Guide

KASP troubleshooting guide

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BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC

Guide

KASP troubleshooting guide

Contents

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1. Introduction

This document is intended as a guide to troubleshooting unexpected results obtained from KASP™ genotyping reactions run in your own laboratory.

Section 2 outlines the common causes of unexpected genotyping results that do not relate specifically to an individual assay. 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. The table in section 2 should be used as a checklist to ensure that all aspects of your laboratory setup are correct, before contacting technical support for guidance.

Section 3 provides a guide to individual assay troubleshooting, and should be used when you are experiencing unexpected results for a specific assay, yet are able to obtain good results for other assays run on the same DNA samples in your laboratory.

Section 4 contains more detailed explanations to some of the suggested solutions for KASP assay troubleshooting, and information regarding the KASP thermal cycling protocols and reaction setup.

2. Common causes of unexpected genotyping results

The tables below outline a wide range of factors that should be considered when troubleshooting KASP chemistry in general in your laboratory. If you are experiencing difficulties with KASP chemistry and are obtaining similar and unexpected results for all of your KASP assays, it is likely that a factor within the laboratory workflow is affecting the results.

Please use these tables as checklists for the common causes of unexpected results. Once you have checked and, if necessary, corrected all possible causes, please re-run some KASP reactions. If you are still experiencing unexpected results, please contact our technical support team: techsupport@lgcgroup.com. Please include full details of the experiments that you have performed, including raw data and screenshots of the cluster plots with your email.

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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 ºC and 1 year at -20 ºC/-80 ºC.
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.

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.

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Experimental set-up

Were all steps of the experimental set-up 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. See section 4.3 for our KASP thermal cycle programmes.

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. See section 4.5 for more details. If you have not run a KASP trial kit (free-of-charge) in your laboratory, please request one via our website.
Plate temperature of post-PCR read is greater than 40 ºC	Completed KASP reaction plates must be read below 40 ºC as KASP chemistry cannot be read above 40 ºC.
Data analysis is performed using real-time read data and/or C_q values	KASP is an endpoint genotyping chemistry. Real-time data and C_q values will not provide any meaningful data. Fluorescence data from KASP should be collected at the end of the PCR programme.

3. Troubleshooting guide

This section will guide you through the range of unexpected data plots that can be observed when running KASP genotyping reactions, the likely causes, and what can be done to resolve the issue.

Before using this section of the guide, LGC Biosearch Technologies strongly recommends repeating your experiment using the same DNA and same KASP Assay Mix to eliminate the possibility of experimental error e.g. two KASP Assay Mixes accidentally pipetted into each well. If the same results are obtained after a second attempt, use this section of the guide to determine the potential cause(s) and suggested solutions.

3.1 Insufficient amplification

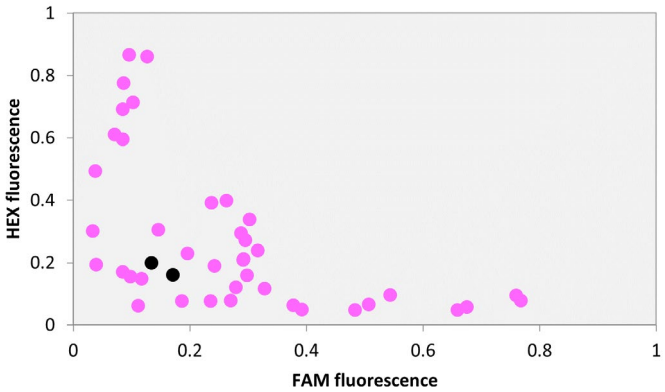

Figure 1. Genotyping data points move more slowly than expected away from the plot origin, and do not form distinct genotyping clusters.

Plot characteristics: genotyping clusters move more slowly than expected away from the origin. Data points do not resolve into separate clusters (figure 1).

Cause 1: insufficient number of PCR cycles performed to bring reactions to completion.
Suggested solution: the reaction plate should be thermally cycled further and re-read (see section 4.4).

Cause 2: low (<30%) or high (>70%) G/C percentage of primers within the SNP assay.
Suggested solution: for low G/C assays, increase the final concentration of $MgCl_2$ in the reaction (see section 4.6). For high G/C assays, add DMSO to the reaction (see section 4.7).

