Manual

KASP genotyping manual

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BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC

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1. Introduction

KASP™ chemistry offers a simple, accurate and flexible method for end-point genotyping. The technology utilises a unique form of competitive allele-specific polymerase chain reaction (PCR) that enables bi-allelic scoring of single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) at specific loci. KASP reactions can be thermally cycled on any standard thermal cycler, and read on most FRET-capable plate readers (with the relevant filter sets) and qPCR machines.

If you are a new KASP user, we highly recommend that you request and run one of our free-of-charge trial kits before commencing your own genotyping experiments. The kit enables you to trial the chemistry in your own laboratory using your existing equipment, without having to purchase your own assays or use your precious DNA. Full instructions are provided with the kit, and complete support from the technical support team is provided for trial kit requests.

Our website contains a range of resources that detail how KASP works, as well as detailed guides for running KASP on common qPCR machines.

2. Kit contents and storage conditions

To perform KASP genotyping reactions, two components are required from LGC, Biosearch Technologies™:

1. KASP Assay Mix – this contains the target-specific primers and is designed to sequence that you have provided
2. KASP-TF Master Mix – this is universal to every KASP genotyping assay. The 2X Master Mix contains *Taq* polymerase in an optimised buffer solution, and the passive reference dye ROX™.

Table 1 details the recommended storage conditions for KASP reagents.

	4 °C	-20 °C	-80 °C
KASP Assay Mix	14 days	≥1 year	n/a
KASP-TF Master Mix	7 days	1 year	≥1 year

Table 1. Storage conditions for KASP reagents.

KASP reagents should be aliquoted upon receipt to minimise the need for repeated freeze-thaw cycles. KASP-TF Master Mix should be aliquoted in light-protective tubes.

3. Customer requirements

To perform KASP genotyping reactions in your laboratory, the following are required:

1. Sample DNA – for each genotyping assay to be run, include a minimum of 22 DNA samples to enable cluster analysis. The minimum final DNA concentration that Biosearch Technologies recommends in KASP genotyping reactions is 2.5 ng/µL (based on human genome size).
2. A FRET-capable plate reader or qPCR machine to read completed KASP reactions. Table 2 details the excitation and emission wavelengths for the fluorophores used to distinguish genotypes (FAM™ and HEX™), and for the passive reference dye (ROX).

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

Table 2. Excitation and emission values for the fluorophores used in KASP.

3. PCR plates – typically 96- or 384-well.
4. Plate seals – these must be PCR-suitable and optically clear to enable fluorescence to be read.

4. Laboratory protocol

4.1 Before you start

- If using KASP for the first time, ensure that you have requested and run a free-of-charge trial kit to familiarise yourself with the protocol, and to confirm your laboratory set-up.
- Prepare DNA sample plate(s). For each genotyping assay to be run, include a minimum of 22 DNA samples to enable cluster analysis. No template controls (NTCs) should be included for every genotyping assay (2 NTCs on 96-well plates and 4 NTCs on 384-well plates).
- Thaw and vortex the required number of aliquots of KASP-TF Master Mix and KASP Assay Mix.

4.2 Step-by-step protocol

4.2.1 Prepare a sufficient volume of KASP genotyping mix (KASP Assay Mix + KASP-TF Master Mix) for each of the assays to be run, including a 10% excess to allow for pipetting. Table 3 details the reagent volumes (per well) required for preparing KASP genotyping mix for both 96-well and 384-well plates.

	Wet DNA method		Dry DNA method	
	96-well plate (µL per well)	384-well plate (µL per well)	96-well plate (µL per well)	384-well plate (µL per well)
DNA	5	2.5	n/a	n/a
2X KASP-TF Master Mix	5	2.5	5	2.5
KASP Assay Mix	0.14	0.07	0.14	0.07
Water	n/a	n/a	5	2.5
Total reaction volume	10	5	10	5

Table 3. Reagent volumes required for preparing KASP genotyping mix. Note that if dry DNA used, water is added to the mix to ensure that KASP-TF Master Mix is at 1X in the final reaction.

