Brilliant HRM Ultra-Fast Loci Master Mix

Part Numbers
5190-7827 (200 reactions)
5190-7909 (15 reactions)

Protocol

Version A0, November 2014

For Research Use Only. Not for Use in Diagnostic Procedures.

Agilent Technologies

Notices

Manual Part Number

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In this Guide...

This document describes how to use the Agilent Brilliant HRM Ultra-Fast Loci Master Mix to perform PCR amplifications with accelerated cycling followed by high resolution melt analysis.

1
Before You Begin

This chapter provides important information on getting started with a high resolution melt assay using the Brilliant HRM Ultra-Fast Loci Master Mix.

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Ordering and kit information

Table 1 shows the part numbers and quantities provided for the Brilliant HRM Ultra-Fast Loci Master Mix.

Table 1 Agilent part numbers for the Brilliant HRM Ultra-Fast Loci Master Mix

Part number	Product name	Quantity
5190-7827	Brilliant HRM Ultra-Fast Loci Master Mix	2 mL (200 20-µL reactions)
5190-7909	Brilliant HRM Ultra-Fast Loci Master Mix, Sample size*	150 µL (15 20-µL reactions)

* The sample size is offered for a limited time.

Storage conditions for the Brilliant HRM Ultra-Fast Loci Master Mix

Store at –20°C upon receipt. After thawing, the master mix may be stored at 4°C for one month or returned to –20°C for long term storage.

The master mix has been demonstrated to be stable after multiple freeze-thaw cycles, but we recommend keeping these at a minimum to preserve optimal performance and avoid the risk of contamination.

The master mix is light sensitive. Protect from light whenever possible.

Required reagents and equipment

Table 2 contains the list of reagents and equipment that are required for the protocol.

Table 2 Required Equipment and Reagents

Equipment or reagent
Agilent Brilliant HRM Ultra-Fast Loci Master Mix, see "Ordering and kit information" on page 6
Agilent AriaMx Real-Time PCR System, or other real-time PCR platform with HRM capability AriaMx Real-Time PCR System, Base Instrument: Agilent part number G8330A AriaMx Real-Time PCR System, 5 Fixed: Agilent part number G8330-64000 AriaMx Real-Time PCR System, 4 Fixed: Agilent part number G8330-64010
Nuclease-free PCR-grade water
Upstream and downstream primers
DNA templates (positive control templates and experimental templates)
QPCR reaction tubes or plates, see "Recommended plasticware" on page 8
(Optional) Agilent Passive Reference Dye (ROX), part number 600536

Recommended plasticware

Table 3 lists the part numbers of Agilent plates, tubes, and other plastic consumables for use in the AriaMx instrument and other real-time PCR platforms.

Table 3 Agilent QPCR plasticware

Agilent Part Number	Description
401490	96-well plate, fully skirted, low profile
401491	96-well plate, rigid, fully skirted, low profile
401494	96-well, non-skirted, low profile
401493	8x strip tubes, without caps, low profile, 120 strips (10 packs of 12 strips)
401425	8x strip tube optical caps, 120 strips (10 packs of 12 strips)
401427	8x strip tube optical caps, 60 strips (5 packs of 12 strips)
401492	Adhesive seal for 96-well plates, 50-pack

Overview of the Brilliant HRM Ultra-Fast Loci Master Mix

High resolution melt (HRM) analysis is a technique used primarily for genotyping samples that include a single nucleotide polymorphism (SNP) in the DNA sequence. Other applications that use HRM analysis include:

- Species identification
- Mutation screening
- Haplotype characterization
- DNA fingerprinting
- DNA methylation analysis
- HLA compatibility typing

The Brilliant HRM Ultra-Fast Loci Master Mix is a single-tube reagent designed for performing HRM assays on the Agilent AriaMx Real-Time PCR System or other suitable platforms.

When performing HRM analysis with the Brilliant HRM Ultra-Fast Loci Master Mix, set up an Allele Discrimination experiment on the AriaMx instrument to amplify all possible alleles for your gene of interest using the same set of primers. The instrument detects the amplicons using the double-stranded DNA-binding dye EvaGreen, which is present in the Brilliant HRM Ultra-Fast Loci Master Mix.

The experiment needs to include positive control samples for each base pair possibility at the SNP location (homozygous as well as heterozygous).

The HRM segment of the Allele Discrimination experiment generates melt curves that can distinguish between amplified alleles that differ by a single nucleotide.

In addition to the EvaGreen dye, the 2× Brilliant HRM Ultra-Fast Loci Master Mix also contains:

- A mutated form of *Taq* DNA polymerase with a chemical hot start mechanism that promotes fast hot start release, high specificity, and short cycling times
- dNTPs
- $MgCl_2$
- A buffer specially formulated for fast cycling

2 Protocol

This chapter provides guidelines and instructions on how to perform real-time PCR with the Brilliant HRM Ultra-Fast Loci Master Mix.

