KASP version 4.0 SNP Genotyping Manual

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26th May 2011

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Introduction

The KBiosciences Competitive Allele-Specific PCR genotyping system (KASP) is a homogeneous, fluorescent, endpoint-genotyping technology.
The technology was initially developed for use at the KBioscience in-house genotyping facility but gradually evolved into a global benchmark technology.
KASP offers the simplest, most cost-effective and flexible way to determine both SNP and insertion / deletion genotypes. Analysis can be carried out in 96, 384 and 1536-well plate formats.

The KASP genotyping system is comprised of two components (1) the SNP-specific assay, separately purchased as a KASP by Design or KASP on Demand (for more details see website), and (2) the universal Reaction Mix, which contains all other required components including the universal fluorescent reporting system and a specially-developed Taq polymerase.

The KASP genotyping system has been used successfully in a wide variety of organisms, achieving around 90% SNP-to-assay conversion rate.

Improvements of KASP version 4.0

KASP version 4.0 represents the result of an intensive development program to further enhance the performance of the KASP genotyping system. The actual modification is to in the formulation and does not require any change to the way the KASP chemistry is used by the customer, it is simply a formulation change that will in many cases confer improvements to genotyping data quality.

Principal of the KASP genotyping assay

(Please go to the following URL to view an animation describing the mechanism of action of the KASP chemistry):
www.kbioscience.co.uk/reagents/KASP.html

The mechanism of action behind KASP is novel; to elucidate, it is necessary to first consider the constituent oligonucleotides:

- Two allele-specific primers (one for each SNP allele). Each primer contains a unique unlabelled tail sequence at the 5' end.
- One common (reverse) primer.
- Two 5' fluor-labelled oligos, one labelled with FAM, one with HEX. These oligo sequences are designed to interact with the sequences of the tails of the allele-specific primers.
- Two oligos, with quenchers bound at the 3' ends. These oligo sequences are complementary to those of the fluor-labelled oligos (and therefore also complementary to the tails of the allele-specific primers). These quenched oligos therefore bind their fluor-labelled complements and all fluorescent signal is quenched until required.

In the initial stage of PCR, the appropriate allele-specific primer binds to its complementary region directly upstream of the SNP (with the 3' end of the primer positioned at the SNP nucleotide). The common reverse primer also binds and PCR proceeds, with the allele-specific primer becoming incorporated into the template. During this phase, the fluor-labelled oligos remain bound to their quencher-bound complementary oligos, and no fluorescent signal is generated.

As PCR proceeds further, one of the fluor-labelled oligos, corresponding to the amplified allele, also gets incorporated into the template, and is hence no longer bound to its quencher-bound complement. As the fluor is no longer quenched, the appropriate fluorescent signal is generated and detected by the usual means.

If the genotype at a given SNP is homozygous, only one or other of the possible fluorescent signals will be generated. If the individual is heterozygous, the result will be a mixed fluorescent signal.

Kit Contents

Reaction Mix (supplied at 2X concentration, containing Taq polymerase enzyme and the passive reference dye, 5-carboxy-X-rhodamine, succinimidyl ester (ROX)
$MgCl_2$ (50mM; for particularly A/T-rich DNA regions)
DMSO (for particularly G/C-rich DNA regions)

Customer Requirements

1 FRET-capable plate reader[1]
2 PCR microtitre plate
3 DNA samples (dissolved in Tris-HCl buffer (10mM; pH 8.3) or PCR grade H_20)
4 10mM Tris-HCl pH 8.3 or PCR grade H_20
5 Optical plate seal

[1]KBioscience are happy to advise on the choice of plate reader.

Storage and Shelf Life

Reaction Mix can be safely stored for one week at 4°C, one year at -20°C or indefinitely at -80°C. If the reaction mix is divided into aliquots, it is recommended that the tubes used are light-protective; assays may also be divided into convenient aliquots. Frequent freeze / thawing of both KASP reaction mix and assays will adversely affect performance.

Important notes

DNA quantity / quality

Most KASP assays will function well with 3-10ng of high quality DNA per reaction. However genome size is also an important consideration as a greater mass of DNA per reaction will be required if genotyping a larger genome; conversely, a smaller DNA mass per reaction will be required for smaller genomes.

