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End-point genotyping compiled

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1. Introduction

An end-point genotyping project starts with the collection of samples, and is completed following the analysis of data. LGC Biosearch Technologies™ can provide everything that is required for the generation of good quality end-point genotyping data in the form of kits or an in-house service, with our support and expertise available throughout the process. You can provide the samples, and we will do the rest, or you can choose to run everything in your own laboratory, requesting support when required – and anything in between. This approach gives you the flexibility to choose what works for you now, and is easily scalable in the future as your projects develop.

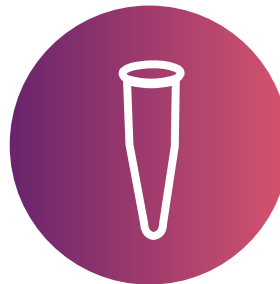
1.1 How to use this guide

The purpose of this document is to outline the end-point PCR genotyping process, from sample collection, to DNA isolation and end-point genotyping, right through to data analysis, and to provide all the information that you need to be able to access the kits and service options that are right for you.

The document is split into four clearly defined sections (sample collection, DNA isolation, end-point genotyping and data analysis), which you can navigate using the links within the contents page. All aspects of these processes are available as a kit or as a laboratory service, and symbols demarcate the relevant sections for each throughout.



Service



Kit

Although the technology is the same, there are differences in the processes for a laboratory service project compared to running the experiments in your own laboratory.

In addition, there is a troubleshooting section at the end of the document which provides advice regarding common issues you may encounter when performing DNA extraction and end-point genotyping in your own laboratory.

Throughout the document you will also find 'Tech tips' that provide helpful technical hints to improve the process and the quality of the data obtained.

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1.2 Schematics of the kit and service processes

Figure 1.1. shows the four key steps involved in an end-point genotyping workflow; these steps are the same for a service project or if running the workflow in your own laboratory.

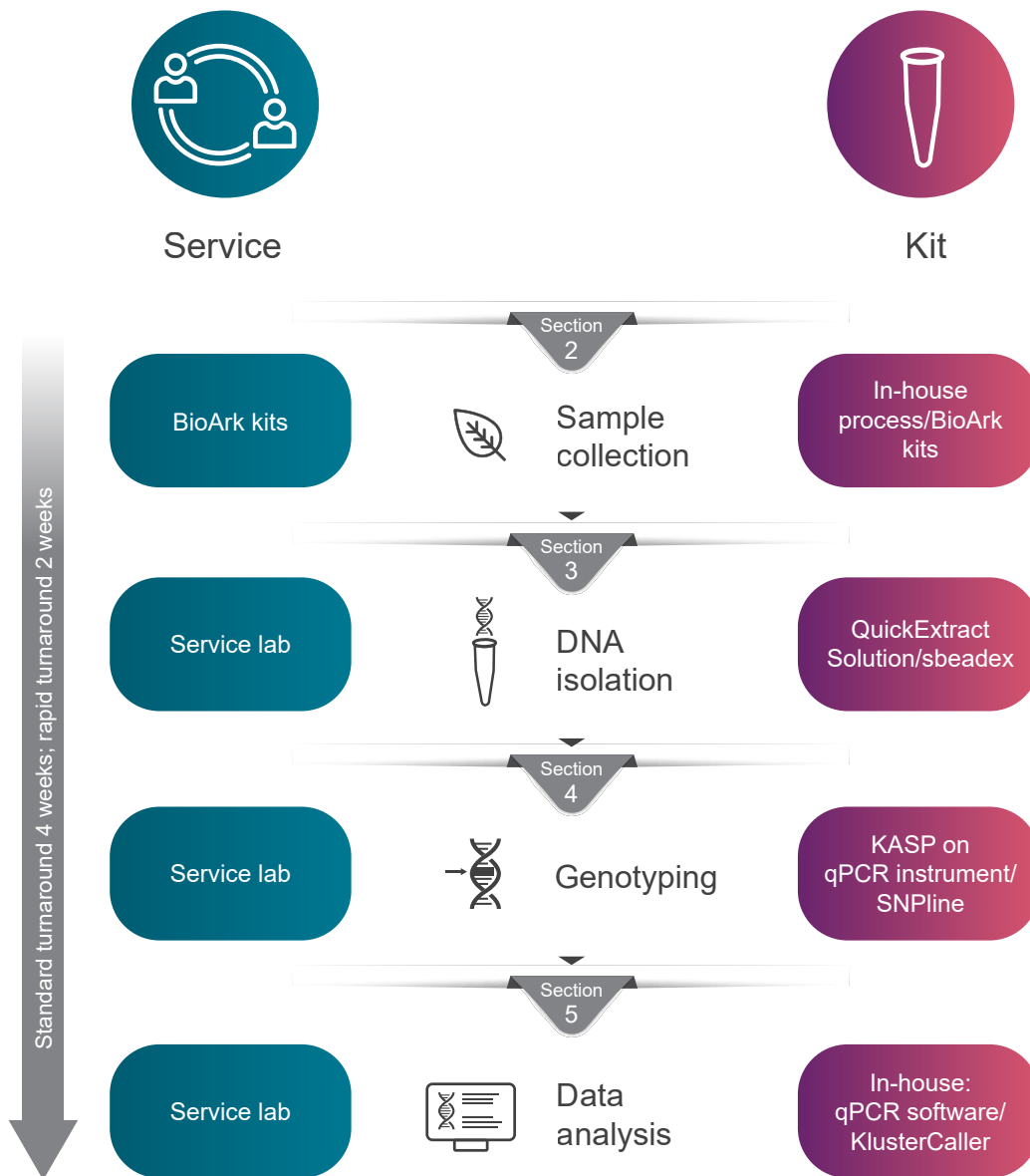


Figure 1.1. The four key stages of an end-point genotyping workflow. The service workflow is shown on the left and the kit workflow is shown on the right.

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The key point to note is that our end-point genotyping workflow offerings can be scaled according to your requirements, so you can start with a service project and then move towards running high throughput in your own laboratory, or start running low throughput in your laboratory and gradually scale this up with our higher throughput instrumentation.



1.2.1 Key steps in setting up a service project

Outlined below are the steps involved in setting up service project:

1. Contact the relevant customer services team ([see Appendix A](#)) for your area (or your Biosearch Technologies Sales Representative directly if known) to discuss your requirements, and to obtain a quotation.
2. Provide a purchase order for the required service project to be carried out
3. Complete a sample submission form (if sending samples for DNA isolation).
4. Ship prepared samples to the location advised by your Project Manager

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2. Sample collection

The process of sample collection is arguably the most important step in the whole end-point genotyping workflow. Without good quality samples, it is much harder to obtain good quality DNA and therefore the value of resultant genotyping data will be reduced. This can have implications on both the cost and the timeframe of a project.