Pre-protocol considerations

Primer design

We recommend using the online primer design tool Primer 3, version 0.4.0, to design suitable primers for your target region (available at http://bioinfo.ut.ee/primer3-0.4.0/).

The default settings (General Primer Picking Conditions) typically result in suitable primers for amplification and HRM.

When possible, design primers with a melting temperature at or near 60°C, and maintain an amplicon size of 50–200 bp.

For targets with a class III or class IV SNP (G>C or A>T, respectively), we recommend designing primers to amplify an amplicon of 100 bp or smaller.

HRM calibration

In order to analyze HRM data from the AriaMx Real-Time PCR System, you must run an HRM calibration plate on the instrument. The purpose of an HRM calibration plate is to normalize temperature variations across the thermal block. This normalization improves the accuracy and clarity of the melt data.

NOTE For each AriaMx instrument, Agilent recommends running a new HRM calibration plate at least once per year.

Agilent offers a pre-aliquoted 96-well plate to be used for HRM calibration on the AriaMx platform. See Table 4 for ordering information.

Table 4 Agilent part number for the HRM calibration plate

Part number	Product name	Quantity
5190-7702	AriaMx HRM Calibration Kit	1 96-well plate

Setting Up and Running an Experiment

Set up the experiment using the AriaMx software

NOTE If using a real-time PCR platform other than the AriaMx system, the cycling conditions in Table 5 are a good starting point, but some temperatures and/or durations may require optimization.

In the AriaMx software, the Allele Discrimination - DNA Binding Dye experiment type is specifically for HRM analysis. Create an experiment of this type and set up the plate as needed. Use the default thermal profile for the experiment, or adjust the thermal profile to meet the needs of your assay. The cycling conditions for the default thermal profile are described in Table 5.

Table 5 Cycling conditions for the Allele Discrimination - DNA Binding Dye experiment

Segment	Cycles	Temperature	Duration
Hot Start	1	95°C	3 minutes
Amplification	40	95°C	5 seconds
		60°C*	10–30 seconds*
High Resolution Melt Resolution: 0.2°C Soak Time: 10 seconds	1	95°C	30 seconds
		65°C	30 seconds
		95°C	30 seconds

* You may need to optimize the temperature and duration of this plateau to meet the needs of your specific assay.

The instrument collects fluorescence data during the Amplification segment (at the 60°C plateau) and during the High Resolution Melt segment (during the ramp between 65°C and 95°C).

Consult the software's help system for instructions on creating and setting up experiments.

Prepare the reactions

NOTE

Once the tube containing the 2× Brilliant HRM Ultra-Fast Loci Master Mix is thawed, store it on ice while setting up the reactions. Following initial thawing of the master mix, store the unused portion at 4°C for up to one month, or return to –20°C for long term storage. Avoid unnecessary freeze-thaw cycles.

Set up positive control reactions for each base pair possibility (homozygous and heterozygous) at the SNP locus under investigation.

The optimal concentration for the primers must be determined empirically. In most assays, the optimal primer concentration is between 150 and 500 nM for each primer.

Keep all solutions containing the master mix protected from light as much as possible.

1 Prepare the reagent mixture for the reactions by combining the components listed in Table 6 *in order*. Prepare a single reagent mixture for all reactions (plus at least one reaction volume excess) using multiples of each component.

Table 6 Real-Time PCR reagent mixture

Component	Volume per reaction
Nuclease-free PCR-grade water	X µL (enough to yield a final reaction volume of 20 µL, including template DNA)
2× Brilliant HRM Ultra-Fast Loci Master Mix	10 µL
ROX Reference Dye (optional)	X µL (appropriate concentration for your real-time PCR platform*)
Upstream primer	X µL (optimized concentration)
Downstream primer	X µL (optimized concentration)

* On the AriaMx Real-Time PCR system, use 30 nM in the final reactions (if including ROX in your reactions).

2 Gently mix without creating bubbles, then distribute the reagent mixture to individual PCR reaction tubes or plate wells.

3 Add x µL of DNA template to each reaction to bring the total volume for each reaction to 20 µL. Table 7 lists a suggested quantity range for different DNA templates.

Table 7 Quantity of DNA template per reaction

DNA	Quantity per reaction
Genomic DNA	1–50 ng
cDNA	1–50 ng*
Microbial DNA	1–50 pg

* Refers to RNA input amount during cDNA synthesis

4 Gently mix the reactions without creating bubbles, then spin the samples in a centrifuge at 1500 × g for 30–60 seconds.

