KAPABIOSYSTEMS

Rapid, high-throughput genotyping using High Resolution Melt (HRM) analysis is limited by laborious genomic DNA extraction methods and time-consuming DNA amplification protocols.

The unique combination of KAPA Express Extract and KAPA HRM FAST PCR Kits with the Illumina Eco™ Real-Time PCR System streamlines SNP genotyping workflows and improves turnaround time.

Introduction

High-resolution melt (HRM) analysis is a fast, cost-effective and broadly applicable method for detecting DNA sequence variation; the technique is ideal for accurate genotyping of multiple SNPs from a large number of samples. Current HRM workflows require laborious DNA extraction methods and time-consuming protocols.

Buffer carryover from the upstream DNA extraction process can introduce variability in amplicon melting profiles and impact the reproducibility of HRM analysis. The KAPA Express Extract Kit contains a novel thermostable protease in a buffer that minimally modifies the melting behavior of amplicons during HRM. The single tube and buffer system eliminate the possibility of residual ethanol and salt contamination, offering consistent, rapid and convenient extraction of PCR-ready DNA from buccal swabs in 15 min.

The KAPA HRM FAST PCR Kit is a ready-to-use master mix developed for the high performance detection of DNA sequence variations. 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 contain EvaGreen®, a next-generation, saturating fluorescent dye specifically chosen because of its unique dye chemistry and maximum sensitivity in the most challenging applications (i.e. genotyping Type IV SNPs).

For the highest accuracy during HRM, the temperature must remain uniform across the entire heating block of the thermocycler. The Illumina Eco™ Real-Time PCR System offers true temperature control, resulting in ±0.1 °C well-to-well thermal uniformity. The 5.5 °C/sec average ramp rate of the instrument allows for amplification and HRM in ~60 min.

The unique combination of KAPA Express Extract kits and KAPA HRM FAST PCR kits with the Illumina Eco™ Real-Time PCR System, streamlines complete genotyping workflows, allowing the extraction of DNA, and the determination of gene mutations or SNPs in less than 90 min.

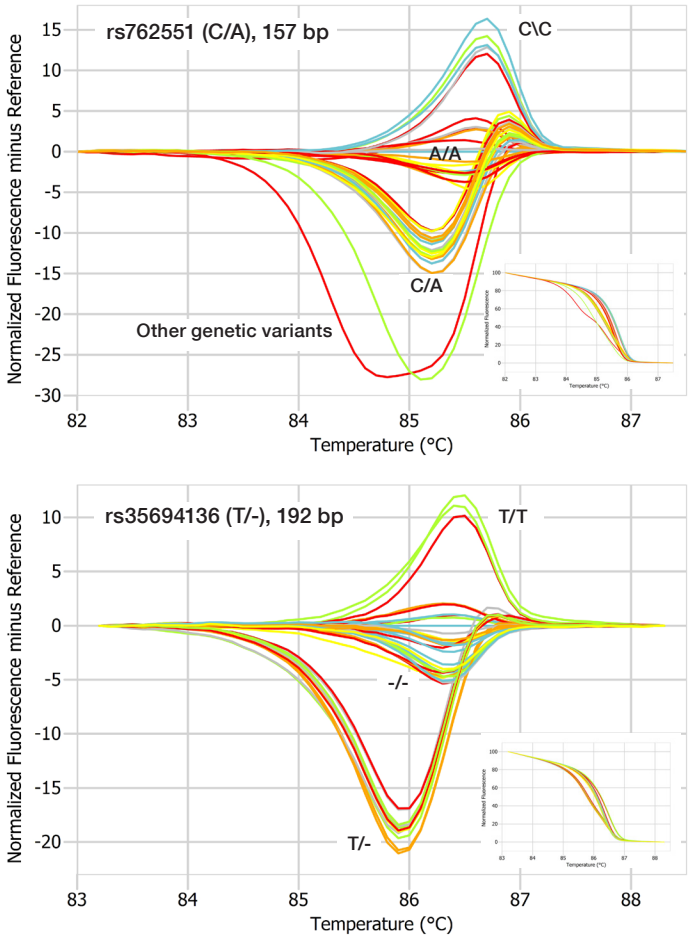


Figure 1. HRM Analysis Data showing the Difference Graphs and Normalized Data (inset) of SNPs rs762551 (C/A) and rs35694136. (T/-) using the Illumina Eco™ Real-Time PCR System. Analysis of 45 gDNA samples, purified from buccal swabs using KAPA Express Extract, and analysis using KAPA HRM FAST on the Illumina Eco™. Labels indicate the genotype of the different melting profiles. SNPs rs762551 and rs35694136 are associated with the cytochrome P450 (CYP) gene involved with the oxidation of organic compounds involved in drug metabolism and bioactivation.