Before commencing the sampling of plant or livestock samples, a number of factors should be considered to ensure that the samples will be suitable for the planned project. These include:

- a. Sampling technique – how exactly are the samples going to be collected? You can think about any equipment that may help improve consistency of sampling, ensure labelling procedures are robust, and carefully consider the type of vessel to sample into.
- b. Time of sampling – what time of the day/year are the samples going to be collected? You can think about times when material will be easier to collect, for example during the growing season for plants. Time of day can also affect sampling, with one example being that leaf material may be damp early in the mornings which can impact sample storage.
- c. Quantity of sample – how much sample is needed? A huge range of factors can affect the quality and quantity of DNA that can be obtained from a specific sample type. It is important to collect sufficient sample for all experiments that you plan to do, but not too much that it causes storage issues and impacts project timelines.
- d. Sample storage – how are samples going to be stored prior to DNA isolation? You can consider options including freezing, storage in ethanol, silica stabilisation, lyophilisation, FTA® cards (Whatman®) and stabilisation buffers. We also offer [BioArk Sample Collection kits](#) (See Section 2.1). Samples must be stored appropriately to prevent any degradation of the tissue prior to DNA isolation. Issues such as mouldy leaf punches can dramatically impact the quality and quantity of DNA that can be obtained.
- e. Shipment of samples – how are samples going to be shipped to Biosearch Technologies? The selected sample storage option will need to be compatible with shipping procedures for your country. We recommend using the appropriate BioArk Sample Collection Kit.

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2.1 BioArk Sample Collection Kits

To assist with sample collection, Biosearch Technologies offer BioArk Sample Collection kits that combine convenient sample collection with better data quality. Using these kits, you can collect samples of consistent sizes directly into a 96-well format, store and then ship at room temperature without the use of buffers, ethanol or freezing.



These kits are currently available for leaf, seed and fish sampling and, although they are primarily used in conjunction with our service provisions, they are available for purchase separately should you wish to use them for processing in your own laboratory.

BioArk Sample Collection Kits offer a number of clear advantages compared to traditional sampling methods:

- Better quality data from better quality of samples
- Environmentally friendly – reduction in hazardous materials, plastic consumption, carbon emissions and energy consumption
- Easy shipment and customs clearing of desiccated material

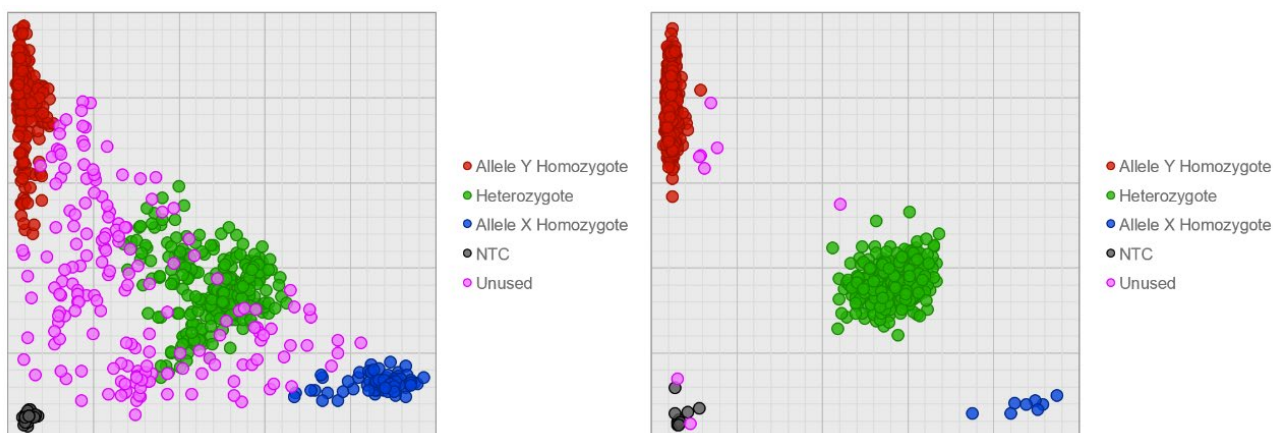


Figure 2.1. KASP™ end-point genotyping results using DNA extracted from leaf tissue with and without good sampling procedures. The plot on the left was generated using DNA from leaf tissue that was collected without using the BioArk leaf collection kit; many data points remain pink (unused/uncallable) and call rate is 78%. The plot on the right was generated using DNA from leaf tissue that was collected using the BioArk leaf collection kit; very few data points are pink (unused/uncallable) and call rate was 99%.

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2.1.1 Contents of a BioArk Sample Collection Kit

All kits contain:

- A 96-well tube storage rack with strip caps
- A detailed instruction manual
- Complete set of tools for collecting plant or animal sample material
- Instructions for return shipment of the filled plate for genetic analysis



2.1.2 Outline protocol for a BioArk Sample Collection Kit

The protocol for each kit (plant, seed, fish) is slightly different due to the difference in nature of the samples involved. The principles are the same though, and all kits follow the same basic protocol.

1. Obtain the samples – cut discs (leaf, fish) or collect seeds
2. Add the samples to the tube storage rack
3. Seal the tubes with supplied strip caps/cap mat
4. Where applicable, place the desiccant sachet on top of the sealed tubes and fix the rack lid in place
5. Place the prepared tube storage rack inside the supplied bag, and seal the bag
6. Package appropriately for shipping (service projects only)

The specific user guides for each kit can be accessed on our [website](#). We also have a [short video](#) that illustrates the process of the BioArk Plant Sample Collection Kit.

TECH TIP: Sampling plant leaf tissue

- **Sample young leaf tissue – the L4 seedling stage is ideal**
- **Sample tissue of a consistent age across plants**
- **Avoid areas of the leaf that are senescing (if older leaves must be sampled)**

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3. DNA isolation

Once suitable biological samples have been collected, the process of isolating the DNA can proceed. Biosearch Technologies offers a DNA extraction service for a diverse range of sample types, and can also supply our extraction and purification chemistries in kit format for use in your own laboratories.



3.1 DNA extraction service

Our nucleic acid extraction team provides DNA extraction services to meet the demands of any end-point genotyping project. We combine our experience and technologies with high-throughput laboratory processes to deliver cost-effective solutions for all project sizes.

