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QUANTIMIX EASY HRM KIT

Kit for High Resolution Melting Analysis

REF.	FORMAT	CONTENT
10.641	100 rxn	Quantimix Easy HRM Kit
10.642	200 rxn	Quantimix Easy HRM Kit
10.643	500 rxn	Quantimix Easy HRM Kit

Store at -20°C

Research Use Only; Not for use in diagnosis procedures

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1. DESCRIPTION

High Resolution Melt (HRM) Assay is a hugely powerful technique for the detection of mutations, polymorphisms and epigenetic differences in double stranded DNA samples. HRM can be used to detect single base sequence variations such as SNPs or to discover unknown genetic mutations. It can be also used to quantitatively detect a small proportion of variant DNA in a background of wild type sequence.

The basis of the HRM assays is the characteristic "melting behaviour" of a particular DNA sample. Real Time instruments with the capacity for HRM analysis enable the precise characterisation of biological samples based on sequence length, GC content and/or DNA sequence heterogeneity. It is used to characterise DNA samples according to their dissociation behaviour during their transition from double stranded DNA (dsDNA) to single stranded DNA (ssDNA) with increasing temperature.

HRM analysis is a simpler and more cost effective way to characterise DNA samples than probe-based genotyping assays and, unlike conventional experiments, it is a closed system requiring no post-PCR. HRM is made possible not only by specialised instrumentation and software, but also by the introduction of third generation fluorescent dsDNA dyes that are brighter than those previously used and do not inhibit PCR reactions at the concentrations necessary to fully saturate the target DNA duplexes.

QUANTIMIX EASY HRM KIT has been optimised to deliver maximum efficiency, precision, and sensitivity during nucleic acid amplification in real time using a third generation intercalating fluorescent dye. The master mix contains a thermo-stable polymerase (Biotools HotSplit DNA Polymerase) that minimises nonspecific product and/or primer-dimer formation, improving HRM sensitivity and specificity. In addition, the master mix which also includes the rest of components for real time amplification assay reduces set up time and liquid handling steps.

2. REAGENTS INCLUDED IN THE KIT

- **QUANTIHHRM:** It is a 2X ready-to-use solution that contains all necessary components for real time amplification assays: Biotools HotSplit DNA Polymerase, dNTPs, Reaction Buffer, and MgCl₂ (final concentration: 4mM).
- **50 mM MgCl₂ Solution:** Used only for assays which require additional optimisation.

The intercalating fluorophore for HRM assay is not provided with the kit

3. STORAGE AND HANDLING INSTRUCTIONS

Store all components of the QUANTIMIX EASY HRM Kit at **-20°C** in a constant temperature freezer (frost-free freezers are not recommended). All reagents must be thawed and handled on ice. For frequent use, divide in aliquots to avoid multiple freeze-thaw cycles.

- **QUANTIHHRM:** Mix thoroughly before use.
- **MgCl₂ Solution:** Mix thoroughly before use.

If properly stored, all kit components are stable through the expiration date printed on the kit label.

4. GENERAL CONSIDERATIONS

Successful HRM is dependent on optimization of the PCR, thus the presence of non-specific amplification products and primer-dimers reduce HRM performance. Small differences in melt curves can arise from sources other than sequences, such as sample quality (e.g. impurities), amplicon length, primer design, and PCR reagents.

Template: Use the same DNA extraction and purification procedure for all the samples. We recommend the use of our Speedtools line for extraction of genomic DNA from blood (*Speedtools DNA Extraction Kit*); from tissue (*Speedtools Tissue DNA Extraction Kit*); from food (*Speedtools Food DNA Extraction Kit*); and from plant material (*Speedtools Plant DNA Extraction Kit*).

To improve HRM results it is recommended that the amount of template used be consistent between samples. Quantify the DNA content in each sample and if possible adjust samples to the same concentration. Results will vary depending upon DNA template quality and the particular sequence analysed. Low-quality DNA may produce nonspecific PCR products reducing the performance of HRM assays.

