KASP genotyping validation kit user guide

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1. Introduction

The validation kit is supplied to ensure that the plate-reader, PCR thermal cycler and process flow are functioning correctly for use with KASP™ chemistry. In general, any qPCR instrument or FRET-capable plate reader should be compatible with KASP. The different instruments have differing requirements for ROX™, the passive reference dye present in KASP Master mix, and you may need to try more than one version to determine the version that is optimal for your instrument. KASP Master mix is available in Low, Standard and High ROX formulations. Please note: these formulations only differ in the level of ROX that they contain and are otherwise identical.

Overview of the procedure

The validation protocol is divided into two parts:

Part 1: Determines that the microplate reader is compatible with the FAM™ and HEX™ fluorophores that are used in the KASP reaction (see Table 1 for excitation and emission values). Fluorophores are dispensed into a microtitre plate by the user and read on the plate reader.

Part 2: This enables the user to trial KASP chemistry in their own laboratory. DNA samples, KASP Master mix and KASP Assay mix are dispensed into a reaction plate. The thermal cycle is performed and the plate read on the plate reader.

2. Kit contents

Part 1 contents

This part of the kit consists of three aliquots of diluted fluorophores. They do not require any thermal cycling and, after being dispensed into a microtitre plate, can be read immediately.

1. 1 tube of FAM
2. 1 tube of HEX
3. 1 tube of HEX / FAM

These tubes of fluors also contain ROX, a passive reference dye, enabling you to normalise your results and thus to remove the effects of variation due to pipetting. ROX is present only as a normalisation dye and KASP genotyping can still be performed successfully if the plate-reader is not capable of reading ROX.

Part 2 contents

This part of the kit contains a KASP genotyping assay comprising of KASP Assay mix, KASP Master mix and DNA.

1. 1 x 96-well microtitre plate containing 33 validation samples (and 3 No-template controls) pre-diluted to a concentration range appropriate for KASP genotyping reactions.
2. 1 x 500 µL tube of KASP Master mix at 2x concentration (sufficient for 100 reactions at 10 µL).
3. 1 x tube of KASP Assay mix (at 72x concentration).

Customer requirements

1. FRET-capable plate reader. Please see Table 1 for details of the excitation and emission values of the fluorophores.
2. Two empty PCR microtitre plates of the type intended for use in the customer's genotyping process.
3. PCR grade water.
4. Optically clear plate seals.

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

Table 1. Fluorophores used in the KASP chemistry and their respective excitation and emission wavelengths.

3. Part 1: Experimental procedure for validation of the plate reader

1. Briefly vortex and spin down the three tubes of fluorophores. These tubes are labelled ‘FAM’, ‘HEX’, and ‘FAM / HEX’.

2. Dispense the three different fluorophores into a microtitre plate. Each fluorophore should be dispensed into separate wells, in triplicate (see Figure 1 for suggested layout). NOTE: use the same plate type that you intend to use for KASP genotyping. For 96-well plates, dispense 10 µL into each well. For 384-well plates, dispense 5 µL into each well.

   a. Pipette the ‘FAM’ fluorophore into wells A1, A2 and A3 of the microtitre plate.

   b. Pipette the ‘HEX’ fluorophore into wells B1, B2 and B3 of the microtitre plate.

   c. Pipette the ‘FAM / HEX’ fluorophore into wells C1, C2 and C3 of the microtitre plate.

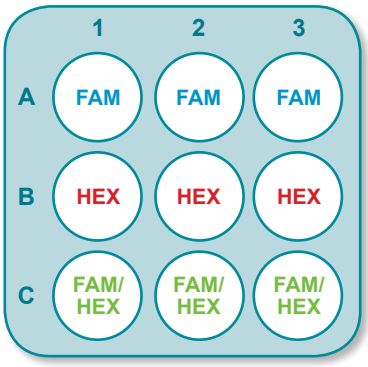

Figure 1. Suggested plate layout for dispense of the three KASP fluorophores supplied in the KASP validation kit.

3. Seal the plate with an optically clear seal.

4. Centrifuge the plate at a minimum of 555 *x* g. Do not spin the plates at a higher speed than is recommended for your rotor.

5. Read the plate on your instrument, ensuring that you have adjusted the settings for the fluorophores as detailed in Table 1.

6. View the data as a cluster plot. The data should give rise to three clusters and closely resemble that shown in Figure 2.

