Perform an HRM Methylation Study

This quick reference card provides brief procedures for performing an HRM methylation study 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 methylated DNA standards

Mix different ratios of 100% methylated and 0% methylated DNA of equal concentration. For example:

- To detect high levels of methylation, prepare methylated DNA standards to represent 100%, 75%, 50%, 25%, 10%, and 0% methylation.
- To detect low levels of methylation, prepare methylated DNA standards to represent 100%, 10%, 5%, 2%, 1%, 0.5%, 0.1%, and 0.0% methylation.

2 Treat the samples and methylated DNA standards with bisulfite

Use the methylSEQr™ Bisulfite Conversion Kit. For instructions, refer to the *Applied Biosystems methylSEQr™ Bisulfite Conversion Kit Protocol* (PN 4374710)

3 Prepare the HRM reactions

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

4 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	• Assay: **Standard Curve (AQ)** • Container: **384 Wells Clear Plate** or **96 Wells Clear Plate** • Template: **Blank Template**	• 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)**	• Assay: **Standard Curve (Absolute Quantitation)** • Container: **96-Well Clear** • Template: **Blank Document**
Detector/ target and plate layout	• Reporter: **MeltDoctor** • Quencher: **Non Fluorescent**	• Reporter: **MeltDoctor** • Quencher: **None**	• Reporter: **MeltDoctor** • Quencher: **Non Fluorescent**
Plate layout	• Task for negative control wells: **NTC** • Passive Reference: **None**	• Task for negative control wells: N • Passive Reference: **None**	• Task for negative control wells: **NTC** • Passive Reference: **(none)**
Thermal profile/run method	• Mode: **Standard** • Sample Volume (µL): **20** (384-well or 96-well Fast) or **50** (96-well standard)	• Reaction Volume Per Well: **20** µL • Expert Mode: Select the checkbox • Click **Select/View Filters**, then select only **Filter-1**	• Sample Volume (µL): **20** • Run Mode: **Fast 7500** • Expert Mode: Select the checkbox • Click **Select/View Filters**, then select only **Filter A**

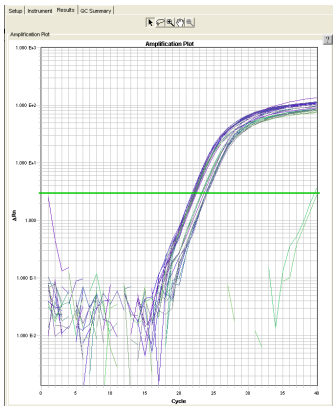
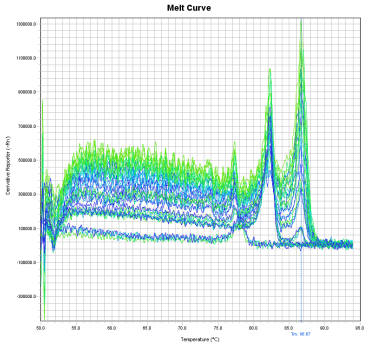
b. Run the plate:

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%

Note: Adjust the annealing temperature during the amplification to increase or decrease the extent of the PCR bias.

4 Amplify and melt the DNA (continued)

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

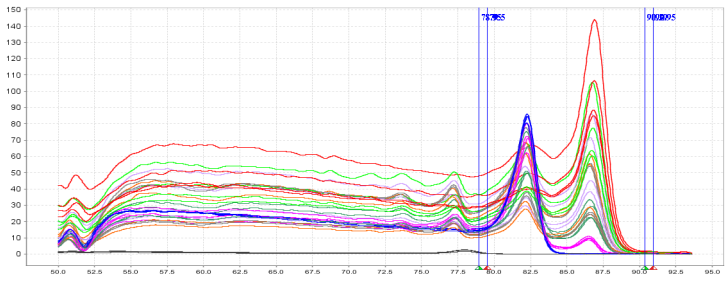
Plot	Example	Review the plot
Amplification Plot		Review the Amplification Plot for normal characteristics: • Fluorescence levels that exceed the threshold between cycles 8 and 35 • An exponential increase in fluorescence **Note:** Note which wells are outliers with C_T values that differ from replicates by more than 2.
Dissociation Curve/Melt Curve		Verify that the Dissociation Curve/Melt Curve shows no unexpected Tm peaks. With methylation experiments, you will likely see multiple peaks. The number of peaks in the melt curve is correlated with the number of methylation sites in the amplicon. **Note:** Unexpected peaks 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 High Resolution Melting Software.

