KAPABIOSYSTEMS

Technical Data Sheet

KAPA HRM FAST qPCR Kit

KR0401_S – v3.20

Product Description

The KAPA HRM FAST PCR Kit is a convenient, ready-to-use, master mix designed for the high-performance detection of DNA sequence variations by High Resolution Melt (HRM) analysis. The kit contains a novel DNA polymerase engineered, via a process of molecular evolution, for fast and efficient DNA amplification in the presence of high concentrations of intercalating fluorescent dyes. KAPA HRM FAST PCR Kits also contain EvaGreen®, a next-generation, saturating fluorescent dye which selectively binds to double-stranded DNA. In contrast to SYBR® Green I dye, EvaGreen can be used at higher concentrations without PCR inhibition and shows equal binding affinity for GC-rich and AT-rich regions. The combination of an engineered DNA polymerase and EvaGreen dye enables amplification and discrimination of even the most challenging sequence differences (i.e. Class IV) without sequence preference.

Product Applications

KAPA HRM FAST PCR Kits are optimized for HRM endpoint analysis and are not recommended for quantitative PCR. Kits are ideally suited for:

- SNP genotyping
- Mutation discovery
- Species identification and genotyping
- DNA fingerprinting
- Genetic association studies.

Kit Codes and Components	
KK4201 100 x 20 µL rxns	KAPA HRM FAST MasterMix (2X) KAPA $MgCl_2$ (25 mM)
KK4202 500 x 20 µL rxns	
KK4203 1000 x 20 µL rxns	

Quick Notes

- KAPA HRM FAST PCR Kits contain EvaGreen, a saturating double-stranded DNA dye optimized for HRM analysis.
- The 2X Master Mix contains a buffer system optimized for HRM analysis and a novel DNA polymerase to enhance amplification efficiency. **The Master Mix does not contain $MgCl_2$.**
- Template DNA from different samples should be prepared using the same method and eluted or diluted in the same buffer (ideally 10 mM Tris-HCl, pH 8.5).
- The optimal amount of template DNA is between 20 ng and 100 pg per 20 µL reaction.
- To maximize the sensitivity of the assay, use the lowest concentration of primers that can be used without compromising the efficiency of the PCR reaction (0.1 µM – 0.3 µM of each primer).
- The concentration of $MgCl_2$ can greatly affect the annealing of heterozygotes and melting of the amplicons during HRM. The recommended starting concentration is 2.5 mM $MgCl_2$, which can be adjusted as required. 1.5 – 3.5 mM final $MgCl_2$ is recommended for most HRM assays.
- Instrument gain settings may have to be adjusted for maximum sensitivity using KAPA HRM FAST PCR Kits.

Product Specifications

Shipping and Storage

KAPA HRM FAST qPCR Master Mix (2X) Kits are shipped on dry ice or ice packs, depending on the destination country. Upon receipt, immediately store all components at -15°C to -25°C in a constant-temperature freezer. When stored under these conditions and handled correctly, the kit components will retain full activity until the expiry date indicated on the kit label.

Handling

Always ensure that components have been fully thawed and thoroughly mixed before use. KAPA HRM FAST qPCR Master Mix (2X) may not freeze solidly, even when stored at -15°C to -25°C.

The EvaGreen dye contained in KAPA HRM FAST qPCR Master Mix (2X) is light sensitive. Exposure to direct light for an extended period of time will result in loss of fluorescent signal intensity.

KAPA HRM FAST qPCR Master Mix (2X) is stable through 30 freeze-thaw cycles. Ensure that all reagents are stored protected from light at -15°C to -25°C when not in use. When protected from light, reagents are stable in the dark at 2°C to 8°C for at least one week and may be stored at this temperature for short-term use, provided that they do not become contaminated with microbes and/or nucleases.