Amplicon Design: The length of the resulting DNA amplicon may impact the sensitivity and specificity of subsequent HRM analyses. Short amplicons, render easier melt profiles, since the effect of a mutation is greater on the overall melt temperature of the fragment. Moreover, the relative change in fluorescence intensity of shorter amplicons is greater than for longer amplicons. Although recommended amplicons size are between 100-300 bp, for SNP analysis the best results are obtained with amplicon lengths of 80-100 bp. Longer products can be analysed successfully with HRM but usually with lower resolution.

The amplicon sequence also affects post-PCR analysis. For instance, sequences with strong secondary structures or rich in GC content could make difficult the detection of single base substitutions.

Primer Design and Concentration: Primer-dimer and other nonspecific products can significantly affect the HRM analysis. Avoid complementarities of 2 or 3 bases at the 3' ends of primer pair to reduce primer-dimer formation. Design primers of 18-30 bp bases long, whenever possible with similar T_m values, with annealing temperature between 55-65°C, and with a GC content of 40-60%. The selected primers will amplify short products and ensure that they flank the SNP of interest.

In order to avoid primer-dimer formation, the concentration of primers must not be too high. Optimise the primer concentration for each experiment, we recommend a range between 0.05-0.5 µM.

Thermal Cycling Conditions: Many parameters influence both the specificity and efficiency of amplification including the temperature and duration of denaturation, annealing and elongation; ramp speed; and total cycle number. Due to the shorter length of real time PCR amplicons, cycling programs for real time PCR are typically shorter than regular PCR. While the number of amplification cycles in HRM assay should be higher than for standard real-time PCR (40-50 cycles).

The resolution of heterozygote melting profiles can be further enhanced by including a *pre-melting step* (denaturation and rapid cooling at 50°C) to ensure that all products have re-associated and encourage heteroduplex formation.

Perform HRM analysis in a melting domain to span about 10°C and centered around the amplicon T_m. To initially determine the melting point for each new PCR product, you can run HRM analysis to span a temperature range from 65-95°C, covering the full range of expected melting points. In future experiments, after determining the T_m, reduce the melt domain to span about 10°C (amplicon T_m ± 5°C).

The use of a "touch-down" cycling protocol for the PCR step is highly recommended to minimise the risk of primers mispriming.

5. STANDARD PROTOCOL

Materials to be supplied by user:

- Intercalating Fluorophore for HRM Assay
- Downstream oligonucleotide primer
- Upstream oligonucleotide primer
- Nuclease-free water

Laboratory workflow must be unidirectional, from pre-amplification to amplification area. Specific equipment for each working area must be used.

KEEP THE REACTION VIALS REFRIGERATED

Proceed to the Reagent Preparation Area in a laminar flow cabinet. Wear disposable gloves and use sterile and nuclease free plastic material. We also recommend the use of filter tips.

- 1.-Thaw and thoroughly mix all the reagents before dispensing
- 2.-**Dilute the fluorophore:** Before using, dilute the stock of your intercalating fluorophore to a working solution (follow the instructions and advice provided by the manufacturer). Protect the dye from prolonged exposure to light.
- 3.-The inclusion of positive and negative controls is highly recommended in each experiment.
- 4.-Prepare the reactions according to Table 1.

TABLE 1. Master Mix preparation

COMPONENT	Final Concentration	20 µl rxn
2X QUANTIHRM	1 X	10 µl
50 mM MgCl ₂ Solution*	4-6 mM	x µl
Primers	0.05-0.5 µM	x µl
Diluted HRM Fluorophore [†]	-	x µl
Nuclease free water	-	Up to 20 µl
Template	2-15 ng genomic DNA	x µl

*Only necessary for concentrations of MgCl₂ >4mM

[†]Final concentration: 0.5-2.0 X for Chromofly™ Dye

Proceed to DNA Purification Area separate from other sources of DNA.