Cause 3: DNA concentration too low (slow to amplify).
Suggested solution: repeat the genotyping with DNA at the appropriate concentration (see section 4.1). The reaction plate could also be thermally cycled further and re-read as this may address the issue (see section 4.4).

Cause 4: DNA concentration too high (high concentration of PCR-inhibitors present).
Suggested solution: repeat the genotyping with diluted DNA such that contaminants are diluted to non-inhibitory levels.

Cause 5: DNA eluted in buffer that contains EDTA (EDTA chelates Mg^{2+} ions).
Suggested solution: increase the $MgCl_2$ to compensate e.g. for samples in 0.5 mM EDTA, increase the magnesium concentration by 0.5 mM. KASP-TF Master Mix contains 2.5 mM $MgCl_2$ and Biosearch Technologies does not recommend exceeding a final $MgCl_2$ concentration of 3.0 mM.

3.2 Scattered grouping of genotyping calls

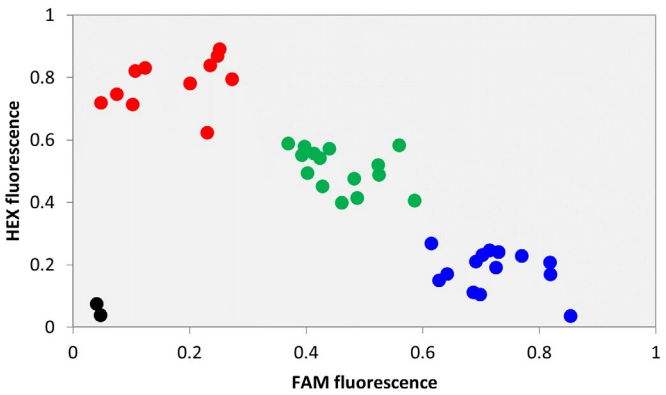

Figure 2. Genotyping clusters are not distinct or tight, and data points are scattered across the plot.

Plot characteristics: genotyping clusters are not distinct or tight, data points are scattered (figure 2).

Cause 1: cross contamination between DNA samples.
Suggested solution: check DNA extraction procedures and sample storage protocols for possible sources of cross contamination.

Cause 2: inconsistent DNA quantity and quality across the plate (this is more likely if the DNA samples being analysed are from different sources).
Suggested solution: normalise DNA samples before using them for KASP genotyping reactions.

Cause 3: insufficient number of PCR cycles performed to bring reactions to completion.
Suggested solution: the reaction plate should be thermally cycled further and re-read (see section 4.4).

Cause 4: magnesium concentration too high for the %GC content of primers within the SNP assay.
Suggested solution: perform a reductive magnesium titration to determine the appropriate magnesium concentration for the specific assay.

Cause 5: non-optimal cycling conditions.
Suggested solution: try alternative cycling conditions, using %GC content as a guide. Increasing the annealing/extension temperature can improve the specificity of the assay in some circumstances. More details about our alternative cycling conditions can be found in section 4.3.

3.3 Little or no separation of the heterozygous and a homozygous group

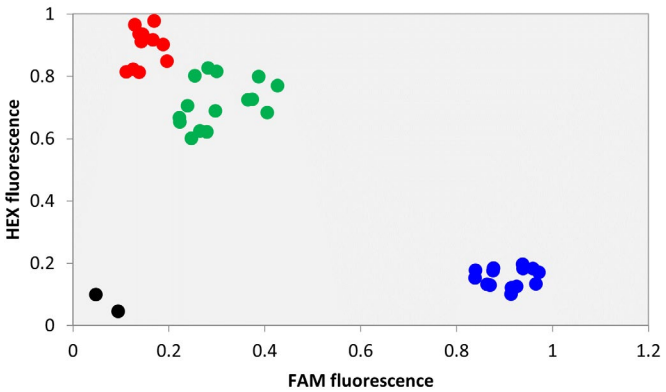

Figure 3. Three clusters are present on the plot, but the heterozygous cluster (green data points) has migrated towards a homozygous cluster (red data points).

Plot characteristics: the heterozygous group migrates towards one of the homozygous groups, making genotype scoring difficult (figure 3).