The purity of DNA is important when using KASP, but no more so than for standard PCR. However, when DNA is crudely extracted, inhibitors of PCR can potentially remain, causing a greater or lesser issue depending on the source of the DNA (and hence the nature of the potential contaminants). Where the DNA has been extracted from plant material, polysaccharides and polyphenols can co-purify with the DNA and interfere with PCR. Addition of polyvinylpyrrolidone (PVP) at around 3% to the DNA samples can bind polyphenols and allow better PCR although this is not deemed necessary in the KBioscience service facility

If the extracted DNA contains PCR inhibitors but is also of high DNA concentration, it should be possible to dilute it such that DNA concentration remains sufficiently high, whilst effectively diluting-out the inhibitors.

Whilst it is feasible to use KASP with DNA derived from such sources, KBiosciences recommends use of the KlearGene family of DNA extraction and purification kits to provide very high quality / quantity, contaminant-free DNA (see Related Products).

KASP can be used in conjunction with a variety of DNA sources: genomic DNA, mitochondrial / bacterial (haploid) DNA, nested PCR amplicons and whole genome amplified (WGA) DNA (by either *Phi* 29 or DOP-based methods).

Assay mix

The Assay mix required to determine SNP genotypes is purchased separately as KASP by Design or KASP on Demand.

KASP by Design represents the most cost effective access to the assay mix, but as such is a non-validated and therefore not guaranteed KASP Assay mix

KASP on Demand is a validated, optimised Assay mix supplied by KBioscience and as such is guaranteed to give good genotyping data.

100µl of assay is sufficient to carry out at least 650 genotypes in 96-well format or at least 1300 genotypes in 384-well format (based on 10µL and 5µL reaction volumes, respectively with plate type).

The assay mix is combined with the Reaction Mix (see below) and added to the DNA samples to be genotyped.

Sample Arraying

DNA samples may be arrayed in any microtitre PCR plate though typically 96, 384 or 1536-well plates are used. The recommended amounts of DNA to use are: 5µl of DNA for 96-well plates and 2.5µl of DNA (1-40ng/µL) for 384- and 1536-well plates. Genotyping should be carried out on at least 24 samples to ensure there are sufficient genotypes to show clustering.

Negative controls

It is recommended that two no-template controls (NTCs) are included on each genotyping plate. A difference in fluorescent signal intensity between the presence and absence of template DNA allows improved confidence in the validity of the genotyping results.

Positive controls

When validating an assay, and particularly when using an assay with low allele frequency, it is advisable to include positive controls i.e. DNA samples of known genotype. If such samples are not available, it is possible to synthesise an alternative positive control comprised of an oligonucleotide representing the amplicon region of the assay. The nucleotide representing the desired SNP allele should be specified in the synthesised oligonucleotide. Please contact KBioscience for more information.

After arraying, the DNA samples can be dried into the wells of the PCR plate whereupon the reaction mix must be diluted to 1X concentration (rather than 2X for liquid samples) to compensate for the absence of liquid in the well (see Table 2). Drying the DNA samples in the plate wells is a useful technique when performing large-scale genotyping, as it allows many plates of DNA to be prepared in advance without the concern of sample evaporation which would otherwise alter the final reagent concentrations. The dried DNA samples are stable at room temperature for at least 3 months track changes turned on if protected from moisture. To dry the DNA samples, after arraying, the plates should be briefly centrifuged and placed in a drying oven at around 60°C for one hour.

It is not necessary to dry the DNA samples in the PCR plate. Samples can simply be arrayed, the plate centrifuged, and genotyping of the samples carried out. However, the arrayed samples should be stored such that any evaporation is minimised, for the reason stated above. Sealing the plate and storing at 4°C (short term) or -20°C (long term) is recommended if genotyping is not to be carried out straight away.

KASP Reaction Mix (in 96- and 384-well plates)

KBioscience recommends carrying out SNP genotyping using total reaction volumes of 4µL for 384-well or 8µL for 96-well genotyping (see Table 2; these volumes can be reduced but the overall robustness of the data quality may also be reduced).