Quality Control

All kit components are subjected to stringent functional quality control, are free of detectable contaminating exo- and endonuclease activity, and meet strict requirements with respect to DNA contamination. Please contact Technical Support at **sigma-aldrich.com/techservice** for more information.

Safety Information

Precautions

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material can vary, the operator must optimize pathogen inactivation and follow the appropriate measures according to local safety regulations.
- Do not eat, drink, or smoke in the laboratory area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats, and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online at www.sigmaaldrich.com, or upon request from www.sigma-aldrich.com/techservice.

KAPA HRM FAST qPCR Protocol

Any existing HRM assay performed efficiently using standard cycling conditions may be converted to a Fast HRM assay using the KAPA HRM FAST Kits. Typically, minimal re-optimization of reaction parameters is required. Care must be taken when optimizing instrument gain settings due to differences in levels of fluorescence emitted from the EvaGreen® dye as compared to SYBR® Green I and other fluorescent dyes.

1. Master Mix Preparation

1.1 Ensure all reaction components are properly thawed and mixed.

1.2 Prepare a master mix containing the appropriate volume of all reaction components common to all or a subset of reactions to be performed. **NOTE:** $MgCl_2$ is not included in the master mix and must be added separately.

1.3 Always include a No Template Control (NTC) to allow for detection of contamination of reaction components.

1.4 Calculate the required volume of each component based on the following table:

Component	20 µL reaction[1]	Final conc.
PCR-grade water	Up to 20 µL	N/A
KAPA HRM FAST Master Mix (2X)	10 µL	1X
25 mM $MgCl_2$	2 µL	2.5 mM[2]
10 µM forward primer	0.2 – 0.6 µL	100 – 300 nM
10 µM reverse primer	0.2 – 0.6 µL	100 – 300 nM
Template DNA[3]	Ideally ≤1.0 µL	100 pg – 20 ng[4]

[1]Reaction volumes may be adjusted from 3 – 25 µL, depending on the block type and instrument used. Reaction volumes >25 µL are not recommended.

[2]Recommended range is 2.5 – 3.5 mM for most HRM assays.

[3]To obtain the best results, template DNA samples should be purified using the same system (to minimize any buffer effect variance in the HRM), be approximately the same concentration and volume added should be kept to a minimum to reduce buffer carryover which can affect detection of Class III and Class IV mutations (G/C and A/T respectively).

[4]Do not exceed 20 ng per 20 µL reaction. For more information, refer to **Important Parameters: Template**.

2. Reaction Setup

2.1 Transfer the appropriate volumes of qPCR master mix, template, and primers to each well of a PCR plate/tube(s).

2.2 Cap or seal the reaction plate/tube(s) and centrifuge briefly.

3. Perform amplification and HRM

3.1 If applicable, select fast mode on the instrument.

3.2 Confirm that the qPCR protocol to be used conforms to the following parameters:

Step	Temp.	Duration	Cycles
Enzyme activation	95°C	20 sec – 3 min[1]	Hold
Denaturation	95°C	5 sec	45
Annealing/ extension/ data acquisition[2]	60°C	≥20 sec[3]	
Dissociation[4]	According to instrument guidelines		

[1]20 sec at 95°C is sufficient time for enzyme activation; however, optimal denaturation of complex targets may require up to 3 min denaturation.

[2]Small amplicon fragments (40 – 200 bp) are recommended for HRM analysis. Longer amplicons may require an additional 10 sec extension time for every 100 bp increase in length. For 3-step cycling protocols, anneal at optimal annealing temperature for 20 sec followed by 1 sec extension and data acquisition at 72°C.

[3]Select shortest time possible for instrument, but not <20 sec.

[4]The temperature of the HRM can be optimized depending on the melting of the amplicon. Smaller or larger temperature increments may be required.