5 Review the high-resolution melting data

a. Using the HRM Software, create an HRM experiment using the *.eds or *.sds run file from your 7900HT Fast or 7500 Fast system.

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

c. 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 Analyze to reanalyze the data.



5 Review the high-resolution melting data (continued)

d. Set up the controls, then click Analyze to reanalyze the data:

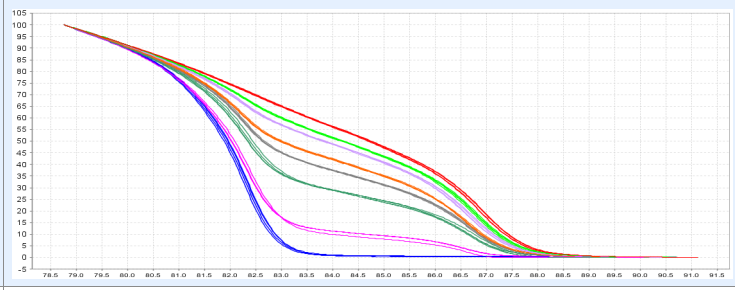
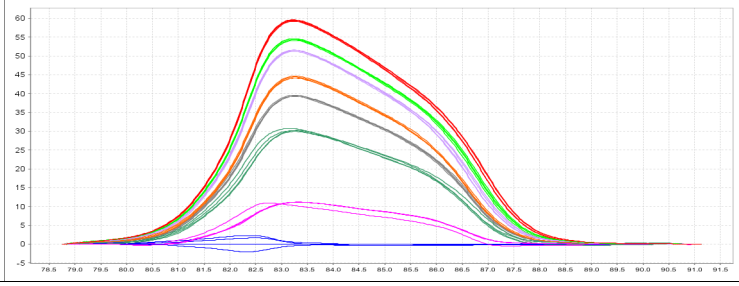
- For each control sample: Enter a Control Name, select the well that contains the control sample, then select the color to display for the control.

  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
0%	C10	
0.1%	C9	
0.5%	C8	
1%	C7	
2%	C6	
5%	C5	
10%	C4	
100%	C3	

e. Review the plots:

Plot	Example	Review the plot
Aligned Melt Curves		• Do the melt curves for the methylated DNA standards cluster well? Are there any outliers? • Which methylated standard melt curves are above and below the melt curves for the unknowns?
Difference Plot		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.

5 Review the high-resolution melting data (continued)

f. Review the software calls:

Sample type	Review the software calls
Methylation standard controls	• 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?
Replicate groups	• 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	

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

6 Sequence the variants

a. Dilute the PCR products of the selected variants to 0.5–1.5 ng/µL with water. If you dilute the PCR product >1:20, go to step c.

b. If you dilute the PCR product <1:20, purify the PCR product:

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

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

c. Perform the sequencing reactions in a Veriti® Thermal Cycler:

Component	Volume
BigDye® Terminator v1.1	2 µL
Forward primer or reverse primer	1 µL
Deionized water	4 µL
BigDye® Terminator v1.1, v3.1 5X Sequencing Buffer	1 µL
Diluted DNA or diluted, purified DNA (3.2 pmol/µL)	2 µL
Total reaction volume	**10 µL**

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

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

6 Sequence the variants (continued)

d. Purify the sequencing reactions:

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

e. Run the sequencing reactions:

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

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 (20×), 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 Reagents Kit	Applied Biosystems PN 4425557
methylSEQr™ Bisulfite Conversion Kit, 48 reactions	Applied Biosystems PN 4379580
BigDye® Terminator v1.1 Cycle Sequencing Kit, 100 reactions	Applied Biosystems PN 4337450
BigDye® XTerminator™ Purification Kit, 2 mL (~100 20-µL reactions)	Applied Biosystems PN 4376486
M13 forward and reverse sequencing primers: • M13 Forward (–20), 2 µg • M13 Reverse, 2 µg **Note:** Use only if the HRM PCR product contains the M13 sequences.	Invitrogen‡ • PN N520-02 • PN N530-02
UltraPure™ DNase/RNase-Free Distilled Water, 500 mL	Invitrogen PN 10977-015
ExoSAP-IT®, 100 reactions	USB Corporation‡ PN 78200

‡ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

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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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