DNA Extraction Protocol

An overview of the KAPA Express Extract protocol is shown in Table 1. Buccal swabs were obtained from 45 individuals. The cotton bud was cut into a 1.5 ml Eppendorf tubes and 400 µl of the KAPA Express Extract master mix was added directly. The lysis protocol was performed as shown in Table 2 using two heat blocks (a single heat block can also be used with the samples stored on ice between the lysis and heat inactivation steps). After the 15 min lysis protocol, each 1.5 ml tube was centrifuged (16,000 g, 1 min) to pellet debris, and ~100 µl of the supernatant removed into a fresh 1.5 ml Eppendorf tube. DNA extracts do not have to be quantified, and may be used directly in PCR.

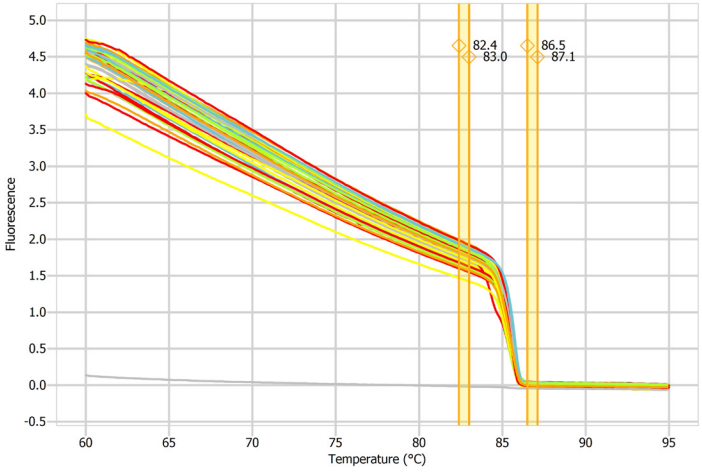
Table 1: KAPA Express Extract protocol for buccal swabs

Step	Description
Sample setup	Take each buccal swab and cut cotton bud into individual 1.5 ml Eppendorf tubes.
Reaction setup	Combine KAPA Express Extract buffer and enzyme in a master mix and add 400 µl to each sample.
Lysis	Incubate at 75 °C for 10 min. During this step, cells are lysed, nucleases and proteins degraded and DNA released.
Heat inactivation	Incubate at 95 °C for 5 min to inactivate the thermostable Express Extract enzyme.
Sample recovery	Centrifuge for 1 min at 16,000 g to pellet debris. Recover DNA-containing supernatant.

Table 2: KAPA Express Extract reaction setup for buccal swabs

Reaction component	Final conc.	Per 400 µl reaction	Master mix (50X)
PCR grade water	–	Up to 400.0 µl	23.9 ml
10X KAPA Express Extract Buffer	0.5X	20.0 µl	1000 µl
KAPA Express Extract Enzyme (1 U/µl)	5 mU/µl	2.00 µl	100 µl
Buccal swab	–	1 x cotton bud	–

Figure 2: Positioning of pre- and post-melt regions on Norm. and Difference Plot



PCR reaction conditions and cycling parameters

Primers were designed using Primer 3 Plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to amplify target SNPs rs762551 (175 bp) and rs35694136 (192 bp). The SNP rs762551 is located close to three other rare SNPs (rs17861150, rs35858607 and rs41279192) and were incorporated into the target. A master mix containing KAPA HRM FAST, $MgCl_2$ and primers was prepared as detailed in Table 3, and 9 µl of the master mix was added to the 48 wells of a plate suitable for the Illumina Eco™. A 1 µl sample from the 45 gDNA samples purified using KAPA Express Extract was added into each well as appropriate, leaving three no template controls (NTC). Total time for amplification and HRM was 58 min. Figure 2 shows how the pre- and post-melt regions were positioned to obtain the Normalization Data and Difference Plots.

Table 3: KAPA HRM FAST reaction setup

Reaction component	Final conc.	Per 10 µl reaction[1]
PCR grade water	–	Up to 10.0 µl
2X KAPA HRM FAST Master Mix[2]	1X	5.0 µl
25 mM $MgCl_2$	2.5 mM	1.0 µl
Forward primer (10 µM)	0.2 µM	0.2 µl
Reverse primer (10 µM)	0.2 µM	0.2 µl
DNA from buccal swab	–	1.0 µl[4]

[1] Reaction size of 10 µl is recommended for the Illumina Eco™.
[2] $MgCl_2$ is not included in the 2X KAPA HRM FAST Master Mix and must be added separately.

Table 4: KAPA HRM FAST cycling parameters for the Illumina Eco™

Cycling step		Temperature and time	
Initial denaturation		3 min at 95 °C	Hold
Denaturation		5 sec at 95 °C	x 45 cycles
Annealing/Extension[1]		30 sec at 60 °C	
HRM[2]	Melt	95 °C	15 sec
	Anneal	60 °C	15 sec
	Melt	95 °C	15 sec

[1] The optimal annealing temperature of the primer set is 60 °C for rs35694136 and 62 °C for rs762551. Extension time may have to be adjusted for other primer sets.
[2] This is the standard setup for all HRM analysis using the Illumina Eco™. The protocol may need to be adjusted for optimal performance with other amplicons and instruments..

Additional information

For more information on HRM and assay optimization please refer to the **Introduction to HRM Analysis Application Guide** (www.kapabiosystems.com)

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