Cause 1: more efficient amplification of one of the allele-specific primers compared to the other.
Suggested solution: dependent upon the type of assay that you have received:

- Primer design only – order an upgrade of the assay to laboratory-validated (this will include redesign to eliminate the bias where required), and include screenshots of the data you have obtained.
- Primer design with validation – contact technical support for assistance (techsupport@lgcgroup.com), and include screenshots of the data that you have obtained.

3.4 Heterozygote group is too close to the origin

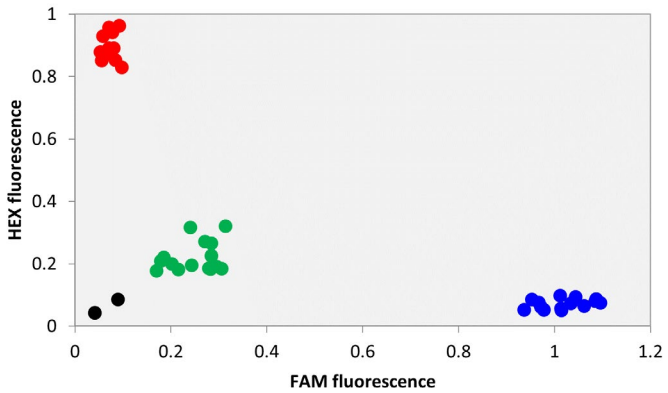

Figure 4. The two homozygous clusters (blue and red data points) appear to amplify correctly, but the heterozygous cluster amplifies less than expected and remains close to the plot origin.

Plot characteristics: the homozygous groups appear to amplify correctly but the heterozygous group amplifies less than expected, remaining close to the origin (figure 4).

Cause 1: KASP Assay Mix has been aliquoted without sufficient mixing or thawed without sufficient mixing.
Suggested solution: mix aliquots thoroughly after thawing and before use.

Cause 2: forward primers are saturating the fluorescent quenching system.
Suggested solution: dependent upon the type of assay that you have received:

- Primer design only – order an upgrade of the assay to laboratory-validated (this will include redesign to eliminate the bias where required), and include screenshots of the data you have obtained.
- Primer design with validation – contact technical support for assistance (techsupport@lgcgroup.com), and include screenshots of the data that you have obtained.

3.5 Too many genotyping groups

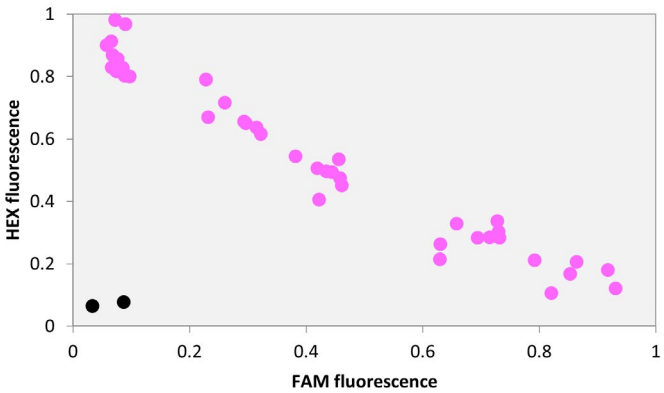

Figure 5. There appear to be more than three genotyping clusters visible on the plot.

Plot characteristics: more than three genotyping clusters are visible on the cluster plot (Figure 5).

Cause 1: contamination in one or more components of the reaction e.g. one KASP Assay Mix has become contaminated with another, or DNA samples have become mixed.
Suggested solution: Try repeating the experiment with fresh aliquots of KASP Assay Mix and/or DNA samples.

Cause 2: presence of polymorphism(s) within the primer binding site.
Suggested solution: where possible, the assay can be re-designed to a region containing no polymorphisms (if available) or to contain a degenerate base in the primer sequence at the site of the neighbouring (non-assayed) SNP:

- Primer design only – identify the additional polymorphism(s) using resources available for your study species and order an upgrade of the assay to laboratory-validated. Include screenshots of the data you have obtained.
- Primer design with validation – identify the additional polymorphism(s) using resources available for your study species prior to contacting technical support for assistance (techsupport@lgcgroup.com). Include screenshots of the data that you have obtained.

