KASP genotyping chemistry
User guide and manual

KASP manual content

1. Introduction

The **K**ompetitive **A**llele **S**pecific **P**CR genotyping system (KASP™) is a homogeneous, fluorescent, endpoint genotyping technology.

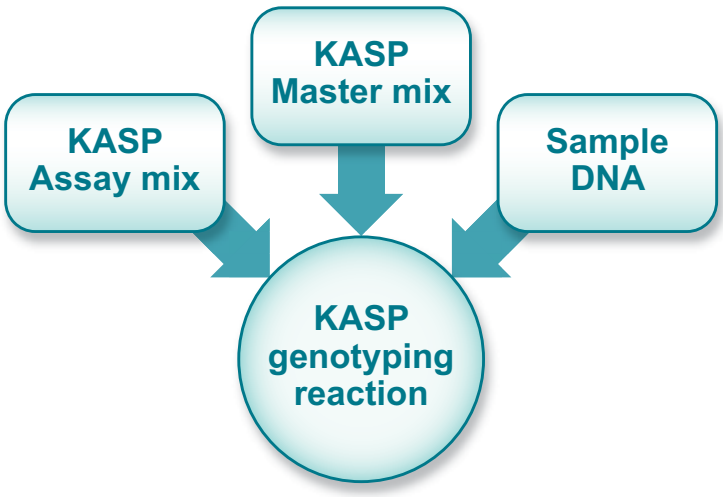
KASP offers the simplest, most cost-effective and flexible way to determine both SNP and insertion/deletion genotypes. Analysis can be carried out in 96-, 384- and 1536-well plate formats.

The KASP genotyping system is comprised of two components:

1. The SNP specific KASP Assay mix, separately purchased as a KASP by Design (KBD) or KASP on Demand (KOD) assay (http://www.lgcgenomics.com/genotyping/kasp-genotyping-reagents/kasp-assays-kbd-kod/)
2. The universal KASP Master mix, which contains all other required components including the universal fluorescent reporting system and a specially developed Taq polymerase (http://www.lgcgenomics.com/genotyping/kasp-genotyping-reagents/).

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

2. Principal of the KASP genotyping assay



Refer to the website for full animation.
http://www.lgcgenomics.com/genotyping/kasp-genotyping-reagents/how-does-kasp-work/

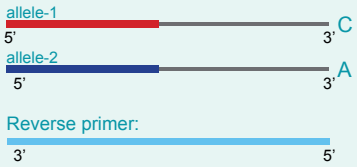
2.1 Mechanism of KASP chemistry

1) Assay components:

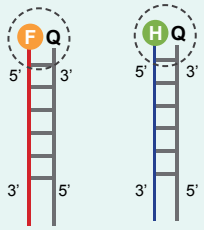
KASP uses three components: test DNA with the SNP of interest; KASP Assay mix containing two different, allele-specific, competing forward primers with unique tail sequences and one reverse primer; the KASP Master mix containing FRET cassette plus Taq polymerase in an optimised buffer solution.

A) KASP Assay mix

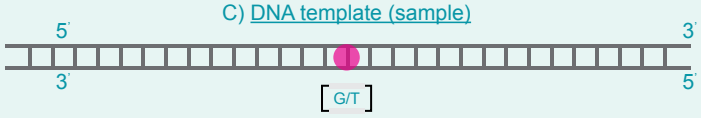
Allele-specific forward primers:



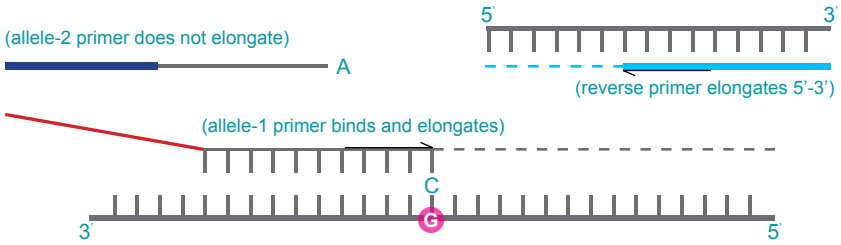
B) KASP Master mix



C) DNA template (sample)