NOTE Bubbles interfere with fluorescence detection.

Run the experiment

Place the reactions in the AriaMx instrument and run the Allele Discrimination - DNA Binding Type experiment.

Analyze the HRM data

At the end of the run, you can view the results of the experiment.

If you ran the experiment on the AriaMx Real-Time PCR System, see the abbreviated instructions below on viewing the HRM data. Consult the software's help system for full instructions.

Step 1. View the Melt Curves in the AriaMx software

The Melt Curve - Raw/Derivative Curve graph displays the fluorescence data collected during the melt segment (Y-axis) as a function of temperature (X-axis). View the normalized raw melt curves to ensure that you see a distinction between the different genotype populations in your experiment.

1. Navigate to the Graphical Displays screen, then select the Melt Curve - Raw/Derivative Curve graph.
2. Set the Fluorescence Term to **R** or **Rn**.
3. Make sure that Normalization is turned on.
4. Adjust the temperature range as needed.
5. Verify that the curves for the different genotype populations have distinguishable shapes. See Figure 1 for an example.

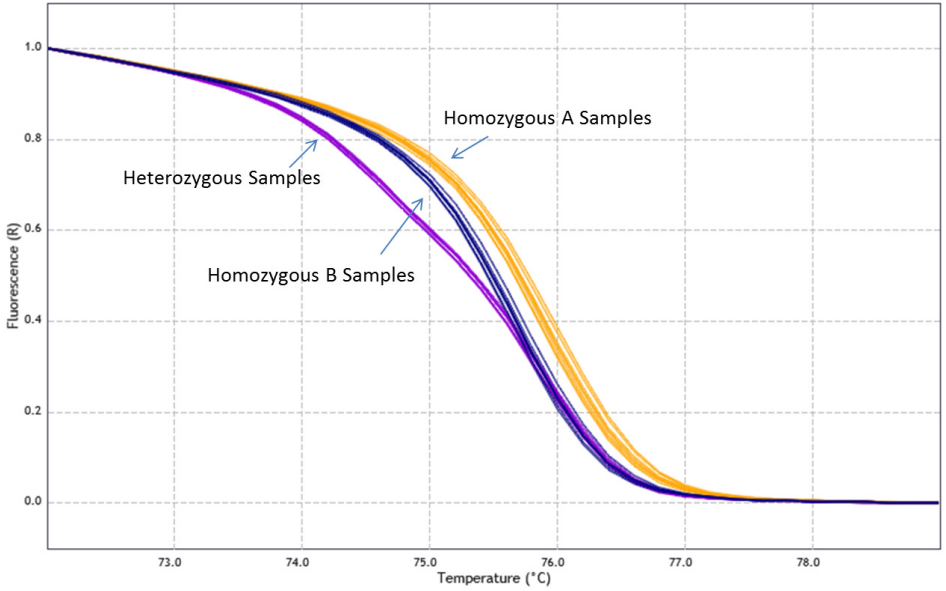

Figure 1 The Melt Curve - Raw/Derivative Curve graph in the AriaMx software, with labels denoting genotype populations

Step 2. View the Difference Plots in the AriaMx software

Difference plots are a useful way of viewing the results of an HRM analysis. The values plotted on the Y-axis are the difference in fluorescence between a sample in one well (or one replicate set) and a control sample from a designated well/replicate set.

Because the plots display the difference in fluorescence, you can detect even slight differences between two plots.

1. Make sure that the experiment has an HRM calibration plate assigned to it. Use the HCP selection button at the bottom of the Analysis Criteria and Graphical Displays screens.
2. Select the Melt Curve - Difference Plots graph on the Graphical Displays screen.
3. Designate the control target. The melt curve for the sample designated as the control target is the one to which all melt curves on the graph are compared.
   a. Make sure that the panel on the right side of the screen is expanded so that the **Control Target** settings are visible.
   b. In the **Well Type** drop-down list, select the well type in which the control target was amplified. Typically, one of the homozygous control wells is selected (Homo Allele A or Homo Allele B).
   c. In the **Well ID** drop-down list (or **Replicate** drop-down list if treating replicates collectively), select the specific well (or replicate set) in which the control target was amplified.
   d. In the **Target** drop-down list, select the dye or target name for the control target.

   The melt curves on the graph now plot the difference in fluorescence compared to the control target. See Figure 2 for an example.