The volumes in Table 2 must be proportionally scaled-up depending on the number of reactions required. The final $MgCl_2$ concentration of KASP v4.0 reaction mix at 1X concentration is 2.5mM, as this is optimal for the large majority of assays. However assays in especially A/T-rich regions may require more $MgCl_2$, which should be added to a final concentration of 2.8mM before use (i.e. an increase of 0.3mM, to increase the concentration from 2.5mM to 2.8mM).

Where the user prefers to dry down the arrayed DNA samples, the reaction mix must be diluted by the addition of water, to bring the overall final mix concentration to 1X. NB., do not use the KASP chemistry at higher final concentrations than 1X as the concentrations of the PCR reagents is critical. Where a solution of DNA is used, the addition of water is not required, as the DNA volume itself will bring the final KASP reagent concentration to 1X.

Table 2. Constituent reagent volumes for making KASP Genotyping Mix. *DNA samples diluted to final concentration of 0.5-20ng per 4µL reaction.

Table 2	KASP Genotyping mix assembly			
Component	Wet DNA method (µL)		Dry DNA method (µL)	
DNA*	2	4	N/A	N/A
2X Reaction Mix	2	4	2	4
Assay	0.055	0.11	0.055	0.11
H_20	N/A	N/A	2	4
Total reaction volume	4	8	4	8

All reagents should be briefly vortex-mixed prior to use. An active version of Table 2 can be found on our website.

KASP Reaction Mix (in 1536-well plates)

Where genotyping is carried out in 1536 well plates, KASP 1536 Reaction Mix should be used in place of the standard mix. KASP 1536 Reaction Mix is specifically-optimised for use with the very low well volumes in 1536 plates (see Related Products).

Dispensing the KASP Genotyping Mix samples

Dispensing can be carried out robotically or manually with a suitable pipette, depending on plate type and sample number. KBioscience is happy to advise on liquid dispensing systems.

Plates and plate sealing

KASP genotyping can be carried out in any plate format. Most commonly however, 96- and 384-well formats are used. For 96- and 384-well plate formats, KBioscience recommends use of the Flexiseal heat-based plate sealer, however plate sealing can be achieved with any optically-clear seal. For black 384- and especially 1536-well plates, KBioscience recommends the Fusion Laser welding system. For further information on these and other KBiosciences products (see Related Products).

Thermal cycling conditions

The KASP chemistry can be used with any standard thermal cycler. Similar results have been obtained on Peltier block-based thermal cyclers and the KBioscience Hydrocycler water bath-based thermal cyclers (see Related Products). KASP uses a set of thermal cycling conditions comprised of two temperature steps, rather than the more traditional three steps. Hence, the DNA is denatured at the higher temperature and annealed and extended at the same (lower) temperature.

The KASP thermal cycling program is as follows:

94°C for 15 minutes Activation	Hot-start
94°C for 20 seconds 65-57°C for 60 seconds (dropping 0.8°C per cycle)	10 cycles
94°C for 20 seconds 57°C for 60 seconds	26 cycles

If using a Peltier block-based thermal cycler, ensure that the PCR plate type is correct for the block being used, as incorrect fit can cause uneven PCR and variation of resultant data quality across the plate.

Plate Reading

Most FRET-capable plate readers (with the relevant filter sets) can be used in conjunction with KASP, though Fluidigm customers should consult the specific manual adjuct entitled 'KASP 4.0 for Fluidigm systems'. Some plate readers can be set to read at a range of temperatures but elevated temperatures (above 40°C), will lead to poor / no data (see the troubleshooting guide).

Because of the underlying mechanism of the KASP chemistry, care should be taken that KASP genotypes are only analysed at (or around) ambient temperature.

Whilst using real time PCR machines, plates should be read at ambient temperature after the completion of the PCR, run rather than using the real time data to generate end point curves.

KASP uses the fluorophores FAM and HEX for distinguishing genotypes. The passive reference dye ROX is also used to allow normalisation of variations in signal caused by differences in well-to-well liquid volume (see Figure 1). The relevant excitation and emission wavelengths are shown in Table 3 below.

Fluorophore	Excitation (nm)	Emission (nm)
FAM	485	520
HEX*	535	556
ROX	575	610

Table 3. Excitation and Emission values for the fluors used in KASPar. *The excitation and emission values for HEX are very close to those of VIC.

