



Precipio, Inc.

High Resolution Melting Protocol

High Resolution Melting of ICE COLD-PCR Products

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High Resolution Melting of the ICE COLD-PCR products

NOTE: For the standard protocol, refer to [Applied Biosystems High Resolution Melting Getting Started Guide \(Part Number 4393102, Rev. D\)](#).

NOTE: Calibrate the 7900HT Fast system according to standard protocol ([Chapter 1](#)) for the MeltDoctor™ HRM Dye if this is the first time performing HRM experiments using MeltDoctor HRM reagents.

IMPORTANT! To avoid cross contamination, set up HRM reactions in an area or room that is separated from DNA extraction and the designated pre-PCR areas. Good laboratory practices, especially cleaning the bench and pipettes after reaction setup, are also required to avoid contamination.

1. Preparation of Template DNA for HRM Analysis
 - a. Make a 1:200 dilution by adding 199 μL water into the wells of a clean, labelled 96-well plate. Add 1 μL of ICE COLD-PCR product from the ICE COLD-PCR plate to the water in the corresponding wells. Maintain the original plate layout. Cap the wells and mix well by vortexing for ~ 10 sec. Spin down briefly.
2. HRM after ICE COLD-PCR:
 - a. Remove the primers from freezer, thaw on ice, vortex each for 3-5 sec, and spin the tubes briefly.
 - b. Remove the 2X MeltDoctor HRM Master Mix from fridge, mix by inverting the tube and briefly spin it down. Keep it on ice.

NOTE: Keep MeltDoctor HRM Master Mix in the dark until ready to use. Once added, keep the plate in the dark until the plate is disposed of following run on the ABI 7900HT. Minimize freeze-thaw cycles.

- c. Prepare reaction mix (30 μL total volume), using [Table 6](#) as a guide.

Table 6: Master Mix calculations for the HRM assay.

Reagents	1X Reaction*
MeltDoctor HRM Master Mix	15.0
HRM Forward Primer (10 μM)	0.9
HRM Reverse Primer (10 μM)	0.9
Deionized water	11.2
ICP product DNA (1:200 dilution) or Deionized water	2.0

*Multiply volumes in this table by the number of samples being tested.

NOTE: See [Table 7](#) for recommended plate layout.

NOTE: A Master Mix volume slightly greater than this calculation will be required to allow for losses during pipetting.

- d. Mix the reaction mix tube by pulse vortexing on low and spin the tube briefly.
- e. Aliquot 28 μL of the reaction mix to the relevant wells of a MicroAmp Optical 96-well Reaction Plate. See [Table 7](#) for plate layout.

- f. To appropriate wells, add 2 μ L of diluted ICE COLD-PCR product from Step 1a, or water (NTC2). See **Table 7** for plate layout. Pipette-mix to ensure sample is well mixed into reaction mixture.

Table 7: Example template for running 28 samples with controls.

	1	2	3	4
A	SMP01	SMP09	SMP17	SMP25
B	SMP02	SMP10	SMP18	SMP26
C	SMP03	SMP11	SMP19	SMP27
D	SMP04	SMP12	SMP20	SMP28
E	SMP05	SMP13	SMP21	WT
F	SMP06	SMP14	SMP22	CTRL_1per
G	SMP07	SMP15	SMP23	NTC1
H	SMP08	SMP16	SMP24	NTC2

NOTE: If it is planned to run the reactions 24 hours after set up, store the plate at 4 °C. Bring the plate to room temperature before running the HRM program.

- g. Place MicroAmp Optical Film over plate and seal. Spin the plate briefly to eliminate any air bubbles from the solution.
- h. Use the MicroAmp Optical Film Compression Pad over the reaction plate while on the 7900HT instrument. Remember the reaction mix is light sensitive so keep the plate in the dark until loaded onto ABI 7900HT.
3. Amplify and melt the DNA

IMPORTANT! HRM_Setup-Table file and HRM_template file are available for download at www.precipiodx.com to facilitate .sds file setup on ABI 7900HT System. It is recommended to use the files and track the user sample names with relevant setup table file sample names. But this is not required.

- a. If HRM_Setup-Table file and HRM_Template file are to be used:
- Make a copy of the downloaded Setup-Table file. Open the copied file in Microsoft Excel. On the "Sample List" sheet, fill in or copy/paste the sample names in the highlighted "Assigned Sample Name" column. Check the resulted plate layout on the right to make sure it is as expected. Save the "HRM_Setup-Table" sheet separately as Text (Tab delimited, .txt) files. Use this .txt file as HRM_Setup-Table later.
- b. In the SDS Software, create a new run file for the HRM reaction by selecting **File > New Plate Wizard**.
- Assay: Standard Curve (AQ)
 - Plate type: 96 Wells Clear Plate
 - Template: Blank Template

NOTE: If the **HRM_Setup-Table** file and **HRM_Template** file are selected here, then all samples, detector and thermal profile have been input into the program click on **Next**. Make sure the sample names, detector and thermal profile are correct. Then skip to Step 3l.