NOTE: Probe annealing. A probe can be used in combination with the KAPA HRM FAST kit for enhanced sequence detection. Asymmetric PCR is recommended for this application using 0.1 µM Forward Primer, 0.5 µM Reverse Primer and 0.5 µM Forward Probe and 50 – 60 cycles for amplification. The T_m of the probe should be at least 5°C lower than the T_m of the forward and reverse primers to minimize the formation of non-specific amplification products. The probe annealing step is performed at 40°C for 1 min after denaturation and before HRM. The optimal annealing temperature of the probe may need to be adjusted.

4. Data Analysis

4.1 Data analysis is dependent on experimental design. Refer to the instrument guidelines for more information on how to perform the appropriate data analysis.

Important Parameters

Template

Genomic DNA, plasmid DNA, or cDNA can be used as template. For best results, template DNA should be prepared using the same method and eluted or diluted in the same buffer (ideally 10 mM Tris-HCl, pH 8.5). Additionally, the template DNA should be the same concentration, this helps reduce background fluorescence that can reduce resolution during the HRM. For optimal results, use up to 20 ng of genomic DNA or plasmid DNA per 20 µL reaction. Using greater amounts of template may increase non-specific amplification. Low concentrations (e.g. <100 pg) of template can result in poor amplification and a greater chance of non-specific amplification. The volume of template added should not exceed 5% of the final PCR volume (e.g. for a 20 µL qPCR reaction, use a maximum 1.0 µL DNA) the buffer has been optimised to discriminate the most difficult Class IV mutations (A/T) and adding additional salts can reduce the detection limits.

Primers

Careful primer design and purification (HPLC-purified primers are recommended, but not essential) is particularly important in order to minimize loss in sensitivity due to the production of nonspecific amplification products. Production of non-specific amplification products can greatly affect the HRM resolution and becomes more prominent at low- and high-target concentrations. To maximize the sensitivity of the assay, use the lowest concentration of primers that can be used without compromising the efficiency of the PCR reaction (50 – 400 nM of each primer). For optimal SNP detection, design primers that specifically amplify PCR products of 40 – 200 bp in length. Clearer differentiation of single mutations is obtained with shorter amplicons, and minimizes the risk of covering multiple mutations. When using longer amplicons (e.g. mutation discovery or species identification), longer amplification products will reduce the possibility of detecting Class III and Class IV mutations (G/C and A/T respectively). The primers should exhibit a melting temperature (Tm) of approximately 60°C, to take advantage of two-step cycling. For best results, use a primer design site such as Primer 3 or Primer 3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

EvaGreen® dye

KAPA HRM FAST Master Mix (2X) contains an optimized concentration of the fluorescent dye, EvaGreen. High signal intensities are achieved as a result of saturation of all double-stranded DNA molecules, emitting a fluorescent signal on binding. The excitation and emission maxima of EvaGreen are at 500/530 nm (DNA bound) and 471 nm (non-DNA bound), respectively, which are compatible with use on any real-time cycler. Gain settings may have to be reduced or increased for proper optimization using KAPA HRM FAST Master Mix with EvaGreen.

DNA Polymerase

KAPA HRM FAST PCR Kits contain a highly engineered version of *Taq* DNA polymerase designed specifically for PCR amplification in the presence of dsDNA binding fluorophores. The DNA polymerase is formulated as a hot start and displays no enzymatic activity at ambient temperature. This prevents the formation of misprimed products and primer-dimers during reaction setup, resulting in high PCR specificity and consistent melting behavior required for HRM. The enzyme is activated at the start of a reaction by a 20 sec, 95°C incubation step. The activation of the enzyme is complete after 20 sec, however complex targets may require up to 3 min for optimal denaturation. The hot start feature enables reactions to be set up rapidly and conveniently at room temperature.

HRM analysis

Following real-time qPCR, HRM analysis should be performed immediately (to minimize any non-specific amplification) using the Green channel. If stationary phase is reached early during amplification (as viewed in the amplification plot), the number of cycles can be reduced as required. If possible, optimise the gain settings prior to HRM analysis. When performing HRM analysis for the first time, a wide range of temperatures (e.g. 65°C – 90°C) is recommended to ensure the DNA melting temperature is detected. In subsequent experiments the temperature range can be narrowed as required. Typical temperature increments can be adjusted depending on the separation of genotypes. Program your thermocycler according to the instructions provided.