A Project Manager will be assigned to you for every service project that you run. They will be your main point of contact for any queries relating to sample shipment, processing and data analysis, and will communicate with you regularly to provide project updates.

Depending on your sample type and throughput requirements, we will determine the most appropriate chemistry for extracting the DNA. Extracted samples will be quality checked, and transferred directly to our genotyping laboratory for the next stage of your project.



3.1.1 Shipment of samples for extraction

Please ensure that all samples are clearly labelled, individually identified and securely packed. The labels should withstand frozen temperatures without detaching from the tubes/plates. If possible, please use tubes with 1D or 2D barcode labels, as these can be scanned directly into our laboratory information management system (LIMS).

To ensure safe sample shipment, please notify Biosearch Technologies when the package is on its way to us, and include details of the courier, date of shipment and waybill number. Your assigned Project Manager will advise you when your samples arrive in our laboratories.

For more specific details regarding shipment of samples for extraction, please view our [webpage](#). We also have detailed [guidance notes](#) to assist you with an extraction or purification service project.



3.2 DNA isolation kits

Biosearch Technologies offers a range of DNA extraction and purification kits, and can provide advice on the most appropriate kit for your sample type and planned downstream applications. We can also provide support to help you get up and running in your laboratory.

For KASP end-point genotyping with plant samples, we recommend either our [QuickExtract™ Plant DNA Extraction Solution](#) or our [sbeadex™](#) purification chemistry. More details on both of these are provided below.

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3.2.1 QuickExtract Plant DNA Extraction Solution

QuickExtract (QE) chemistry facilitates simple, rapid extraction of PCR-ready DNA that is suitable for screening and genotyping applications. The one-tube protocol does not require any centrifugation steps, and only requires between 3 and 8 minutes to complete. The method is also compatible with robotic automation, should you wish to automate throughput.



3.2.1.1 Storage

QuickExtract Plant DNA Extraction Solution should be stored at -20 °C. Minimise the number of freeze-thaw cycles by aliquotting as necessary. Thawed QuickExtract DNA Extraction Solution can be stored at 4 °C for 1 month.



3.2.1.2 Outline protocol for QuickExtract Plant DNA Extraction Solution

1. Aliquot 0.5 mL QuickExtract DNA Solution into the appropriate number of tubes, and label
2. Place one sample into each prepared tube. Please note that seed samples may require grinding for optimal results.
3. Mix by vortexing for 15 seconds
4. Incubate at 65 °C for 6 minutes
5. Mix by vortexing for 15 seconds
6. Incubate at 98 °C for 2 minutes
7. Store the DNA at -20 °C for up to 1 week, or at 70 °C for longer periods

Each extraction will yield 0.5 mL DNA; use 5 µL or less in KASP genotyping reactions.

TECH TIP: The impact of seed grinding prior to performing the QuickExtract protocol varies depending on the plant species. Please see our [application note](#) for more details. We recommend determining the impact of seed grinding for your species of interest before proceeding with large-scale extractions.



3.2.2 sbeadex purification kits

Our sbeadex chemistry facilitates high-throughput purification of high quality DNA using magnetic-bead separation. Super paramagnetic particles coated with sbeadex surface chemistry are used to capture nucleic acids from a sample. The novel two-step binding mechanism, combined with water-based washing buffers, removes impurities present in the sample matrix and minimises inhibitor carryover into the eluate. DNA purified using sbeadex is suitable for all downstream applications including Next Generation Sequencing. If you plan to perform a wider range of downstream applications than just KASP end-point genotyping, sbeadex may be more appropriate for your needs than QuickExtract.

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3.2.2.1 Range of kits

Biosearch Technologies offer a number of different sbeadex purification kits for different types of samples.

The [sbeadex Plant DNA Purification Kits](#) are suitable for extraction from a range of fresh or lyophilised plant tissues including leaf, seed, bark, pulp and root. We offer support in protocol development for samples from challenging materials and plant species. Depending on the quantity of plant material that you need to extract from per sample, you can choose between either the mini or the maxi sbeadex plant kit (see table 3.1).

sbeadex plant kit	Fresh material	Lyophilised material*
Mini	10-30 mg	5-10 mg
Maxi	30-120 mg	10-30 mg

Table 3.1. Starting material for sbeadex mini and sbeadex maxi plant kits. *We recommend lyophilisation of plant material prior to homogenisation.

Prior to performing isolation from plant tissue, samples must be homogenised to enable high molecular weight DNA to be obtained. Lyophilised plant tissue should be homogenised without the addition of lysis buffer. Fresh tissue should be snap frozen in liquid nitrogen prior to grinding, or homogenised after the addition of lysis buffer.

Homogenisation (grinding) of a small number of samples should be performed in one of the following ways:

- Using a mortar and pestle. Sea sand can be added to aid disruption.
- In a 1.5 mL microcentrifuge tube, using a micro pestle.
- In a 1.5/2 mL sample tube, using a steel ball and ball mill.

If you are working with large numbers of samples, homogenisation should be performed using steel balls and a ball mill plate/rack of tubes format.

The [sbeadex Livestock DNA Purification Kit](#) has been developed to enable purification to be performed on a range of sample types such as ear punches, hair, saliva, and fish fins, in parallel using just one central kit.

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3.2.2.2 Outline protocol for sbeadex purification kits

Depending on the type and quantity of starting material, the volumes and incubation periods in the sbeadex purification protocol will vary. The overall process remains the same, and follows the six key steps shown in figure 3.1. and outlined in more detail below.

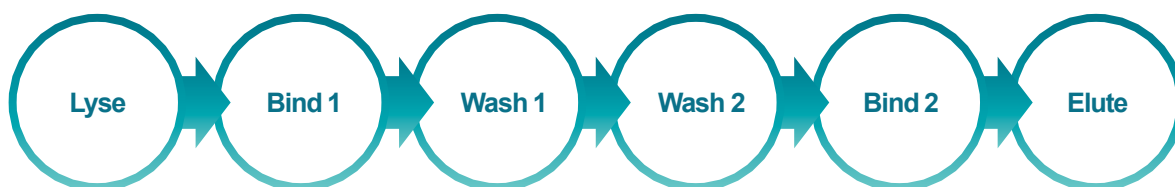


Figure 3.1. The six key steps in the sbeadex purification protocol. sbeadex technology utilises a unique two-step binding mechanism.