- iv. Click on **Next**
- c. Enter the samples names by double-clicking in the sample field to activate the cursor. Then type in the sample name. After typing in the name, press **Enter** and a new row will appear. Make sure to press **Enter** after the very last sample row. If a same set of samples was used in an existing plate, click **Existing Plate** to import the existing sample names.
- d. Click on **Detector Manager** and select MeltDoctor detector (This detector should have been made during the HRM dye calibration as indicated in the beginning of HRM protocol) for the assay(s) and then click on **Copy to Plate Document**. Select **OK** in the pop-up window, then click **Done**.
- e. Once the detector has been added to the plate, click on **Next**.
- f. Assign samples and detector to the appropriate wells. From the plate layout drop-down list, select **Individual Wells**.
- g. Select the well(s) that contain the first sample and then select the **Add Samples and Detectors tab**. Then select the appropriate sample and MeltDoctor detector that corresponds to that well. In the Task field drop-down list, click on NTC for the NTC1 and NTC2 and Unknown for the controls and samples.
- h. Repeat for all samples.
- i. Select the **Well Inspector** tab to check that all the wells are correctly assigned. Click on **Finish**.
- j. On the Set Up tab, select **None** from the Passive Reference dropdown menu.
- k. Select the instrument > Thermal Cycler > Thermal Profile tabs, the set the thermal cycler protocol.
 - i. Mode: Standard
 - ii. Sample Volume (uL): 30 uL for standard 96-well plates
 - iii. Thermal profile:

Stage	Step	Temp	Time	Ramp rate
Holding	Enzyme activation	95C	10 min	100%
Cycling (40 cycles)	Denature	95C	15 sec	100%
	Anneal/extend	60C	1 min	100%
Melt curve/dissociation (IMPORTANT! Set up this part of the program using "Dissociation steps")	Denature	95C	10 sec	100%
	Anneal	60C	1 min	100%
	High resolution melting	95C	15 sec	1%
	Anneal	60C	15 sec	100%

- l. Select the Real-Time tab. Spin the plate briefly, load the plate into the instrument, then start the run.
- m. Save the melt curve file at the prompt.
- n. When the run completes, click OK as a dialog box opens
- o. Save the file, and then unload the plate.
- 4. Review the high-resolution melting data in SDS software
 - a. Review the Amplification Plot for normal characteristics:
 - i. Fluorescence levels that exceed the threshold between cycles 8 and 35
 - ii. An exponential increase in fluorescence

NOTE: Document which wells are outliers with CT values that differ from replicates by more than 2. The outliers may produce erroneous HRM results.

- b. Verify that the Dissociation Curve/Melt Curve shows no unexpected T_m peaks.

NOTE: If the sequence you amplified contains more than 1 variant or a more complex mutation, you may see more than 1 T_m peak

NOTE: Unexpected peaks may indicate contamination, primer dimers, or nonspecific 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. Analyzing melt curve file with HRM v. 2.

- a. Once open the Software, click **Open** on the main window to import the saved *.sds run file saved in the previous step.

NOTE: If this is your first time creating an HRM experiment with the HRM software, select the default HRM calibration file at prompt.

- b. In the **Results Pane**, select specific samples to omit if multiple targeted regions/amplicons are run on the same plate.
- c. In the **Derivative Melt Curves Plot**, 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 melting transition region:
 - i. The pre-melt Start and Stop temperature lines (green and red arrows on the left) should be approximately 0.2 to 0.5 °C apart from each other.
 - ii. The post-melt Start and Stop temperature lines (green and red arrows on the right) should be approximately 0.2 to 0.5 °C apart from each other.
- d. Click **Analyze** on the top of the main window. Variant calls for each sample will be shown in the **Results Pane**. Any samples that were not assigned the same call as the wild type control are considered bearing mutation or some type of polymorphism.
- e. Review the **Difference Plot**.
 - i. In the **Analyzed Data Pane**, select the **Aligned Data - Difference Plot** tab.
 - ii. From the **Reference** dropdown menu, select a control or any well as the reference, and then review variant clusters and outliers.

NOTE: Try selecting different reference samples to find the optimal display of the clusters. Changing reference will not affect variant calls.

NOTE: Further information can be found in the Amplify [and melt the DNA](#) and [Review the high-resolution melting data](#) sections in [Chapter 4. Perform an HRM Mutation Scanning Experiment](#) of the Getting Started Guide.

Mutation Confirmation of HRM Positive Samples

1. It is recommended that secondary confirmation of any HRM mutation positive samples be confirmed by a secondary method such as Sanger Sequencing or Real-Time PCR.
 - a. A sequencing primer is present in the respective HRM kit.



Technical Support/Questions

For any questions regarding the kit, or IFU, please contact us at Techsupport@precipiodx.com or call 1.203.787.788 ext. 509.