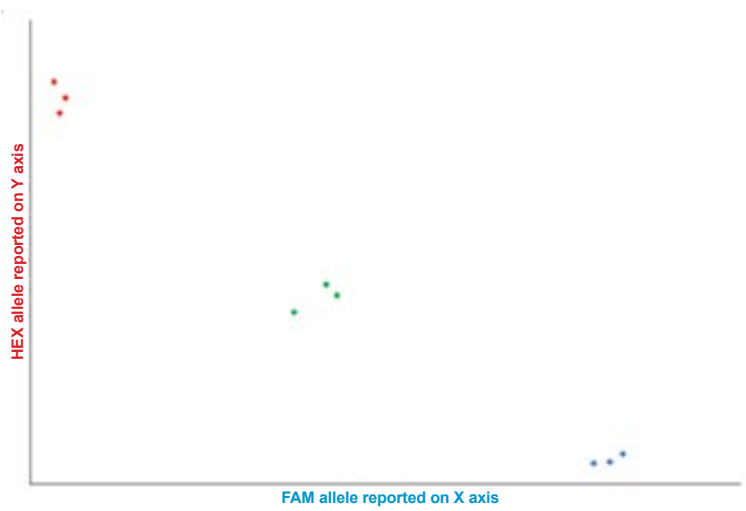

Figure 2. Cluster plot of expected results when reading the FAM (blue), HEX (red) and FAM/HEX (green) fluorophores. NB. Please be aware that some systems may plot FAM and HEX on the opposite axes to that shown in Figure 2.

4. Part 2: Experimental procedure for performing KASP genotyping reactions

4.1 KASP genotyping reactions

The following reagents are used to prepare the KASP reaction:

- 2x KASP Master mix
- 72x KASP Assay mix
- DNA samples (sample DNA is provided at the correct concentration for using at a 2x dilution)
- Water to be added if necessary to make the final reaction volume

4.2 Kit assembly

A 5 µL total reaction volume is required for 384-well plates and a 10 µL total reaction volume for 96-well plates.

Table 2 is an example of how to construct 60 reactions for KASP for both 384- and 96-well plate formats. This will enable you to make sufficient genotyping mix for the 33 samples and 3 no template controls (NTCs), with an additional spare volume.

4.3 KASP genotyping mix assembly

	Wet DNA method (µL) for 60 reactions	
Plate format	384-well plate	96-well plate
2x KASP Master mix	150	300
Assay mix	4.2	8.4
Total reaction volume	5	10
Total	300	600

Table 2. An example of KASP reaction assembly.

4.4 Experimental procedure

1. Thaw the KASP Master mix and KASP Assay mix tubes. Vortex each tube, and store on ice.
2. Thaw the DNA plate. Briefly vortex and spin down the plate.
3. Use a single- or multi- channel pipette to transfer DNA to the empty reaction plate (supplied by customer).

   For a 96-well plate: transfer 5 µL of the DNA provided to each well.

   For a 384-well plate: transfer 2.5 µL of the DNA provided to each well.

4. Prepare the genotyping mix (2x KASP Master mix plus Assay mix) as outlined in Table 2. Add the required amount of genotyping mix to each DNA sample in the reaction plate. A single- or multi-channel pipette can be used for this procedure.

   For a 96-well plate: add 5 µL of the prepared genotyping mix to each DNA sample.

   For a 384-well plate: add 2.5 µL of the prepared genotyping mix to each DNA sample.

5. Seal the plate with an optically clear seal.

6. Centrifuge the plate at a minimum of 550 *x* g. Do not spin the plates at a higher speed than is recommended for your rotor.

Thermal cycling conditions

KASP chemistry can be used with any standard thermal cycler. The thermal cycling conditions are detailed in Table 3. Please note that a two-step touchdown PCR method is used, with the elongation and annealing steps incorporated into a single step. It is recommended to cycle the reaction plates as soon as possible after the genotyping mix and DNA have been dispensed. If this is not feasible, prepared plates can be stored for up to 1 hour at 4°C. Plates should be sealed with an optically clear seal immediately after dispensing to prevent any evaporation.

Step	Description	Temperature	Time	Number of cycles per step
1	Activation	94°C	15 minutes	1 cycle
2	Denature	94°C	20 seconds	10 cycles
	Annealing / Elongation	61 - 55°C	60 seconds (drop 0.6°C per cycle)	
3	Denature	94°C	20 seconds	26 cycles
	Annealing / Elongation	55°C	60 seconds	

Table 3. Thermal cycling conditions for the KASP chemistry.

After thermal cycling, read the plate. Please note all plates should be read below 40°C. If the plate is not read below 40°C, it will not be possible to analyse the genotyping data.

Additional cycling conditions

If you have not obtained clear genotyping clusters, the plate should be thermally cycled for an additional 3 cycles and read again. Please see Table 4 for recycling conditions.

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

Table 4. Conditions for further thermal cycling of the KASP chemistry.

Further cycling and reading can be performed until tight genotyping clusters have been attained.

Note: This protocol pertains to all three of the following validation kit part numbers. The only difference between the three kits is the concentration of the passive reference dye ROX in the KASP Master mix, which varies based upon the type of reader being used.

Please visit www.lgcgenomics.com/mastermixcheck to confirm that you have the correct Master mix for your instrument.

5. Ordering information

Product code	Description
KBS-1014-101	Standard Rox validation kit
KBS-1014-102	High Rox validation kit
KBS-1014-103	Low Rox validation kit

For any queries about this guide or running KASP reactions in your laboratory please contact the technical support team:
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