1. Lysis of the sample – the protocol for this differs depending on the type of starting material. All protocols are clearly outlined in the full user manuals for the plant and the livestock kits. We recommend following the sample-specific initial lysis stage to ensure that optimal DNA purification is performed.
2. Bind 1 – add appropriate volumes of Binding buffer PN and sbeadex particle suspension, mix well, and incubate at room temperature for the appropriate time period. Pellet the sbeadex particles using a magnet and discard supernatant.
3. Wash 1 – add appropriate volume of Wash buffer BN1/PN1, mix well, and incubate at room temperature for the appropriate time period. Pellet the sbeadex particles using a magnet and discard supernatant.
4. Bind 2 – add appropriate volume of Wash buffer TN1/PN2, mix well and incubate at room temperature for the appropriate time period. Pellet the sbeadex particles using a magnet and discard supernatant.
5. Wash 2 – add appropriate volume of ultrapure water/Wash buffer TN2, mix well and incubate at room temperature for the appropriate time period. Pellet the sbeadex particles using a magnet and discard supernatant.
6. Elute - add the appropriate volume of Elution buffer AMP/PN. Incubate at 55 °C for 10 minutes with periodic shaking. Pellet the sbeadex particles using a magnet. Transfer the eluate to a new tube by pipetting. To avoid particle transfer, it is recommended to only transfer 90% of eluate.

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3.2.2.3 Storage

All sbeadex buffers are premade and can be shipped and stored at room temperature. After opening, the sbeadex particle suspension, Wash Buffer PN2 (plant kit), Wash buffer TN2 (livestock kit), Elution Buffer EB (plant kit) and Elution Buffer AMP (livestock kit) should be stored at 4 °C.



3.2.2.4 Automation of the sbeadex purification protocol

Once the sbeadex purification protocol has been trialled effectively for your sample type manually, it is possible to automate the procedure to increase throughput. Biosearch Technologies can offer support with automation in your laboratory, including advice, pilot studies and standard pre-written programs for both the KingFisher Flex and our own [oKtopure](#) platform.

Key points to note:

- To mix samples effectively using an automated liquid handling system:
 - Set the mixing volume to between 50% and 80% of the volume to be mixed
 - For each mixing step, aspirate and dispense between 5 and 10 times
 - Use low aspirate and dispense speeds for steps involving lysis buffers and Binding buffer PN/SB to prevent frothing
 - Increase aspirate and dispense speeds for resuspension of pellets to ensure complete resuspension
- Using a KingFisher Flex (or similar) open platform
 - Keep all volumes the same as for manual extraction, with the exception of Elution buffer; use an additional 20 µL to account for evaporation
 - Ensure all incubation steps are a minimum of 5 minutes long to account for diffusion-dependent wash effects
 - Prior to mixing for the binding, washing and elution steps, use the 'Release Beads' function with a 'bottom mix' for 10 seconds. Automated mixing should then be performed using the 'Fast' setting.
- Using the oKtopure platform
 - The oKtopure platform is supplied with standard sbeadex templates that can be used for automation
 - When testing, it is important to observe for blocked tips due to clumping of beads, and effective re-suspension of the pellet after addition of buffers
 - The oKtopure is not intended for use with blood

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3.3 DNA requirements

Two key factors need to be considered when determining the appropriate amount of DNA for performing KASP end-point genotyping; quality and quantity. The quality of DNA is important, but no more so than for standard PCR. Depending on the nature of the starting sample, crudely extracted DNA may contain inhibitors to PCR. 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. The quantity of DNA also needs to be considered in relation to the genome size of the organism in question. Most KASP assays will function well with 5-50 ng of DNA per reaction, but a greater mass will be required per reaction for larger genomes and a smaller mass for smaller genomes. More details on the amounts of DNA to ship to Biosearch Technologies for a service project can be found in the KASP genotyping service section ([4.3](#)), and information on how to determine the appropriate concentration to use if running KASP in your own laboratory can be found in the KASP in your own laboratory ([4.4](#)).



3.4 DNA quantification

A range of methods can be used for the quantification of DNA samples, including spectrophotometry (measuring UV absorbance), fluorescent dyes (e.g. PicoGreen™, Thermo Fisher Scientific), and agarose gel electrophoresis. Spectrophotometers have a tendency to overestimate the quantity of DNA present whilst fluorescent dye quantitation assays are more exact. Please consider the method used for quantification when determining the volumes of DNA to use in-house or to ship to us.



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4. Genotyping

Once DNA has been isolated from your samples, the next step is to perform end-point genotyping reactions to determine the individual genotypes. Biosearch Technologies offers KASP genotyping as a service, and the reaction components can also be purchased in kit format for use in your own laboratories. 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 genotyping technology offers simplicity, flexibility and accuracy to the agrigenomics community, with the following key features:

- Universal reporting system – this simplifies assay design and eliminates time-intensive probe design steps
- Flexible primer design – this increases the rate of successful assay development
- Supports low-, medium-, and high-throughput studies
- Compatible with a wide range of liquid handling systems and thermal cyclers – signal can be read on most FRET (fluorescent resonant energy transfer)-capable plate readers and qPCR instruments.
- Low reaction volumes keep reagent costs to a minimum

4.1 How does KASP work?

A KASP genotyping reaction comprises of three components (figure 4.1.):

- KASP Assay Mix – containing the target-specific primers
- KASP-TF Master Mix – universal to every KASP genotyping assay
- Sample DNA - with the sequence of interest

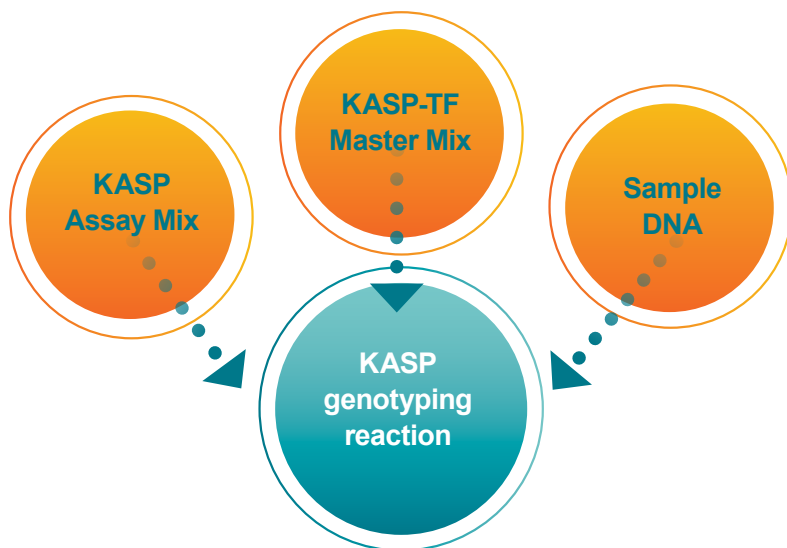


Figure 4.1. Components of a KASP end-point genotyping reaction.