3.6 Fewer genotyping groups than expected

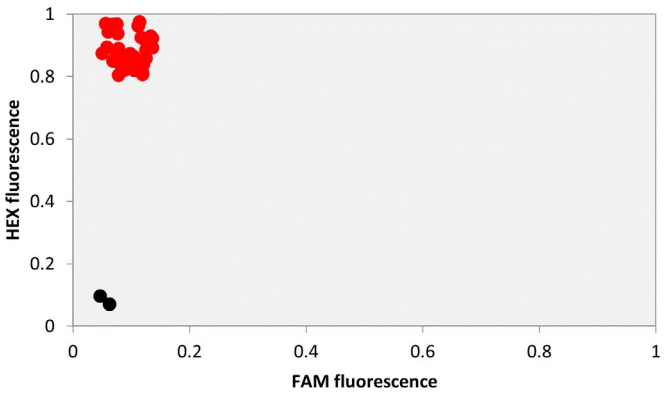

Figure 6. All data points cluster together in one position on the plot – in this case, samples are monomorphic for the allele detected by HEX.

Plot characteristics: monomorphic results on the genotyping plot (figure 6).

Cause 1: the population being analysed contains only one genotype with respect to the SNP being studied. One of the expected genotypes has a low frequency (minor allele frequency) and is therefore not present within the assayed population.

Suggested solution: no solution required the result is genuine. For minor allele frequency, include a positive control on every plate. This should be a DNA sample that is known to contain the minor allele (i.e. it has been sequenced). It is also possible to order synthetic positive controls alongside your KASP assay.

Cause 2: primers are annealing to a homologous region within the genome.

Suggested solution: where possible, the assay can be redesigned to ensure that it is specific to the region of interest and not to homologous regions. See section 4.6 for more information on homology:

- Primer design only – investigate homology using resources available for your study species. Identify bases that are unique to the region of interest and order an upgrade of the assay to laboratory-validated. Include screenshots of the data you have obtained.
- Primer design with validation – investigate homology using resources available for your study species. Identify bases that are unique to the region of interest prior to contacting technical support for assistance (techsupport@lgcgroup.com). Include screenshots of the data that you have obtained.

Cause 3: the SNP is not real.

Suggested solution: SNP is not present within the population being analysed. You should choose an alternative SNP for analysis.

3.7 Some samples do not amplify

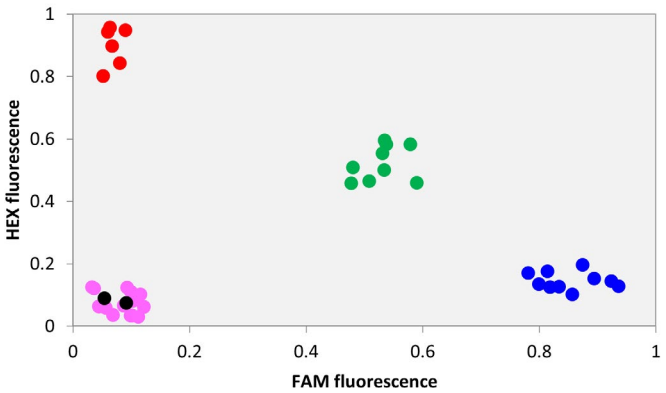

Figure 7. Some samples amplify and form genotyping clusters as expected (blue, green and red data points), whilst other samples fail to amplify (pink data points).

Plot characteristics: some samples amplify as expected whilst others do not amplify at all and remain clustered at the origin (figure 7).

Cause 1: inconsistent DNA quantity or quality (this is more likely if the DNA samples being analysed are from different sources).
Suggested solution: normalise DNA samples before using them for KASP genotyping reactions.

Cause 2: arraying of DNA into the PCR plates was not performed as expected i.e. little or no DNA in the wells has resulted in no amplification.
Suggested solution: investigate DNA arraying procedures.

Cause 3: poor dispensing of reaction mix into wells, resulting in insufficient mix for amplification in some wells.
Suggested solution: investigate the ROX levels in each of the wells across the plate as this relates directly to the volume of KASP-TF Master Mix that was dispensed into the plate.