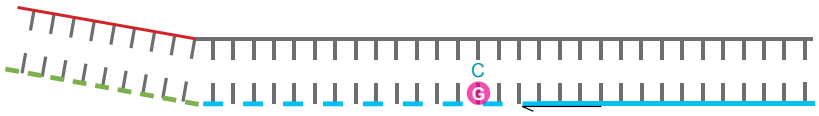


2) Denatured template and annealing components – PCR round 1:



In the first round of PCR, one of the allele-specific primers matches the target SNP and, with the common reverse primer, amplifies the target region.

3) Complement of allele-specific tail sequence generated – PCR round 2:

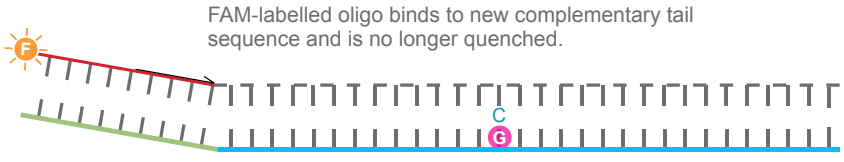


(Reverse primer binds, elongates and makes a complementary copy of the allele-1 tail.)

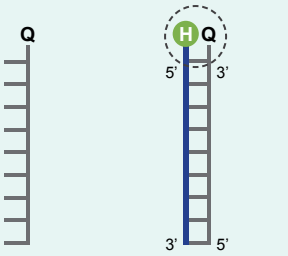
Legend

- Allele-1 tail FAM-labelled oligo sequence
- Allele-2 tail HEX-labelled oligo sequence
- Common reverse primer
- FAM dye
- HEX dye
- Target SNP
- Quencher

4) Signal generation – PCR round 3:



In further rounds of PCR, levels of allele- specific tail increase. The fluor labelled part of the FRET cassette is complementary to new tail sequences and binds, releasing the fluor from the quencher to generate a fluorescent signal.



Fluor for incorporated G allele no longer quenched.

Fluor for non-incorporated T allele remains quenched.

3. The KASP reaction components

The constituents of the KASP assay are:

KASP Assay mix

- 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.

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

KASP by Design (KBD) represents the most cost-effective access to the Assay mix, but is designed *in-silico* and not functionally validated prior to shipment.

KASP on Demand (KOP) is a validated, optimised Assay mix supplied by LGC and is guaranteed to generate good genotyping data when used in conjunction with DNA of suitable quality and KASP Master mix.

100 µL of Assay mix is sufficient to carry out at least 650 genotypes in 96-well format or at least 1300 genotypes in 384-well format (based on 10 µL and 5 µL total assay volumes, respectively with plate type). The KASP Assay mix is combined with the KASP Master mix (see Table 1) and added to the DNA samples to be genotyped.

KASP Master mix

- FAM™ and HEX™ specific FRET cassette
- Taq polymerase specially modified for allele-specific PCR
- Optimised buffer.

Please check that the ROX level of your KASP MAter mix is compatiböe with your qPCR machine / plate reader.

3.1 Kit contents

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

3.2 Customer requirements

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

[1]Please contact LGC for advice on the choice of plate reader.

3.3 Storage and shelf life

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

3.4 Sample arraying

DNA samples may be arrayed in any microtitre PCR plate though typically 96-, 384- or 1536-well plates are used. The recommended amount of DNA to use per reaction is 5 - 50 ng (see section 3.9 for details). Genotyping should be carried out on at least 22 samples + 2 No-Template-Controls (NTCs) to ensure there are sufficient genotypes to show clustering.

3.5 Negative controls

It is recommended that two 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.

3.6 Positive controls

When validating KASP Assay mix, and particularly when working with a SNP that has low allele frequency, it is advisable to include positive controls i.e. DNA samples of known genotype.