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The components of the KASP reaction are explained in detail in [this short video](#).

The SNP-specific KASP Assay mix and the universal KASP-TF Master Mix are added to DNA samples, a thermal cycling reaction is then performed, followed by an end-point fluorescent read. Bi-allelic discrimination is achieved through the competitive binding of two allele-specific forward primers, each with a unique tail sequence that corresponds with two universal FRET (fluorescence resonant energy transfer) cassettes; one labelled with FAM™ dye and the other with HEX™ dye.

In addition to the schematic in figure 4.2, [this video](#) explains how the KASP reaction works.

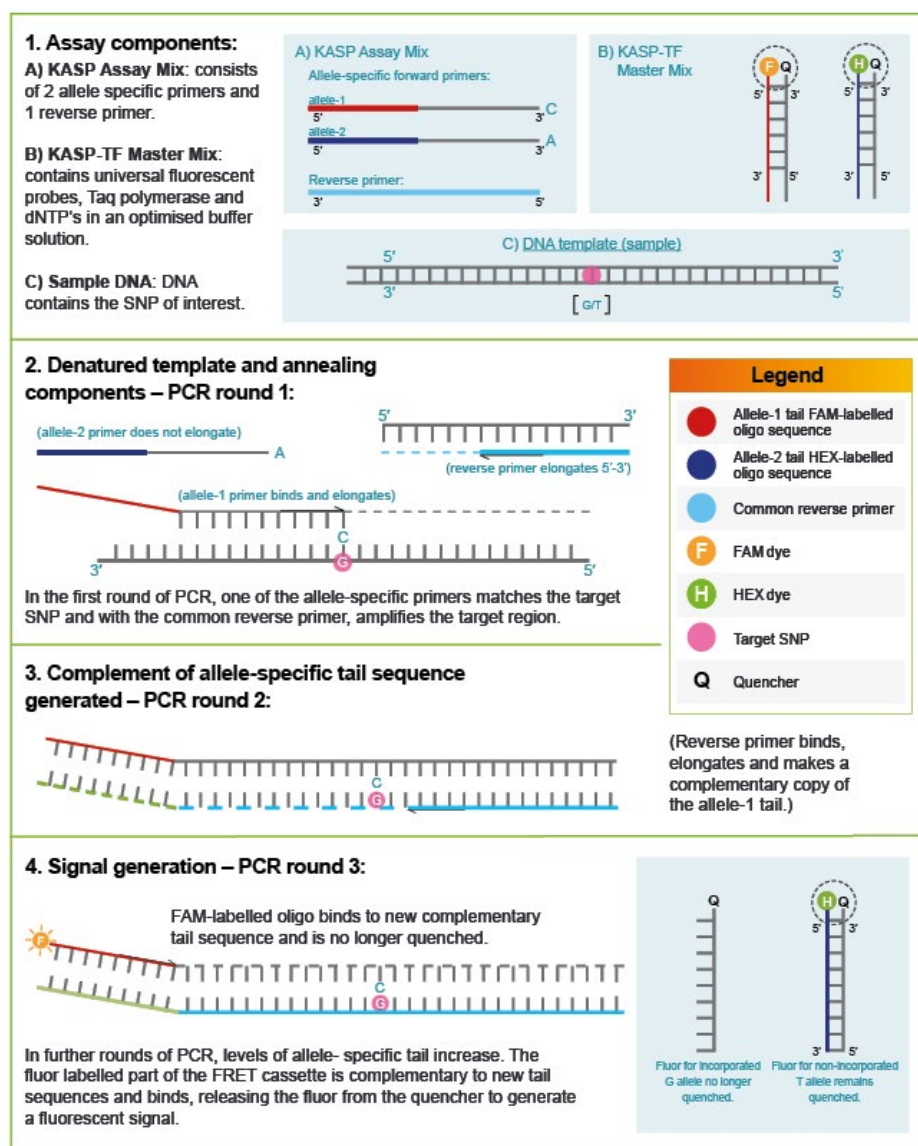


Figure 4.2. Schematic of how KASP works. Bi-allelic discrimination is achieved through the competitive binding of two allele-specific forward primers, each with a unique tail sequence that corresponds with one of two universal probes; one labelled with FAM™ dye and the other labelled with HEX dye.

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4.2 KASP chemistry: assay design capabilities

KASP assays can be used for the detection of the following polymorphism types:

- Single nucleotide polymorphisms (SNPs)
- Insertions or deletions (indels)
- Multi-nucleotide variants (MNVs)
- Haplotypes



Please note that for all assay design requests LGC Biosearch Technologies™ require a minimum of 50 bp of sequence on each side of the polymorphism.

Examples of the sequence format that should be used for each type of polymorphism are illustrated below.

4.2.1 Single nucleotide polymorphisms

KASP assays can be designed to detect bi-allelic single nucleotide polymorphisms (SNPs) within any organism. The SNP of interest should be submitted within [square brackets] and can either be formatted as [allele1/allele2] or [IUPAC code].

Example SNP ID	Sequence
SNP_alleles	GCTCTCTGTGCTGATCCAAGAGGCGAGGCCAGTTTCATTTGAGCATTAA [A/G] TGTC AAGTTCTGCACGCTATCATCATCAGGGCCGAGGCTTCTCTTTGTT
SNP_IUPAC	GCTCTCTGTGCTGATCCAAGAGGCGAGGCCAGTTTCATTTGAGCATTAA [R] TGTC AAGTTCTGCACGCTATCATCATCAGGGCCGAGGCTTCTCTTTGTT
rs12913832	GCTCTCTGTGCTGATCCAAGAGGCGAGGCCAGTTTCATTTGAGCATTAA [A/G] TGTC AAGTTCTGCACGCTATCATCATCAGGGCCGAGGCTTCTCTTTGTT

Figure 4.3. Examples of how to format sequence for KASP assay design for a SNP. Alleles are coloured teal and pink in the first example, and the appropriate IUPAC code is coloured orange in the second example.

When submitting SNPs that are identified with RefSNP (rs) numbers the sequence is optional. It is advisable to submit the sequence in addition to the rs number to ensure that Biosearch Technologies are using exactly the sequence that you require.