If using a plate reader optimised for use with the dye VIC (e.g. Applied Biosystems), no modification of settings will be necessary as the excitation and emission values for VIC and HEX are extremely close. However, if required, KASP formulations can be made with most fluorescent dyes to customer specification.

Graphical viewing of genotyping data

KBiosciences offer a data analysis software package either as part of a full Workflow Manager (Kraken) or a version with reduced functionality (KlusterKaller) (see Related Products). In KBioscience's software, the FAM and HEX data are plotted on the *x*- and *y*- axes, respectively. Inclusion of a passive reference dye (ROX) allows data to be normalised by dividing FAM and HEX values by the passive reference value for that particular well, thus removing the variable of liquid volume. Genotypes can then be determined according to sample clusters (Figure 1). The inclusion of a passive reference leads to tighter clustering and, as a result, more accurate calling of data.

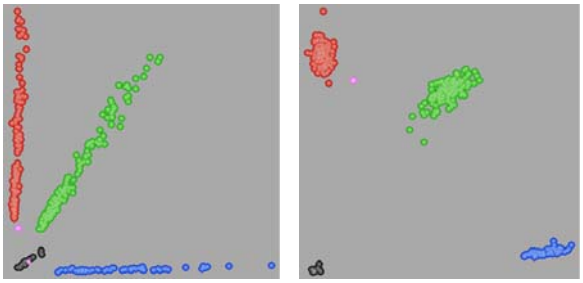

Figure 1. Genotyping data plotted using KBiosciences KlusterKaller software. The same data can be viewed without normalisation with ROX (left) or with normalisation (right).

From Figure 1, genotyped samples marked red are homozygous for the allele reported with HEX, those marked blue are homozygous for the FAM allele; those marked green are heterozygous.

Validating the genotyping process

Before using the KASP genotyping chemistry for the first time, it is recommended (though not necessary) to contact KBioscience for a free-of-charge validation kit. Using the validation kit, the following procedures will determine whether the plate reader and the PCR machine are functioning correctly for the KASP chemistry.

Determining correct functionality of the plate reader for the KASP chemistry

The KASP validation kit contains three separate tubes of fluors representing the three observable genotyping groups. The tubes are labelled 'FAM', 'HEX' and 'FAM/HEX'. The appropriate volume (for the plate type) of each of these samples should simply be dispensed into the required PCR plate (several wells for each of the three samples: use the same volume as you plan to use in your experiment) and the plate sealed, centrifuged and read on the plate reader (no thermocycling required). When viewed graphically, the reader output should reveal three tightly-clustered, well-separated groups of genotypes, demonstrating that the plate reader is correctly set up to read KASP-genotyped samples correctly (Figure 2).

Read the plate below 40C.

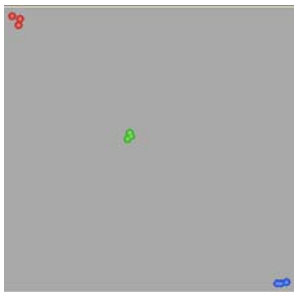

Figure 2. Cluster plot showing the three KASP genotyping clusters of HEX (red; homozygote), FAM (blue; homozygote) and HEX / FAM (green; heterozygote). Some plate readers (and their associated software) may plot the HEX and FAM data the opposite way around.

Determining correct functionality of the thermal cycler for the KASP chemistry

Please follow the procedures set out in the section entitled 'KASP reaction mix'.

The KASP validation kit contains a plate of 44 DNA samples (25 µL at a working concentration) and 4 no-template controls (NTCs) in wells D9, D10, D11 and D12. If the validation DNA is to be used in liquid form, 25 µL of water (dd or RO) should be added to the NTC wells; this is not necessary if the DNA is to be dried down.

Also included in the kit is an aliquot (50 µL) of test KASP assay in a 2-D barcoded tube. When genotyped with the test assay, the validation DNA samples will give rise to the three observable genotyping groups, respectively FAM (homozygote), HEX (homozygote) and FAM / HEX (heterozygote). Successful completion of these tests demonstrates that the plate reader, thermocycler and genotyping procedure are suitable for use with the KASP chemistry.