3.7 96- and 384-well plates

We recommend carrying out SNP genotyping using total reaction volumes of 5 µL for 384-well or 10 µL for 96-well genotyping (see Table 1; any reduction to these volumes may reduce the data quality and robustness). The volumes in Table 1 must be proportionally scaled up depending on the number of reactions required. It is essential to combine KASP Master mix and KASP Assay mix in sufficient quantities for all samples, rather than attempting to pipette very small volumes of KASP Assay mix and KASP Master mix into each well.

The final $MgCl_2$ concentration of KASP Master mix (v4.0) at 1x concentration is 2.5 mM. This is optimal for the large majority of KASP Assay mixes. SNPs that are located in particularly A/T-rich regions may require more $MgCl_2$, which should be added to a final concentration of 2.8 mM.

Where the user prefers to dry down the arrayed DNA samples (see section 3.10), the KASP Master mix must be diluted by the addition of molecular biology grade water, to bring the overall final mix concentration to 1x.

NB. Do not use the KASP chemistry at higher final or lower final concentrations than 1x as the concentrations of the PCR reagents are critical.

3.8 1536-well plates

Where genotyping is carried out in 1536-well plates, KASP 1536 Master mix should be used in place of the standard mix. KASP 1536 Master mix is specifically optimised for use with the very low well volumes in 1536 plates.

3.9 DNA quantity / quality

Most KASP assays will function well with 5-50 ng of high quality DNA per reaction. 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. When DNA is crudely extracted, inhibitors of PCR can potentially remain, causing a greater or lesser issue depending upon the source of the DNA and hence the nature of the potential contaminants.

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. 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.

For details of DNA extraction kits recommended by LGC, please visit our website: http://www.lgcgenomics.com/nucleic-acid-extraction/kits/

3.10 Drying down DNA samples

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. Dried DNA samples are stable at room temperature for at least 3 months 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 55°C for one hour.

*Table 1. Constituent reagent volumes for making KASP genotyping mix. *DNA samples diluted to final concentration of 5 - 50 ng per reaction.*

	KASP genotyping mix assembly			
Components	Wet DNA method (µL)		Dry DNA method (µL)	
DNA*	2.5	5	N/A	N/A
2x Master mix	2.5	5	2.5	5
Primer mix	0.07	0.14	0.07	0.14
H_2O	N/A	N/A	2.5	5
Total reaction volume	5	10	5	10

All reagents should be briefly vortex-mixed prior to use.

4. Running KASP reactions

4.1 Dispensing the KASP genotyping reagents

Dispensing can be carried out robotically or manually with a suitable pipette, depending on plate type and sample number. Please contact customer service about our liquid dispensing systems.

4.2 Plates and plate sealing

For 96- and 384-well plate formats, we recommend the use of the Kube™ heat-based plate sealer. Plate sealing can also be achieved with an optically-clear seal. For black 384- and especially 1536-well plates, we recommend the Fusion3™ laser welding system. For further information on these and other products please contact us.

4.3 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 our Hydrocycler™ water bath-based thermal cyclers. KASP uses a set of thermal cycling conditions comprised of two temperature steps, rather than the more traditional three steps. In this protocol 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:

Table 2: Thermal cycling conditions for KASP chemistry

94°C for 15 minutes	Hot-start activation
94°C for 20 seconds 61-55°C for 60 seconds (dropping 0.6°C per cycle)	10 cycles
94°C for 20 seconds 55°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. If clear genotyping clusters have not been obtained, the plate should be thermally cycled further and read again. Please see Table 3 for recycling conditions. Further cycling and reading can be performed until tight genotyping clusters have been obtained.

Table 3: Conditions for further thermal cycling of the KASP chemistry

Step	Temperature	Time	Number of cycles per step
Denature	94°C	20 sec	3 cycles
Annealing / Elongation	57°C	60 sec	

4.4 Plate reading

Most FRET-capable plate readers (with the relevant filter sets) can be used in conjunction with KASP. Some plate readers can be set to read at a range of temperatures but elevated temperatures (above 40°C) will lead to poor quality data.

Because of the underlying mechanism of the KASP chemistry, care should be taken that KASP genotypes are only analysed at ≤ 40°C.