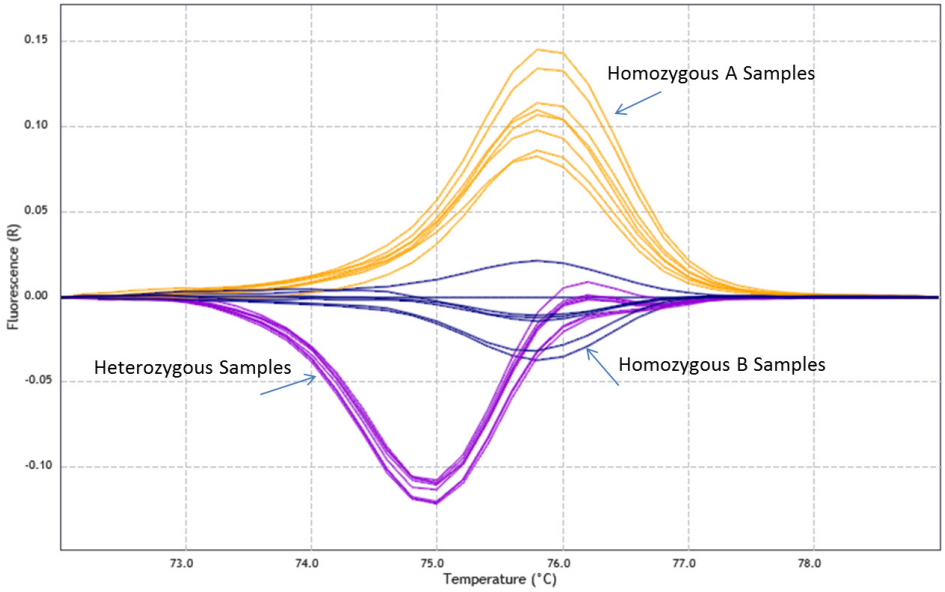

Figure 2 The Melt Curve - Difference Plots graph in the AriaMx software, with labels denoting genotype populations

Step 3. Assign experimental samples to a genotype call in the AriaMx software

1 Add the Call column to the Results Table, if it is not already being displayed.

2 In the Results Table, locate the row for experimental sample of interest. Right-click directly on that row to open a shortcut menu.

3 In the shortcut menu, click **Apply Call to Current Item**, then click the genotype that you want to assign (Homozygous A, Homozygous B, or Heterozygous).

If the Results Table is displaying the Call column, then the call that you applied appears in that column.

3 Troubleshooting FAQs

Table 8 Troubleshooting suggestions based on potential observations

Observation	Possible Cause	Suggestion
The difference plots do not show a distinction between homozygous allele A and homozygous allele B	The control DNA template samples do not have the correct genotype.	Verify that the control samples are correct by mixing the post-PCR reactions for the two samples and then re-melting in a high resolution melt segment. The resulting melt curve on the Difference Plots graph should align with the heterozygous control sample.
	The genetic variation is a particularly difficult one to detect.	Design primers to amplify a shorter amplicon.
	DNA templates are not sufficiently pure.	Check the purity of the DNA templates, and repurify if possible. Variations in salt concentrations, in particular, can shift the melting temperature.

Table 8 Troubleshooting suggestions based on potential observations

Observation	Possible Cause	Suggestion
The increase in fluorescence with cycling is low or nonexistant	The efficiency of PCR is low because the PCR product is too long.	Design the primers so that the PCR product is <200 bp in length.
	The melting temperatures of the primers are lower than the annealing temperature used during cycling.	Use a lower annealing/extension temperature or redesign the primers with a higher melting temperature.
		Increase the primer concentrations in the reactions.
	The DNA polymerase is not functioning optimally.	Make sure that the thermal profile starts with a 3-minute incubation at 95°C to activate the DNA polymerase in the master mix.
	The reaction is not optimized and insufficient product is formed.	Test for formation of enough specific product by gel electrophoresis.
There is increased fluorescence in no-template control reactions	The reaction has been contaminated.	Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips.
	Primer dimers were formed during cycling.	Redesign the primers.
The Cq in the no-template control sample is less than the total number of cycles but the amplification plot is flat	The background level of fluorescence is varying from cycle to cycle.	Review the amplification plot and, if appropriate, adjust the threshold or the baseline cycle range.

Table 8 Troubleshooting suggestions based on potential observations

Observation	Possible Cause	Suggestion
Multiple peaks are observed when plotting derivative melt data on the Melt Curve - Raw/Derivative Curve graph	A nonspecific amplicon was formed during cycling.	Determine the size of the nonspecific product by gel electrophoresis or on the Agilent BioAnalyzer. If possible, increase the annealing/extension temperature above the melting temperature of the nonspecific product.
	Primer dimers were formed during cycling.	Increase the annealing/extension temperature above the melting temperature of the primer dimers.
		Redesign the primers.
	Regions within the amplicon melt at different temperatures due to local variations in GC content.	Redesign the primers to amplify a region with a more uniform GC content.
	A rare genetic variation is present in the DNA template.	Redesign primers to amplify a region that avoids the problematic variation.

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In this book

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