	Applied Biosystems 7500 Fast Real-Time PCR System with SDS Software v1.4
Document/experiment	<ul style="list-style-type: none"> Assay: Standard Curve (AQ) Container: 384 Wells Clear Plate or 96 Wells Clear Plate Template: Blank Template 	<ul style="list-style-type: none"> Instrument: 7500 Fast (96 Wells) Experiment type: Quantitation - Standard Curve Reagents: Other, then select the Include Melt Curve checkbox Ramp speed: Standard (~ 2 hours to complete a run) 	<ul style="list-style-type: none"> Assay: Standard Curve (Absolute Quantitation) Container: 96-Well Clear Template: Blank Document
Detector/target and plate layout	<ul style="list-style-type: none"> Reporter: MeltDoctor Quencher: Non Fluorescent 	<ul style="list-style-type: none"> Reporter: MeltDoctor Quencher: None 	<ul style="list-style-type: none"> Reporter: MeltDoctor Quencher: Non Fluorescent
Plate layout	<ul style="list-style-type: none"> Task for negative control wells: NTC Passive Reference: None 	<ul style="list-style-type: none"> Task for negative control wells: N Passive Reference: None 	<ul style="list-style-type: none"> Task for negative control wells: NTC Passive Reference: (none)
Thermal profile/run method	<ul style="list-style-type: none"> Mode: Standard Sample Volume (μL): 20 (384-well or 96-well Fast) or 50 (96-well standard) 	<ul style="list-style-type: none"> Reaction Volume Per Well: 20 μL Expert Mode: Select the checkbox Click Select/View Filters, then select only Filter-1 	<ul style="list-style-type: none"> Sample Volume (μL): 20 Run Mode: Fast 7500 Expert Mode: Select the checkbox Click Select/View Filters, then select only Filter A

2 Amplify and melt the DNA (continued)


b. Run the plate:

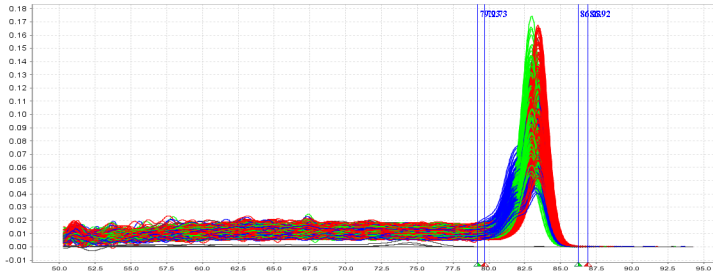
Stage	Step	Temp	Time	Ramp rate (7900HT only)
Holding	Enzyme activation	95 °C	10 min	100%
Cycling (40 cycles)	Denature	95 °C	15 sec	100%
	Anneal/extend	60 °C	1 min	100%
Melt curve/dissociation	Denature	95 °C	10 sec	100%
	Anneal	60 °C	1 min	100%
	High resolution melting	95 °C	15 sec	1%
	Anneal	60 °C	15 sec	100%


c. Using the instrument system software, verify that the samples amplified and review the peaks in the melt curve:

Plot	Example	Review the plot
Amplification Plot		<p>Review the Amplification Plot for normal characteristics:</p> <ul style="list-style-type: none"> • Fluorescence levels that exceed the threshold between cycles 8 and 35 • An exponential increase in fluorescence <p>Note: Note which wells are outliers with C_T values that differ from replicates by more than 2.</p>
Dissociation Curve/Melt Curve		<p>Verify that the Dissociation Curve/Melt Curve shows no unexpected T_m peaks. If the sequence you amplified contains more than 1 SNP or a more complex mutation, you may see more than 1 T_m peak.</p> <p>Note: Unexpected peaks may indicate contamination, primer dimers, or non-specific amplification.</p> <p>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 High Resolution Melting Software.</p>

3 Review the high-resolution melting data

- Using the HRM Software, create an HRM experiment using the *.eds or *.sds run file from your 7900HT Fast or 7500 Fast system.
- Make sure the HRM calibration file that is assigned to the HRM experiment is correct.
- 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, then click  to reanalyze the data.



- Set up the controls, then click  to reanalyze the data:
 - For each control sample: Enter a Control Name, select the well that contains the control sample, then select the color to display for the control.

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

- For control replicates: Enter a Control Name and select a color that are identical to the other control replicates. You can enter up to 5 replicates for each control.

Control Name	Well		Color	
Hom - wild type	A4	▼	■	▼
Hom - wild type	P18	▼	■	▼
Hom - variant	A3	▼	■	▼
Hom - variant	P21	▼	■	▼
Heterozygote	A2	▼	■	▼
Heterozygote	E22	▼	■	▼

- Review the plots:

Plot	Example	Review the plot
Aligned Melt Curves		<ul style="list-style-type: none"> How many different variant groups are displayed? Does this number correspond to the number of variants 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		<p>Select the wild type control as the reference, then review:</p> <ul style="list-style-type: none"> Variant clusters – How many distinct clusters are displayed? Outliers – How tight are the curves within each variant cluster? <p>Note: Try selecting different reference samples to find the optimal display of the clusters.</p>

3 Review the high-resolution melting data (continued)

f. Review the software calls:

Sample type	Review the software calls
Positive controls	<ul style="list-style-type: none"> Variant Call column – Do all of the positive control replicates have the correct 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?
Replicate groups	<ul style="list-style-type: none"> 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?
All samples	Sort the results according to the variant call. For each variant call, review the samples that were assigned that call.

Note: If you omit outliers or change calls, remember to click  to reanalyze the data.

Ordering information

Item	Source
MeltDoctor™ HRM Calibration Plate, 96-Well	Applied Biosystems PN 4425618
MeltDoctor™ HRM Calibration Plate, 384-Well	Applied Biosystems PN 4425559
MeltDoctor™ HRM Calibration Standard (20X), 1 mL	Applied Biosystems PN 4425562
MeltDoctor™ HRM Master Mix: <ul style="list-style-type: none"> 5 mL bottle 5 × 5 mL bottle 10 × 5 mL bottle 	Applied Biosystems <ul style="list-style-type: none"> PN 4415440 PN 4415452 PN 4415450
MeltDoctor™ HRM Positive Control Kit	Applied Biosystems PN 4410126
MeltDoctor™ HRM Reagents Kit	Applied Biosystems PN 4425557

For Research Use Only. Not for use in diagnostic procedures.

NOTICE TO PURCHASER: PLEASE REFER TO THE APPLIED BIOSYSTEMS HIGH RESOLUTION MELTING GETTING STARTED GUIDE FOR LIMITED LABEL LICENSE OR DISCLAIMER INFORMATION.

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