Whilst using real-time PCR machines, plates should be read at ≤ 40°C 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 4.

Table 4. Excitation and Emission values for the fluors used in KASP. If using a plate reader optimised for use with the dye VIC, no modification of settings will be necessary as the excitation and emission values for VIC and HEX are extremely similar.

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

4.5 Graphical viewing of genotyping data

We offer a data analysis software package either as part of a full Workflow Management system (Kraken™) or a scaled-down version (KlusterCaller™). In our 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.

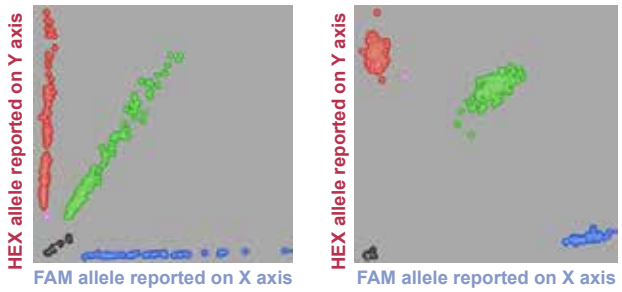


Figure 1. *Genotyping data plotted using KlusterCaller software. The same data can be viewed without normalisation with ROX (left figure) or with normalisation (right figure). Genotyped samples marked red are homozygous for the allele reported with HEX, those marked blue are homozygous for the FAM allele and those marked green are heterozygous.*

5. KASP trial kit

5.1 Validating the genotyping process

Before using the KASP genotyping chemistry for the first time, it is recommended (though not necessary) to contact us for a free-of-charge KASP trial kit. In general any qPCR plate reader instrument that is capable of reading FRET at the wavelengths in Table 4 should be compatible with KASP.

The KASP trial kit contains a plate of 33 DNA samples prediluted to a concentration range appropriate for KASP genotyping reactions and 3 No-Template-Controls (NTCs) in wells A12, B12 and C12.

Also included in the kit is an aliquot (50 µL) of test KASP assay in a 2-D barcoded tube. To run the test assay on the trial kit DNA samples, please follow the procedures set out in Section 4 of this manual and refer to the KASP genotyping trial kit guide for full instructions.

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.

Please visit our website to request your free-of-charge KASP trial kit: http://www.lgcgenomics.com/tryit/

For any queries about this guide please contact:
All locations except USA:
email tech.support@lgcgenomics.com
or call +44 (0)1992 476 486
USA only: email us-support@lgcgenomics.com
or call +1 978 338 5317

Products and services overview

- **Genotyping**
  - KASP™ genotyping reagents and services
  - SNPline™ high-throughput PCR workflow instrumentation
- **Extraction**
  - DNA and RNA extraction services
  - DNA extraction products (sbeadex™, Kleargene™ and mag™ kits)
  - Extraction automation instruments (oKtopure™ and Genespin™)
- **Sequencing**
  - Sanger sequencing
  - Next-generation sequencing services (Roche 454, Illumina HiSeq & MiSeq)
- **Other valuable tools**
  - Enzymes and PCR reagents (KlearKall, KlearTaq™, KlearTaq™ HiFi)
  - Whole Genome Amplification (WGA) kits and services
  - DNA shearing instruments (Covaris)
  - Heat and laser sealing films (96-, 384- & 1536-well plates)

For further information please visit our webpage

www.lgcgenomics.com

LGC
Germany
Ostendstr. 25 • TGS Haus 8
12459 Berlin

Tel: +49 (0)30 5304 2200
Fax: +49 (0)30 5304 2201
Email: info.de@lgcgenomics.com

United Kingdom
Unit 1-2 Trident Industrial Estate • Pindar Road
Hoddesdon • Herts • EN11 0WZ

Tel: +44 (0) 1992 470757
Fax +44 (0) 1438 900670
Email: info.uk@lgcgenomics.com

USA
100 Cummings Center • Suite 420H
Beverly • MA 01915

Tel: +1 (978) 232 9430
Fax: +1 (978) 232 9435
Email: info.us@lgcgenomics.com

245/1.00/2013-07