4.2.2 Insertions or deletions

KASP assays can be designed for the detection of insertions or deletions (indels) of any size. One allele-specific primer will be designed to incorporate the indel sequence and the second allele-specific primer will be designed to the same target sequence excluding the indel. The common reverse primer will be designed to sequence present in all samples.

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Example SNP ID	Sequence
1bp_indel	ATTGTCAATAATATGTGGAGGTCGTGTTGGTACAACATGATAGCATAAAG [A/] GTGATGATTAGTTTCCGTAATGCGTCCGCTGGGCTGATGGGCCTTCTGGA
1bp_indel_alt	ATTGTCAATAATATGTGGAGGTCGTGTTGGTACAACATGATAGCATAAAG [A/-] GTGA TGATTAGTTTCCGTAATGCGTCCGCTGGGCTGATGGGCCTTCTGGA
15bp_indel	ATTGTCAATAATATGTGGAGGTCGTGTTGGTACAACATGATAGCATAAAG [AGATCCATACATTTA/] GTGATGATTAGTTTCCGTAATGCGTCCGCTGGGCTGATGGGCCTTCTGGA
15bp_indel_alt	ATTGTCAATAATATGTGGAGGTCGTGTTGGTACAACATGATAGCATAAAG [AGATCCATACATTTA/-] GTGATGATTAGTTTCCGTAATGCGTCCGCTGGGCTGATGG GCCTTCTGGA

Figure 4.4. Examples of how to format sequence for KASP assay design for indels. The 'insertion' allele is shown in teal in all examples. The 'deletion' allele is omitted and may optionally be represented by a dash as shown in the second and fourth examples (pink dash).

Detection of large indels is also possible using KASP. There is no upper size limit for the indel, aside from the requirement for the sequence to all be present on the same strand of DNA. To submit an indel that is larger than 100 bp you should provide 50 bp of sequence at the start and end of the indel region within the square bracket and add an N in the centre to represent the remainder of the indel sequence.

Example SNP ID	Sequence
Large_indel	ATTGTCAATAATATGTGGAGGTCGTGTTGGTACAACATGATAGCATAAAG [AGATCCATACATTTAAGTCGGTCAAATTATGGATCCTTCAAGATCTTAAGNGCCTTAA GGTAACCTTGACTTAGTGCATAGGTGGAAGGGTTTATAGTAGCT/] GTGATGATTAGTTT CCGTAATGCGTCCGCTGGGCTGATGGGCCTTCTGGA

Figure 4.5. Example of how to format sequence for KASP assay design for a large indel. Within the square brackets, 50 bp at the start and end of the indel should be specified (shown in teal) with an N in the centre (shown in purple) to represent the remainder of the sequence. The 'deletion' allele is omitted in this example.

4.2.3 Multi-nucleotide variants

Detection of multi-nucleotide variants (MNV) is possible using KASP. Sequence information for MNVs should be submitted using the [allele1/allele2] format.

Example SNP ID	Sequence
MNV	TGGGCAGCATTAGTAGAAGAAAGTACAAGACCGTGTGTAGAGGATACTCT [GATATACTTGAG/CAGTCC] AGCAGATAGCGTTGGATAGGCGACAGGATTATTGGAG CGCCGTCGAGAAC

Figure 4.6. Example of how to format sequence for KASP assay design for a MNV. Alleles are coloured teal and pink in this example.

If either or both of the MNV alleles are longer than 100 bp then you should submit 50 bp of sequence at the start and end of the allele with an N in the centre to represent the remainder of the MNV allele sequence.

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Example SNP ID	Sequence
Large_MNV	TGGGCAGCATTAGTAGAAGAAAGTACAAGACCGTGTGTAGAGGATACTCT [GATATACTTGAGGTGTCAGTTGATGCTTGATATACTTGACGTTCTCTACANTGTGCGA GCTACGCCGATGGGCGGTCCACACGGAATTCAGAGGTGGGATT /CAGTCCGCAGCTAGGGGGGTGATGGTTCGCAAGAAAATATACGGTCTATGNATGTAAC TTTATCATAAAGGTAGACACCAGTTATTGTTTCGGGTCGTAGA] AGCAGATAGCGTTGG ATAGGCGACAGGATTATTGGAGCGCCGTCGAGAAC

Figure 4.7. Example of how to format sequence for KASP assay design for a large (≥ 100 bp) MNV. Alleles are coloured teal and pink in this example. The N representing the remainder of the MNV allele sequence is coloured purple.

4.2.4 Haplotypes

KASP assays can be designed to detect haplotypes of any size. Sequence information for haplotypes should be submitted using the [allele1/allele2] format. The [square brackets] should surround all SNPs that are part of the haplotype. Any nearby SNPs that are not considered to be part of the haplotype should be outside of the square brackets and should be identified using the appropriate IUPAC ambiguity code.

Example SNP ID	Sequence
Haplotype	CAAACACCAAACCTGGTGAGTCGTGGTTTACAACACGGGAGTTCAAACCTG [TATCCGAATGACGAATGTTTACGTCCTTAAAC /CATCCGAATCACGAATGTTTCAGTCCTTTAAG] CATCATGAAATGAGTTTAGTTTGGG TGGCTCGTAAGTAGACATAAGGCAC

Figure 4.8. Example of how to format sequence for KASP assay design for haplotypes. Alleles are coloured teal and pink in this example.

4.3 Formatting guidelines for sequence submission

Sequence information for assay design should be submitted using the appropriate SNP submission template (see Section 4).

4.3.1 SNP ID

Use this field to provide a unique identifier for the assay (i.e. an assay name).

- Can be up to 20 characters in length
- Use alphanumeric and underscore characters only e.g. ABCD1234_ab
- Do not begin the name with a hyphen (-)

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4.3.2 Sequence information

Sequence orientation

Enter the sequence in the 5' – 3' orientation.

Upper case and lower case bases

Enter the sequence information using upper case characters (ATCG). If particular regions of the surrounding sequence are less certain, use lower case characters to identify the uncertain bases (atcg). A combination of both upper case and lower case characters is acceptable.

Identify the target SNP using square brackets

The SNP of interest (target site) must be identified by surrounding it with square brackets. Use the appropriate IUPAC code (e.g. [R]) or a forward slash (/) character to denote the two possible alleles (e.g. [A/G]). The IUPAC codes for ambiguous nucleic acids are detailed in table 1 for your reference.

Symbol	Translation
R	A or G
Y	C or T
M	A or C
K	G or T
S	C or G
W	A or T
N	A or C or G or T

Table 4.1. IUPAC codes for ambiguous specification of nucleic acids.