Cause 4: the SNP being investigated is not present in some samples and the non-amplification is therefore a genuine result. For example, the SNP is a Y chromosome SNP but the population being tested contains both male and female samples (in this instance, heterozygotes would not be present).
Suggested solution: not required – genuine result.

3.8 No pattern to the results

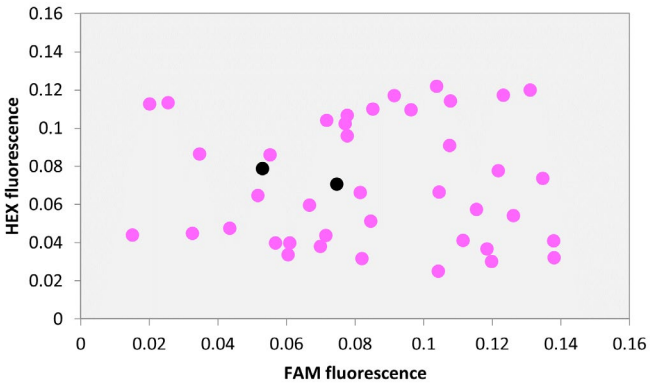

Figure 8a. Data points are scattered across the plot and there is no apparent pattern to the genotyping results.

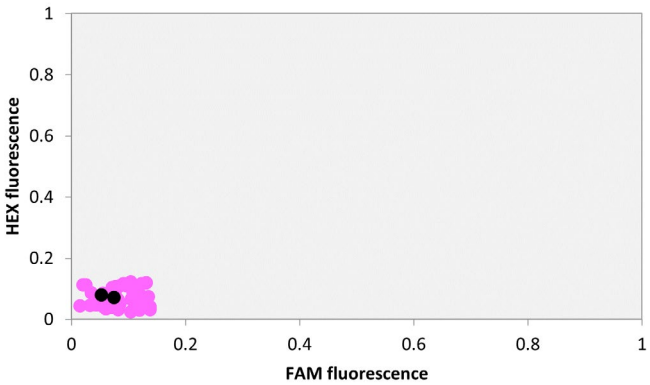

Figure 8b. The X and Y axes have been rescaled and all data points group with the NTCs, indicating that no amplification has occurred.

Plot characteristics: no pattern to the results, scattered data points (figure 8a). Rescaling of the X and Y axes can help to clarify if amplification has occurred or not (figure 8b).

Cause 1: plate reader fault.
Suggested solution: investigate plate reader and re-read plate.

Cause 2: arraying of DNA into the PCR plates was not performed as expected i.e. little or no DNA in the non-amplified wells.
Suggested solution: investigate DNA arraying procedures.

Cause 3: poor dispensing of reaction mix into wells, resulting in insufficient mix for amplification in some wells.
Suggested solution: investigate the ROX levels in each of the wells across the plate as this relates directly to the volume of KASP-TF Master Mix that was dispensed into the plate.

Cause 4: the SNP being investigated is not present in some samples and the non-amplification is therefore a genuine result. For example, the SNP is a Y chromosome SNP but the population being tested contains both male and female samples (in this instance, heterozygotes would not be present).
Suggested solution: not required – genuine result.

Cause 5: very slow amplification due to insufficient DNA or inherent assay properties.
Suggested solution: repeat the genotyping with DNA at the appropriate concentration (see section 4.4). The reaction plate could also be thermally cycled further and re-read as this may address the issue (see section 4.1).

3.9 Genotyping groups merging

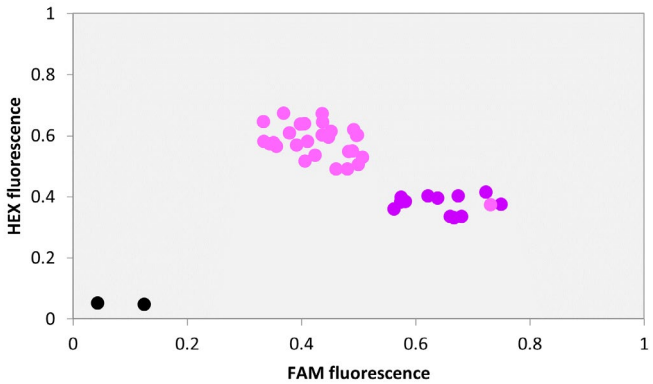

Figure 9a. Distinct genotyping clusters are apparent but their position on the plot is skewed.

