HRM Methylation Studies 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 methylated DNA standards

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

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

2 Treat the samples and methylated DNA standards with bisulfite

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

3 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**

4 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**

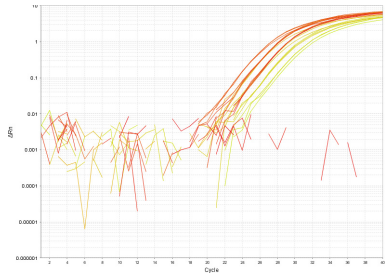
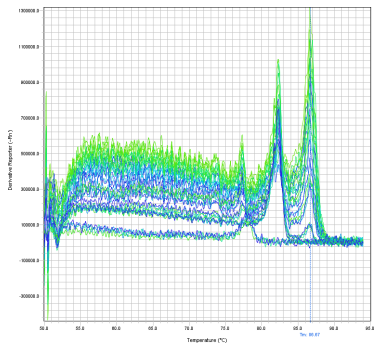
4 Amplify and melt the DNA (continued)

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

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

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. 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 HRM Software.

5 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.

Note: Set up and define controls in the software for each methylated DNA standard with known percentages of methylated DNA.

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

5 Review the high-resolution melt data (continued)

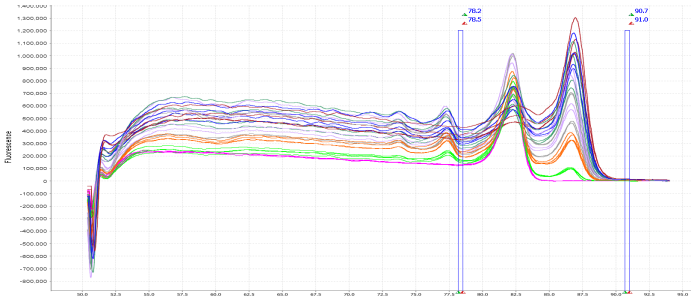
d. For each assay in the plate, select an assay, then specify the analysis settings:

- (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** – Make sure the checkbox is selected so that the software will automatically determine the number of variant groups.

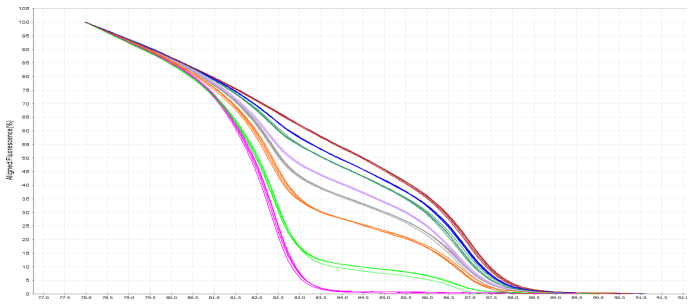
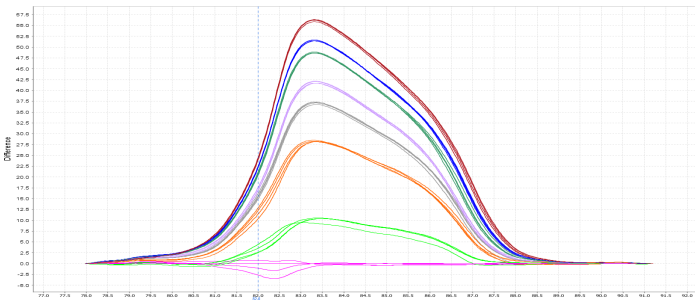
e. Click **Apply Analysis Settings** to close the analysis settings and reanalyze the experiment.

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		• 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? The estimated percent methylation in the unknowns should lie between the percent methylation in those standards.
Difference Plot		Select a control or any well as the reference, then review: • Variant groups – How many distinct clusters are displayed? • Outliers – How tight are the curves within each variant group? **Note:** Try selecting different wells as the reference to find the optimal display of the groups.

5 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
Methylation standard controls	• Variant Call column – Do all of the methylation standard controls have the correct call? • Silhouette Score column – Are the silhouette scores close to 1.0 (0.8 to 1.0)?
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	The curves for unknown samples should fall within the controls of known %methylation. Estimate the range of %methylation in your unknown samples based on the two flanking control curves.

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.

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, proceed with the sequencing reactions.
- If you dilute the PCR product <1:20, purify the PCR product before you proceed with the sequencing reactions:

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	∞

b. Prepare the sequencing reactions:

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

6 Sequence the variants (continued)

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

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.

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 products on a capillary electrophoresis instrument:

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

Ordering information

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

Item	Source
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
Bisulfite conversion kits and control kits: • Cells-to-CpG™ Bisulfite Conversion Kit (50) • Cells-to-CpG™ Bisulfite Conversion Kit (2x96) • Cells-to-CpG™ Bisulfite Conversion and Quantitation Control Kit • Cells-to-CpG™ Methylated and Unmethylated gDNA Control Kit	Applied Biosystems • PN 4445555 • PN 4445554 • PN 4445553 • PN 4445552
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 (if the HRM PCR product contains the M13 sequences): • M13 Forward (–20), 2 µg • M13 Reverse, 2 µg	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

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NOTICE TO PURCHASER: PLEASE REFER TO THE HRM EXPERIMENTS USING MELTDOCTOR™ HRM REAGENTS AND HIGH RESOLUTION MELT SOFTWARE v3.0 USER GUIDE FOR LIMITED LABEL LICENSE OR DISCLAIMER INFORMATION.

4457856B 11/2010

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