Identify the target indel using square brackets

The location of the indel of interest (target site) must be identified by surrounding it with square brackets.

See figures 2, 3 and 4 for examples of how to format sequence for indels of varying sizes.

Surrounding SNPs and indels

If there are additional known SNPs in the sequence surrounding the SNP of interest, these must be identified using IUPAC codes. These surrounding SNPs must not be surrounded by square brackets. Do **not** mark surrounding SNPs as Ns.

If there are known indels in the sequence surrounding the SNP of interest, these must be identified as Ns.

These surrounding indels must not be surrounded by square brackets.

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Avoiding bases

If there are particular regions of sequence that should be completely avoided during assay design, replace these bases with an N.

TECH TIP: Before submitting sequence information, double (and triple!) check to ensure that it is correct. We design to the sequence that is submitted, and if there is an issue (e.g. pasted incorrectly, wrong orientation, homology) the assay will not work on your samples, however hard you try.

Acceptable sequence submission formats

Acceptable sequence submission formats	Example
[First allele/second allele]	ATAACTACTTTTAAAGGCACATTATTCAACCTCACTGTGCATTTTCATCCTC [A/T] TGCATA ATGACAGTTATTCTCTCCCAAGTCTCCTTCTGGGACAGTTAAAG
IUPAC code	ATAACTACTTTTAAAGGCACATTATTCAACCTCACTGTGCATTTTCATCCTC [W] TGCATAA TGACAGTTATTCTCTCCCAAGTCTCCTTCTGGGACAGTTAAAG
Rs Number	rs123456* *NOTE: It is advisable to submit the sequence in addition to the rs number to ensure that Biosearch Technologies are using exactly the sequence that you require.
indels	ATAACTACTTTTAAAGGCACATTATTCAACCTCACTGTGCATTTTCATCCTC [/T] TGCATAAT GACAGTTATTCTCTCCCAAGTCTCCTTCTGGGACAGTTAAAG

Unacceptable sequence submission formats

Unacceptable sequence submission formats	Example
Multiple IUPAC codes without marking the target site in square brackets	ATAACTACTTTTAAAGGCACATTATSCAAACCTCAYYTGCATTTTCATCCTCWTGCATAAT GACAGTTRTTCTCTCCCAAGTCTCCWWCTGGCACAGTTAAAG
Insufficient surrounding sequence (<50 bases either side)	CACTGTGCATTTTCATCCTC [A/T] TGCATAATGACAGTTATTCTCT
Surrounding SNPs marked with square brackets	ATAACTACTTT [T/G] AAAGGCACATTATT [C/G] AACCTCACTGTGCATTTTCATCCTC [A/T] TGCATAATGACAGTTATTCTCTCCCA [A/C] GTCTCCTTCTGGGACAGTTAAAG

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Marking special design requirements

Marking special requirements	Example	Design
Surrounding polymorphisms – mark these with IUPAC codes (without square brackets)	ATAACTACTTTT K AAAGGCACATTATT S AACCTCACTGTGCATTTCATCCTC [A/T] TGCATAATGACAGTTATTCTCTCCCA M GTCTCCTTCTGGGACAGTTAAAG	Primer design software will take surrounding SNPs into account during primer design.
Avoiding bases – designate as Ns	ATAACTACTTTTAAAGGCACAMTATTCAACCTCACTGTG CATTTNATCC NN [A/T] TGCATAATGACAGTTATTCTCTCCC AAGTCTCCTTCTGG	Primer design software will avoid the bases designated as Ns in assay primers.
Suspicious bases – user lower case characters	ATAACTACTTTTAAAGGCACATTATTCAACCTCA ctgtgcat ttcatcct C [A/T] TGCATAATGACAGTTATTCTCTCCCAAGTCT CCTTCTGG	Bases in lower case characters will be avoided by primer design software.

Tables 4.2, 4.3 and 4.4. Acceptable and unacceptable submission formats, and the marking of special requirements.

4.4. Homologous sequences

When designing KASP assays, homologous sequences should be avoided. If this is not possible, anchoring may be used to increase the specificity of the assay. For more details on anchoring, please view our [guide to anchoring](#).

4.5. Submitting sequence information to Biosearch Technologies

Sequence information for KASP assay design must be submitted to Biosearch Technologies using the appropriate SNP submission template (Excel format).

4.5.1 Genotyping service projects

Please [download](#) the sequence submission form for genotyping service projects.

4.5.2 Assays to be run in your own laboratory

Please [download](#) the sequence submission form for KASP assays to run in your own laboratory. To run KASP genotyping in your own laboratory you will require both KASP Assay Mix and KASP Master Mix. Biosearch Technologies manufacture KASP Master Mix with three different levels of ROX; these are the Low, Standard, and High ROX formulations. These formulations only differ in the level of ROX that they contain and are otherwise identical. To determine the appropriate version of KASP Master Mix for your qPCR instrument or plate reader, please visit [our website](#).

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4.6 KASP genotyping service

A KASP genotyping service project includes labour, consumables, all reagents, data analysis and reporting. A Project Manager will be assigned to you for every service project that you run. They will be your main point of contact for any queries relating to sample shipment, processing and data analysis, and will communicate with you regularly to provide project updates.

4.6.1 DNA requirements for service projects

DNA can be shipped to Biosearch Technologies in a dried down or liquid format. As service projects are run using our robotics instrumentation, an excess of DNA is required (dead volume) if shipping wet.

Before shipping DNA, please ensure that you have read our [service project DNA guide](#) which explains in detail the masses and volumes required for dry and wet DNA, and how to adjust this to account for the number of SNPs to be run. Please note that the minimum mass of DNA that we accept per sample is 2 ng, and the minimum concentration that we accept per sample is 5 ng/μL (based on human genome size). We always advise sending more DNA than the minimum requirement. All DNA will be tested in-house prior to commencing your genotyping project to determine the most suitable dilution.

Allow a minimum of two no template control (NTC) wells per 96-well plate and four NTC wells per 384-well plate. Please leave these wells empty.



4.6.2 Plate types

DNA samples should be shipped to Biosearch Technologies in 96- or 384-well plates. Please note that we are unable to accept 96-well plates with a well volume of greater than 0.8 mL as these are not compatible with our robotics systems. We can transfer samples on arrival but this will incur an additional charge.