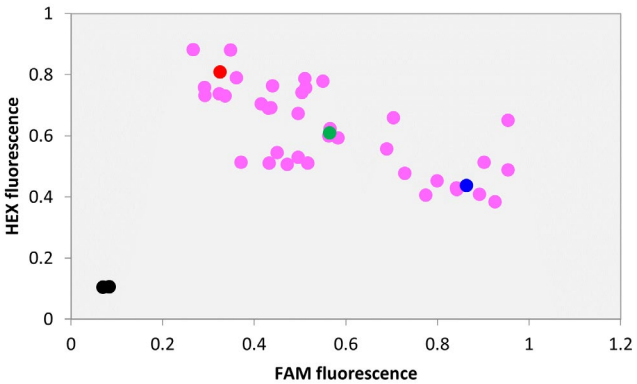

Figure 9b. It is difficult to discern clear genotyping groups, even when positive control samples for each genotype (blue, green and red data points) are included.

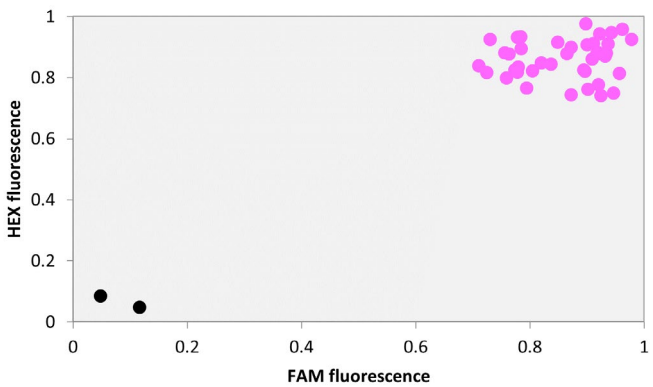

Figure 9c. All of the data points cluster in one position on the plot.

Plot characteristics: the genotyping groups are drawing too closely together, or merging, on the data plot. Distinct genotyping groups are apparent but their position on the plot is skewed (figure 9a), it is difficult to discern clear genotyping groups (figure 9b), or all of the data points cluster in one position (figure 9c).

Cause 1: primers are annealing to identical homologous regions, and the allele-specific primers are not able to bind specifically to their target template.

Suggested solution:

1. Experimentally increase the temperature of the annealing stage in the thermal cycle to force improved specificity.

2. Where possible, the assay can be redesigned to ensure that it is specific to the region of interest and not to homologous regions. See section 4.6 for more information on homology.
    - Primer design only – investigate homology using resources available for your study species. Identify bases that are unique to the region of interest and order an upgrade of the assay to laboratory-validated. Include screenshots of the data you have obtained.
    - Primer design with validation – investigate homology using resources available for your study species. Identify bases that are unique to the region of interest prior to contacting technical support for assistance (techsupport@lgcgroup.com). Include screenshots of the data that you have obtained.

4. Useful information

This section contains information on a range of topics that may be helpful when troubleshooting KASP assays run in your own laboratory.

4.1 DNA concentration for KASP genotyping

The minimum final DNA concentration that Biosearch Technologies recommends in KASP genotyping reactions is 2.5 ng/µL. For example, if you were preparing a 10 µL reaction consisting of 5 µL of DNA and 5 µL of genotyping mix (KASP-TF Master Mix + KASP Assay Mix – see section 4.2), then the input DNA would need to be at 5 ng/µL to ensure a final concentration of 2.5 ng/µL.

This value is based on the human genome size (~3,000 Mbp). If the genome size of your study organism is larger than human, you will need to adjust final DNA concentration (and hence input concentration) accordingly. (Please note that we do not recommend reducing the input DNA concentration for genomes smaller than human).

For genomes larger than human, a higher concentration of DNA is required. To calculate this, divide the genome size of your organism by the size of the human genome (3,000 Mbp), and use the resulting number to multiply the final concentration of DNA that should be used in your KASP reactions.

e.g. *Triticum aestivum* (wheat): 15,966 Mbp

15966 Mbp/3000 Mbp = 5.3

You will need a final DNA concentration that is 5.3 times more concentrated = 2.5 ng/µL DNA × 5.3 = 13.25 ng/µL final concentration.