4.2.2 Dispense the required volume of prepared KASP genotyping mix into each well of the DNA plate.
4.2.3 Seal the prepared reaction plate with a PCR-suitable, optically clear seal.
4.2.4 Centrifuge the plate at a minimum of 550 *x* g to ensure all reaction volume is at the bottom of each well.
4.2.5 Load the reaction plate into the thermal cycler or qPCR instrument.
4.2.6 Run the KASP thermal cycle as detailed in Table 4.

Protocol stage	Temperature	Duration	Number of cycles for each stage
Stage 1 Hot-start *Taq* activation	94 °C	15 minutes	× 1 cycle
Stage 2 Touchdown	94 °C	20 seconds	× 10 cycles
	61 °C (61 °C decreasing 0.6 °C per cycle to achieve a final annealing/extension temperature of 55 °C).	60 seconds	
Stage 3 Amplification	94 °C	20 seconds	× 26 cycles
	55 °C	60 seconds	
Optional* stage 4 (read stage for qPCR instruments only)	30 °C (any temperature below 40 °C is suitable for the read stage)	60 seconds	× 1 cycle

Table 4. KASP thermal cycle protocol. *Please note that Stage 4 of the program is only required if running and reading KASP genotyping reactions on a qPCR machine. If running the KASP thermal cycle program on a Peltier block or a Hydrocycler^{2TM}, only Stages 1, 2 and 3 are needed although you must ensure that reaction plates are cooled to <40 °C before performing the plate read.

4.2.7 Before performing a plate read, ensure that the plate is cooled to below 40 °C. If the plate is not read below 40 °C, it will not be possible to analyse the genotyping data.
4.2.8 Perform the end-point plate read using a FRET-capable plate reader or qPCR machine. Table 2 details the fluorophores for KASP.
4.2.9 Perform further PCR cycling of the reaction plate if necessary. The KASP recycle program is detailed in Table 5.

Step	Description	Temperature	Time	Number of cycles per step
1	Denaturation	94 °C	20 sec	3 cycles
2	Annealing/ elongation	57 °C	60 sec	

Table 5. Conditions for further cycling (recycling) of KASP reactions. This should be performed when data points have not separated into distinct clusters following the standard KASP thermal cycle. The maximum number of recycling steps (3 PCR cycles each) that Biosearch Technologies recommend is four. This equates to an additional 12 PCR cycles to the standard KASP thermal cycle. If tight clusters are not attained after four recycle steps, the assay will require further troubleshooting.

4.2.9 Store completed reaction plates in a dark fridge (~4 °C for a maximum of 1 week) until data has been analysed. This will allow you to perform additional read(s) or recycle steps if required, to ensure you have obtained the best possible data.

4.2.10 Analyse raw data using cluster plots to enable genotypes to be assigned to the DNA samples.

5. Troubleshooting

If you are obtaining the same, or similar, unexpected results for all of your KASP assays, it is likely that a factor within the laboratory workflow is affecting the results. Before contacting technical support for guidance, please use the tables (6.a-d) below as a checklist to ensure that all aspects of your laboratory setup are correct.

Reagents

Were the reagents (KASP-TF Master Mix and KASP Assay Mix) stored and prepared correctly?

Common errors include:

Incorrect storage of KASP reagents	Reagents should be aliquoted upon receipt to minimise the need for repeated freeze-thaw cycles. KASP-TF Master Mix should be stored in light-protective tubes. KASP-TF Master Mix is stable for 1 week at 4 °C and 1 year at -20 °C/-80 °C.
Insufficient thawing of reagents	All reagents must be thoroughly thawed before use. This is because components of the reagents thaw at different rates, hence the whole aliquot must be thawed before using to prepare KASP genotyping reactions.
Insufficient mixing of reagents	Once completely thawed, all reagents should be thoroughly mixed before use. Insufficient mixing can result in issues such as not all of the primers being incorporated into the reaction mix.
Incorrect KASP-TF Master Mix version	Different qPCR instruments have different requirements for ROX (passive reference dye). Ensure that you are using the optimal version of KASP-TF Master Mix for your instrument – please see our website for more details.

