KAPA™ HRM FAST PCR Kit

KK4201 100 x 20 µl reactions	**1 x 1 ml - KAPA HRM FAST Master Mix (2X)** **1 x 1.6 ml - 25 mM $MgCl_2$** (not included in mix)
KK4202 500 x 20 µl reactions	**1 x 5 ml - KAPA HRM FAST Master Mix (2X)** **2 x 1.6 ml - 25 mM $MgCl_2$** (not included in mix)
KK4203 1000 x 20 µl reactions	**2 x 5 ml - KAPA HRM FAST Master Mix (2X)** **4 x 1.6 ml - 25 mM $MgCl_2$** (not included in mix)

1. 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. Type IV) without sequence preference.

2. Product Applications

KAPA HRM FAST PCR Kits are optimized for endpoint HRM 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

3. Product Specifications

3.1 Shipping and Storage

Upon arrival, store kit components protected from light at –20 °C in a constant-temperature freezer. When stored under these conditions and handled correctly, full activity of the master mix is retained for at least one year, or until the expiry date indicated on the kit.

3.2 Handling

Minimize exposure of the Master Mix (2X) to direct light. Exposure to direct light for an extended period of time may result in loss of fluorescent signal intensity. Always ensure that the product has been fully thawed and mixed well before use.

3.3 Quality Control

KAPA HRM FAST Master Mix (2X) is free of contaminating DNase and RNase. It is functionally tested to demonstrate detection of various SNPs by HRM analysis.

3.4 Product Use Limitations

KAPA HRM FAST PCR Kits are sold exclusively for research purposes and *in vitro* use. Neither the product, nor any individual components, was tested for use in diagnostic applications or for drug development, nor is it suitable for administration to humans or animals.

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. For most HRM assays 1.5 – 4.0 mM final $MgCl_2$ is recommended.
- Instrument gain settings may have to be reduced or increased for maximum sensitivity using KAPA HRM FAST PCR Kits.

Instruments suitable for HRM analysis*

- QIAGEN Rotor-Gene® 6000
- QIAGEN Rotor-Gene® Q
- Applied Biosystems® 7500 Fast System
- Applied Biosystems® 7900
- Applied Biosystems® *ViiA™ 7* Real-Time PCR System
- Roche LightCycler® 480
- Bio-Rad CFX 96
- Bio-Rad CFX 384
- Illumina Eco™ Real-Time PCR System

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

4. KAPA HRM FAST PCR Protocol

Any existing HRM assay performed efficiently using standard cycling conditions may be converted to a Fast HRM assay using the KAPA HRM FAST PCR Kit. 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.

4.1 Step 1: HRM Reaction Setup

- Before preparing the amplification and HRM reactions, **thoroughly mix** the KAPA HRM FAST Master Mix (2X), 25 mM $MgCl_2$, PCR-grade water, primers and template DNA. **Note:** $MgCl_2$ is not included in the master mix and must be added separately.
- For optimal results, template DNA samples should be purified using the same system (in order to minimize DNA melting variance due to buffer differences) and be diluted to approximately the same concentration. The volume added should be kept to a minimum to reduce buffer carryover which can affect detection of Type III and Type IV mutations (G/C and A/T resepectively).
- The recommended setup is given in the table below, but for maximum sensitivity this may need to be adjusted.

Component	Recommended volumes	Final conc.	Recommended conc. range
PCR grade water	Up to 20 µl	–	–
2X KAPA HRM FAST Master Mix	10.0 µl	1X	1X
25 mM $MgCl_2$	2.00 µl	2.5 mM	1.5 – 4.0 mM
Forward Primer (10 µM)	0.40 µl	0.2 µM	0.1 µM – 0.3 µM
Reverse Primer (10 µM)	0.40 µl	0.2 µM	0.1 µM – 0.3 µM
Template DNA	Ideally ≤1.0 µl	1 – 10 ng	100 pg –20 ng

4.2 Step 2: Plate Setup

- Transfer the appropriate volume of reaction mixture to a PCR tube or each well of a PCR plate. Reaction volumes may be scaled down from 20 µl to 10 µl if low volume tubes/plates are used.
- Cap or seal the reaction tubes/plate and centrifuge briefly.