4.2 KASP reaction assembly

KASP genotyping reactions consist of three components: universal KASP-TF Master Mix, SNP-specific KASP Assay Mix, and DNA template. The reactions can be prepared using either wet DNA (table 1) or dried down DNA (table 2), depending on your preferred laboratory workflow.

KASP genotyping reaction assembly (wet DNA)

Component	5 µL reaction (384-well plate)	10 µL reaction (96-well plate)
DNA*	2.43 µL*	4.83 µL*
KASP-TF Master Mix (2X)	2.5 µL	5 µL
KASP Assay mix (72X)	0.07 µL	0.14 µL
Water	n/a	n/a

Table 1. The constituent volumes of each component for both 5 µL and 10 µL reaction volumes when using wet DNA.

*Please see section 4.1 for details of the final DNA concentration that is required for KASP genotyping.

KASP genotyping reaction assembly (dried down DNA)

Component	5 µL reaction (384-well plate)	10 µL reaction (96-well plate)
DNA*	n/a*	n/a
KASP-TF Master Mix (2X)	2.5 µL	5 µL
KASP Assay mix (72X)	0.07 µL	0.14 µL
Water	2.43 µL	4.86 µL

Table 2. The constituent volumes of each component for both 5 µL and 10 µL reaction volumes when using dried down DNA.

*Please see section 4.1 for details of the final DNA concentration that is required for KASP genotyping – ensure that sufficient DNA has been dried down into the plate to meet the final DNA concentration requirements.

The volumes outlined in the tables above give the exact ratios of the reaction components that should be used when preparing KASP reactions. It is expected that reactions will not be assembled individually, but that larger volumes (sufficient for all planned reactions plus an additional percentage to account for pipetting error) will be prepared prior to dispensing into the reaction plate.

Please note that it is possible to round up the volumes of DNA/water used in the reactions if preferred (e.g. from 2.43 µL to 2.5 µL for 5 µL reactions). The below example details how to prepare reactions in this way, if setting up a full 96-well plate of reactions using wet DNA.

Dispense 5 µL of template DNA at the appropriate concentration into each well of the 96-well plate. Please note that at least two wells of the plate should contain water instead of DNA – these wells will act as the no template controls (NTCs).

Combine KASP-TF Master Mix and KASP assay to create KASP genotyping mix. The table below details how to prepare sufficient KASP genotyping mix for 96 reactions plus 10% excess.

KASP genotyping mix assembly

Component	10 µL reaction	96 × 10 µL reactions	96 × 10 µL reactions plus 10% excess
KASP-TF Master Mix (2X)	5 µL	480 µL	528 µL
KASP Assay mix (72X)	0.14 µL	13.44 µL	14.78 µL
Total volume	**5.14 µL**	**493.44 µL**	**542.78 µL**

Table 3. Preparation of sufficient KASP genotyping mix for 96 reactions with a 10% excess.

Pipette 5 µL of KASP genotyping mix (see table above) into each well of the reaction plate. Combined with the template DNA, this gives a final reaction volume of 10 µL per well.

4.3 KASP thermal cycling conditions

The standard KASP thermal cycling conditions are referred to as the '61-55 °C touchdown protocol', and are detailed in table 4 below. Design-only assays should be run using the 61-55 °C touchdown protocol in the first instance.

Protocol Stage	Temperature	Duration	Number of cycles for each stage
Stage 1 Hot-start *Taq activation*	94 °C	15 minutes	x 1 cycle
Stage 2 Touchdown	94 °C	20 seconds	x 10 cycles
	61 °C (61 °C decreasing 0.6 °C per cycle to activate a final annealing/extension temperature of 55 °C)	60 seconds	
Stage 3 Amplification	94 ºC	20 seconds	x 26 cycles
	55 ºC	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	x 1 cycle

Table 4. The standard KASP thermal cycling conditions.