Never introduce DNA in the laminar flow cabinet from the reagent preparation area. Amplification must start within 10 minutes of adding template and primers to the amplification mix. Keep all reagents on ice until their introduction in the thermal cyclor.

- 5.-Add template DNA to each reaction tube. Close the tubes and mix gently.
- 6.-Centrifuge the amplification vials briefly.

Proceed to Amplification Area

- 7.-Place the tubes in the thermal cyclor and begin cycling.
- 8.-Program your HRM thermal cyclor following the recommendations provided by the manufacturer (see suggestions on Table 2).

TABLE 2. PCR cycling parameters for QUANTIMIX EASY HRM Kit

	STEP	Temperature	Time
Initial Denaturation*	Enzyme activation	95-98°C	5-8 min
	Denaturation	95-98°C	5-15 sec
Amplification cycling (40-50)	Annealing	T _m of primers (55-65°C)	5-30 sec
	Extension** #	72°C	15-40 sec
	Denaturation	95-98°C	30-45 sec
Pre-Melting***	Denaturation	95-98°C	30-45 sec
	Rapid cooling	50°C	30 sec
HRM Melting Curve		Amplicon T _m ± 5°C (increments: depend on each assay)	

*HotSplit DNA Polymerase is activated by this heating step

**Optional: In 2-step protocol fluorescence acquisition during the Annealing/Extension Step

Fluorogenic data should be collected during this step in the appropriate channel.

***Optional but highly recommended

The interpretation of the results is performed with the help of specific software (follow the instructions and advice provided by the manufacturer). Placement of the pre- and post-melt regions can affect resulting clusters and classifications of the HRM analysis results. The pre-melt region is the most critical, as correct placement of these bars can maximize differences between the variants.

6. TROUBLESHOOTING

Prior to running HRM, examine real-time plot data for abnormal amplification curve shape. Problems with HRM experiments are usually evidenced by abnormal PCR characteristics (high Ct values, post-amplification artifacts, primer-dimers, etc) that trigger inconclusive HRM analysis.

PCR Problems

1. **Check template quality and quantity.** Check the quality of template DNA by agarose gel electrophoresis or fluorimetry. Use sufficient template and maintain consistent amount between samples. Amplification plots should have a Ct≤30 cycles.
Take care with some inhibitors such as ethanol carry-over. Dilution of the sample can help to solve these problems.
2. **Check primer design and concentration.** Although lower primer concentration can prevent primer-dimer formation, sufficient primers are needed for successful real-time PCR (≤ 0.5 µM). Increase primer concentration in increments of 0.05 µM, and run product on gel to ensure single band.
3. **Optimise the fluorphore concentration.** Increase the proportion of Dye in the reaction.
4. **Revise cycling conditions:**
-Increase the number of amplification cycles (beware of nonspecific amplification).
-Check detection step accuracy. Ensure the fluorescence detection step takes place during the correct step of the PCR cycling program.
-Choose the appropriate filter. For a real-time instrument that is equipped with a multiple dye detection system, ensure that the appropriate channel is activated.
-Select a "touch-down" cycling protocol for the PCR step to minimize the risk of primer mispriming

HRM Analysis Inconclusive

1. **Replicate samples show wide spread in HRM curves.** Ensure similar DNA starting concentrations between samples.
2. **Melt curves with multiple melt domains.** Presence of more than one mutation site: re-optimize PCR and/or redesign primers to decrease amplicon length. Confirm mutation by sequencing.
3. **HRM curves do not clearly discriminate between homozygous and heterozygous samples.** Check by PCR followed by melt curve analysis to look for nonspecific products and primer-dimers.

7. ORDERING INFORMATION

DESCRIPTION	Size	Reference
QUANTIHRM	1100 µl	10.641
	2 x 1100 µl	10.642
	5 x 1100 µl	10.643
50 mM MgCl₂ Solution	1.8 ml	10.641
	1.8 ml	10.642
	1.8 ml	10.643