Table 6.2.a

DNA

Was the DNA template of sufficient quantity and quality?

Common errors include:

Insufficient DNA template used in reactions	Ensure that sufficient DNA template has been used. The optimum concentration will vary based on genome size of the study organism (larger genomes require more input DNA). If the concentration used is too low, the reactions will not amplify sufficiently. See Section 4.1 for more details.
Poor DNA quality	Use of poor quality DNA (containing contaminants or in a degraded state) will affect the efficiency of KASP reactions. If the DNA works well as template in standard PCR, then it should be suitable for KASP genotyping reactions.

Table 6.2.b

Experimental set-up

Were all steps of the experimental setup correct?

Common errors include:

Incorrect reaction assembly	Ensure that prepared KASP reactions contain all of the required reagents in the correct proportions. See Section 4.2 for details of KASP reaction assembly.
Inappropriate reaction volume for plate type	Ensure that the appropriate total reaction volume is used for the plate type. For 96-well plates, a reaction volume of 10 µL should be used. For 384-well plates, a reaction volume of 5 µL should be used.
Inaccurate or inconsistent pipetting of genotyping mix into reaction plate	Inconsistent pipetting can result in poor genotyping results. Review the ROX levels across your reaction plate as these are indicative of the accuracy of pipetting.
Incorrectly programmed KASP thermal cycle programme	Ensure that the cycling conditions have been programmed correctly on your PCR block or qPCR instrument.

Table 6.2.c

Plate read

Was the plate read performed correctly?

Common errors include:

Inappropriate plate seal used	A PCR-suitable optically clear seal must be used to enable fluorescent signal to be read properly. The reaction plate must also be sealed sufficiently to prevent evaporation as evaporation will affect efficiency of the reaction and the signal that is generated.
Plate reader or qPCR instrument not configured correctly to read fluorescent signal	Ensure that the correct excitation and emission values are programmed on the plate reader or qPCR instrument. If you have not run a KASP trial kit (free-of-charge) in your laboratory, please request one here.
Plate temperature of post-PCR read is greater than 40 °C	Completed KASP reaction plates must be read below 40 °C as KASP chemistry cannot be read above 40 °C.
Data analysis is performed using real-time read data and/or Ct values	KASP is an end-point genotyping chemistry. Real-time data and Ct values will not provide any meaningful data. Fluorescence data from KASP should be collected at the end of the PCR programme.

Table 6.2.d

Tables 6.a-d. Common causes of unexpected results for KASP end-point genotyping.

If you are obtaining unexpected results for one specific assay, but are able to obtain good results for other assays run on the same DNA samples in your laboratory, the assay-specific troubleshooting section (Section 3) in our KASP troubleshooting guide should be used to determine the potential cause(s) and suggested solutions. We strongly recommend repeating your experiment using the same DNA and same KASP Assay Mix first to eliminate the possibility of experimental error.

6. Automating the KASP genotyping protocol

High-throughput users may wish to consider our SNPline™ PCR genotyping system for partial or full automation of end-point genotyping laboratory workflows. The RepliKator™ can be used to stamp copies of DNA plates, or reformat plates from 96- and 384-well into 1536-well. We have options for robotic sealing of plates, including the Kube™ thermal plate sealer. The Hydrocycler^2 provides a waterbath-based option for thermal cycling, suitable for all plate types, and offering faster cycling times and increased throughput.

7. Further support

There are a wide range of support materials for KASP end-point genotyping on our website, covering aspects such as KASP assay design, submission of sequence information, alternative thermal cycling protocols, and user guides for running KASP on specific qPCR machines.

If you require further support, please contact our technical support team at techsupport@lgcgroup.com or submit a request for support directly into our case system.

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