Instruments compatible with HRM analysis*

Instrument
QIAGEN Rotor-Gene® 6000, Q
Applied Biosystems® 7500 Fast, 7900HT Fast, ViiA™ 7, StepOnePlus™, StepOne™
Illumina Eco™
Roche LightCycler® 480, 96, Nano
Bio-Rad CFX96, CFX384

*Not a comprehensive list. Please refer to manufacturer's manual for these and other qPCR instruments for full details.

Magnesium chloride

The concentration of $MgCl_2$ can greatly affect the annealing of heterozygotes and melting of the amplicons during HRM. The recommended starting concentration is 2.5 mM $MgCl_2$ which can be adjusted as required. The concentration of $MgCl_2$ can help the determination of heterozygosity by helping annealing of mispaired bases. Therefore KAPA HRM FAST Master Mix (2X) does not contain any $MgCl_2$, and is supplied separately at a 25 mM concentration. It is recommended that a final concentration of 1.5 – 3.5 mM is used for best results with HRM analysis.

Troubleshooting

Symptom	Possible Cause	Solution
Replicate samples show a wide variance during HRM analysis (i.e. results do not allow accurate genotyping, particularly between homozygotes)	Variations in reaction mixture (e.g. salt)	• Check the purity of the template solution. • Try to dilute all template samples in the same buffer (10 mM Tris-HCl, pH 8.5) to equalize template buffer. • Re-purify the DNA using a new method. • Ensure all reagents and master mixes are vortexed well prior to mixing and starting analysis.
	Concentrations of template is very different	Ensure all samples are the same DNA concentration for maximum resolution.
	Rare or new genetic variants may generate results outside of the expected ranges	• Sequence the unusual results. • Redesign primers to produce a shorter amplicon, hopefully avoiding the genetic variation. • Mixing the amplicons (9.5 µL from each of two amplicons) can help identify potential new variants.
	Secondary structure in the amplicon	Use a Melting Profile software system to ensure no secondary structures are present.
Multiple melting peak(s) appear(s) for one product	Two products of the same length	• Check the products on an agarose gel. • Heterozygosity can result in two peaks.
	A HRM melt uses more readings than a standard melt that can give the impression of ≥2 products on a melting peak	• Check the products on an agarose gel. • Adjust the filter settings of the melt peak analysis to minimize noise. • Increase the annealing/extension temperature to help minimize non-specific amplification.
Only one homozygote is detected during HRM	Difficult to detect mutation (typically Class IV, A/T)	May need to sequence to confirm or use a probe or snapback primers.
	Only one homozygote is present	Mix together 9.5 µL of each of the homozygotes and run an HRM analysis (include an initial melt at 95°C for 3 min). The melting profile should change to the heterozygote which is usually easier to detect.
Different genotypes cannot be detected during HRM	$MgCl_2$ concentration needs to be optimized	Perform amplification and HRM at 1.5, 2.5 and 3.5 mM $MgCl_2$ to find the optimal concentration for HRM analysis.
	Non-specific amplification	• Check the melting curve to see if multiple peaks are present (adjust filter settings if necessary). • Run on a 2 – 3% agarose gel to see if multiple bands are present. • Reduce primer concentration to minimize primer-dimers and non-specific priming. • Increase the annealing/extension temperature. • Redesign primers.
	Template contains HRM inhibitors (e.g. high salt)	Re-purify DNA template.
	Difficult to detect mutation (typically Class IV, A/T)	• Shorter amplicons are better for detecting Class III and Class IV mutations. • Redesign primers to produce a smaller amplicon. • Use a probe or snap-back primers.

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