4.3 Step 3: Amplification and HRM

- If applicable, select fast mode on the instrument.
- Program the following cycling protocol:

Step	Temperature	Duration	Cycle	Comments
Enzyme activation	95 °C	1 min - 3 min	Hold	Use 3 min for complex genomic templates
Denaturation	95 °C	5 sec	x 40 - 45	Activate data acquisition (green channel)
Annealing/extension	60 °C	30 sec[1]		
Denaturation	95 °C	1 min	Hold	Amplicon melt
HRM	65 – 90 °C[2]	2 sec	N/A	Data acquisition (green channel)

[1] Small amplicons (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 temperature for 20 sec followed by 1 sec extension and data acquisition at 72 °C.

[2] The temperature range 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, 0.5 µM Forward Probe (with a blocked 5′ end) and 50 – 60 cycles for amplification. The Tm of the probe should be at least 5 °C lower than the Tm of the forward and reverse primers to minimize the formation of non-specific amplification products. Probe annealing time and temperature must be determined empirically based on the Tm of the probe.

4.4 Step 4: Data Analysis

- Data analysis varies depending on the instrument used. Please refer to your instrument user guide for information.

5. Important Parameters

5.1 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 at the same concentration; this helps reduce background fluorescence that can reduce resolution during the HRM. For optimal results, use up 100 pg - 20 ng of genomic DNA per 20 µl reaction. Higher or lower concentrations of DNA may result in increased 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 of 1.0 µl DNA). The buffer has been optimised to discriminate the most difficult Type IV mutations (A/T) and adding additional salts can reduce the detection limit.

5.2 Primer design and amplicon length

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 nonspecific amplification products. 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 designing website such as *Primer 3* or *Primer 3 Plus* 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). For optimal SNP detection, design primers that specifically amplify PCR products 40 – 200 bp in length. Clearer differentiation of single mutations is obtained with shorter amplicons, and minimizes the risk of covering multiple mutations. Using longer amplicons (e.g. mutation discovery or species identification) will reduce the possibility of detecting Type III and Type IV mutations (G/C and A/T respectively).

5.3 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, enabling reaction setup at room 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 1 -3 min, 95 °C incubation step.

5.4 HRM analysis

Following amplification, HRM analysis should be performed immediately (to minimize any non-specific amplification), acquiring data in 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 setting 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 that the DNA melting temperature is covered. 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.

5.5 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.

5.6 Magnesium chloride

The KAPA HRM FAST Master Mix (2X) does not contain any $MgCl_2$, and is supplied separately at a 25 mM concentration. The concentration of $MgCl_2$ can greatly affect the annealing of sequence variants, amplicon melting, and the specificity of amplification during HRM. The recommended starting concentration is 2.5 mM $MgCl_2$ which can be adjusted as required. The concentration of $MgCl_2$ can facilitate the determination of heterozygosity by promoting the annealing of mispaired bases. It is recommended that a final concentration of 1.5 – 4.0 mM is used for best results with HRM analysis.

For technical support please contact: support@kapabiosystems.com or visit the FAQs at www.kapabiosystems.com/products/name/kapa-hrm-fast-pcr-kits

A comprehensive application guide "Introduction to High Resolution Melt Analysis" is also available for download at www.kapabiosystems.com/products/name/kapa-hrm-fast-pcr-kits

6. 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 are 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 peaks appear 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 Type 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 a 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 and 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 Type IV, A/T)	• Shorter amplicons are better for detecting Type III and Type IV mutations. • Redesign primers to produce a smaller amplicon. • Use a probe or snap-back primers.

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Kapa Biosystems Inc. is licensed by Biotium Inc. to sell reagents containing EvaGreen dye for use in real-time PCR, for research purposes only.

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KAPABIOSYSTEMS
www.kapabiosystems.com

Boston, Massachusetts, United States
600 West Cummings Park, Suite 2250
Woburn, MA, 01801 U.S.A.
Tel: +1 781 497 2933 Fax: +1 781 497 2934

Cape Town, South Africa
2nd Floor, Old Warehouse Building, Black River Park,
Fir Road, Observatory, 7925 Cape Town, South Africa
Tel: +27 21 448 8200 Fax: +27 21 448 6503