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

If you have purchased a laboratory-validated assay, you will have received information regarding the optimal thermal cycling conditions for that assay. For design-only assays, it is not possible for Biosearch Technologies to provide information regarding the optimal cycling conditions as the assays will not have been wet-lab validated by ourselves.

There are two additional KASP thermal cycling protocols that may either be recommended as optimal (for laboratory-validated assays) or may be worth trying (for design-only assays) if non-optimal results are obtained.

The '68-62 ºC touchdown protocol' may be beneficial for assays that have a high %GC content.
The '2-step 57 ºC protocol' may be beneficial for assays that have a low %GC content.
Full details of all KASP thermal cycling conditions can be found in the 'KASP thermal cycling conditions' document.

4.4 KASP recycling protocol

The efficiency of KASP genotyping reactions is dependent on a number of factors including concentration of sample DNA and composition of the DNA sequence surrounding the SNP site. DNA sequence composition will impact the efficiency of primer binding and hence affect the rate of the PCR reaction. For this reason, different KASP assays will reach completion at different rates and can require additional PCR cycles to produce clear genotyping clusters.

Following completion of the standard KASP thermal cycle (10 cycles of touchdown PCR and 26 cycles of standard PCR), it is possible that your data points will not have separated into distinct clusters. Rather than indicating that the KASP genotyping assay is not working, it is more likely that the PCR reactions have not undergone a sufficient number of cycles to reach completion. At this stage, Biosearch Technologies recommends further cycling of reaction plate – termed 'Recycling'.

If clear genotyping clusters have not been obtained after completion of the standard KASP thermal cycle, the reaction plate should be thermally cycled further.

One KASP recycling step comprises of three additional PCR cycles, as outlined in table 5.

Protocol Stage	Temperature	Duration	Number of cycles for each stage
Stage 1 Amplification	94 °C	20 seconds	x 3 cycles
	57 ºC	60 seconds	
Optional Stage 2 (read stage for qPCR instruments only)	30 ºC (any temperature below 40 ºC is suitable for the read stage)	60 seconds	x 1 cycle

Table 5. Conditions for further cycling (recycling) of KASP chemistry.

4.5 Excitation and emission wavelengths for KASP

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. The required excitation and emission wavelengths for reading completed KASP genotyping reactions are detailed in table 6 below.

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

Table 6. Excitation and emission wavelengths for reading completed KASP genotyping reactions.

*If using a qPCR instrument or plate reader optimised for the fluorophore VIC, no modification of settings is required as the excitation and emission values for VIC and HEX are very similar.

**Please note that it is not essential to read ROX values of KASP reactions, but these values will enable normalisation of results.

4.6 Adjusting $MgCl_2$ concentration

The final $MgCl_2$ concentration of KASP-TF Master Mix (v4.0) at 1X concentration is 2.5 mM. This is optimal for the large majority of KASP assays. All assays should first be run with the standard 2.5 mM $MgCl_2$ unless they are laboratory-validated KOD assays that are received with specified conditions that include additional $MgCl_2$. Target SNPs that are located in particularly low G/C regions may require more $MgCl_2$. This should be added to a final concentration of 2.8 mM.

4.7 Addition of DMSO to the reaction

KASP assays that target SNPs that are located in particularly high G/C regions may benefit from the addition of 5-10% DMSO to the final volume of the reaction. When adding DMSO to the reaction, there is no need for a concomitant reduction in water volume to compensate for the disturbed reaction volume. Reactions with added DMSO should be run at the standard $MgCl_2$ concentration (2.5 mM final concentration).

4.8 Homology

If there are highly homologous regions within the genome of your study species, it is possible that primers designed to the region of interest may not actually be unique to that region. As a result, they will be able to bind to both the region of interest and the homologous region(s), and hence will affect the genotyping results that are obtained.

If there are any bases present close to the SNP itself (within 50 bp either side) that are unique to the region of interest, these can potentially be used to increase the specificity of the assay. The common reverse primer can be designed with the unique base at the 3′ end and will preferentially amplify only the region containing this base or anchoring point.

If you believe that homology may be affecting your genotyping results, Biosearch Technologies recommends that you identify bases that are unique to the region of interest and either order an upgrade (from primer design-only to primer design with validation) or contact technical support (techsupport@lgcgroup.com) for assistance.

5. Further support

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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