4.6.3 DNA sample plate data

Biosearch Technologies requires information about the layout of samples in your DNA plates, and of the corresponding plate names and/or barcodes. This information should be supplied to your Project Manager using the appropriate DNA plate template. This information is imported directly into our proprietary Kraken software and enables your samples to be fully tracked through the laboratory workflow.

- [Excel grid 96](#) - well plates – please use this template to submit DNA sample information for samples submitted in 96-well plates
- [Excel grid 384](#) - well plates – please use this template to submit DNA sample information for samples submitted in 384-well plates
- [Excel list](#) – please use this template to submit DNA sample information in a list format (can be used for both 96-well and 384-well plates)

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If your plates do not have barcodes, they will be barcoded upon arrival at our laboratories. All non-barcoded plates must be labelled clearly with unique plate names.

- The minimum plate name length is 1 character. Shorter plate names will make your results file more easily readable.
- The maximum plate name length is 15 characters.
- Only alphanumeric and underscore or dash characters should be used. Please avoid space, comma and full stop characters.
- If you are numbering your plates and have more than 10 plates, please use a leading zero e.g. P01, P02 to facilitate data sorting.
- If the plate seal hides the well location identifiers, please mark the A1 corner of the plate.



4.6.4 Plate sealing

It is essential that all DNA sample plates that are shipped to our laboratories are sufficiently sealed to prevent sample leakage and contamination during transit. The use of sticky seals is typically not sufficient, and can result in substantial sample leakage. Biosearch Technologies recommend either:

- a. Heat sealing – the use of a thermal weld achieved by an automated or semi-automated heat sealer will ensure that each individual well of the sample plate is sealed. You may be interested in the [Kube™](#), our heat sealing instrument.
- b. Cap mat sealing – cap mat seals are available from a wide range of scientific suppliers and provide an individual seal for each well of the sample plate. Do not overfill plate wells when using cap mat seals as, when samples are frozen, the expansion of the ice can disrupt the seal.



4.6.5 Plate shipping

Please include a cardboard spacer between each plate. This cardboard layer will prevent the bottom of one plate piercing the seal of the plate stacked below it. Wrap plate stacks in bubble wrap to protect during transit.

Biosearch Technologies recommend that sample plates are shipped on dry ice. This will ensure that samples remain frozen during transit, thus minimising the possibility of sample leakage. Shipping address:

LGC Genomics Ltd.
Unit 1-2, Trident Industrial Estate
Pindar Road
Hoddesdon, Herts
EN11 0WZ
UK

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4.6.6 Laboratory workflow

DNA samples provided by the customer are stamped into reaction plates in preparation for KASP genotyping reactions. These can be either 384-well or 1536-well format plates depending upon the scale of the project. DNA samples are stamped using our [repliKator™](#) instrument. All reaction plates are barcoded and tracked to your project within our [Kraken](#) database. DNA samples are dried down into reaction plates to ensure stability of samples during storage.

Once the KASP assays within your genotyping project have been assembled and validated (where applicable), they can be dispensed across the DNA sample plates. Dispensing is performed using our [Meridian³™](#) dispensing robot. Plates are laser-sealed using the [Fusion³™](#) and are thermally cycled in one of our [Hydrocycler²™](#) instruments. Following completion of the initial 35 cycles of PCR, all genotyping reaction plates run at Biosearch Technologies are read on a BMG PHERAStar plate reader. This initial read data is visually inspected by a member of the genotyping team to assess the progression of the PCR reaction. The plates are then recycled (3 cycles per recycle step) and read after each recycle step, before proceeding to the data analysis stage.



4.7 KASP in your own laboratory (kit)

It is straightforward to perform KASP end-point genotyping in your own laboratory. Once you have your DNA samples, and have ordered your SNP-specific [KASP assays](#) and universal [KASP-TF Master Mix](#) from Biosearch Technologies, all that is required to read the fluorescent signal from completed KASP reactions is a FRET-capable plate reader or a qPCR instrument.



4.7.1 Outline KASP protocol

The steps below outline the protocol for setting up and running KASP end-point genotyping in your own laboratory.

1. Run a free-of-charge trial kit to confirm your laboratory set-up and to familiarise yourself with the protocol. See [section 4.7.2](#).
2. Prepare sample DNA plates. 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). See [section 4.7.3](#) and [section 4.7.4](#) for more details about DNA requirements.
3. Prepare genotyping mixes for each of the assays to be run. Thaw and thoroughly vortex KASP Assay Mix(es) and KASP-TF Master Mix. Combine appropriate volumes of assay and master mix for the number of samples to be genotyped (See [table 4.7](#) for guidance). See [section 4.7.5](#), [section 4.7.6](#), and [section 4.7.7](#) for details on ordering and storing KASP reagents.
4. Dispense prepared genotyping mix into the prepared DNA sample plate. See [section 4.7.8](#) for information on dispensing reagents.
5. Seal the plate with a PCR-suitable plate seal, and briefly centrifuge to bring all reagents to the

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bottom of the wells.

6. Load plate into the Peltier block or qPCR machine, and run the KASP thermal cycle. See [section 4.7.9](#) for information on thermal cycling.
7. At the end of the thermal cycle, cool the plate to 40 °C or below.
8. Read the fluorescence on a FRET-capable plate reader or qPCR machine. See [section 4.7.10](#) for information on reading completed reactions.
9. Recycle the reaction plate if required and repeat steps 6 and 7. See [section 4.7.11](#) for more details regarding recycling of KASP reactions.



4.7.2 KASP trial kits

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.

A KASP trial kit contains:

- DNA samples (pre-diluted)
- A KASP Assay Mix
- KASP-TF Master Mix (correct version for your instrumentation – see [section 4.7.6](#))

[Full instructions](#) are provided with the kit. You can set up a KASP genotyping reaction plate, run the PCR, and then read the plate on your instrument. The samples will give rise to three clear genotyping clusters. Complete support from the technical support team is provided for trial kit requests.

TECH TIP: Request one of our free-of-charge trial kits. These are an excellent way to check your laboratory process and equipment, and to familiarise yourself with our support procedure in case you should need it in the future.



4.7.3 DNA requirements for genotyping in your own laboratory

The minimum final DNA concentration that Biosearch Technologies recommend 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) 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.

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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 (3000 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): 15966 Mbp
 $15966 \text{ Mbp} / 3000 \text{ Mbp} = 5.3$

It is also advisable to set up and run a dilution test when using your DNA for the first time. If PCR inhibitors are present in your samples, diluting can reduce their impact on the reaction and hence improve the quality of the data generated. Diluting DNA also means that you can potentially run more experiments using your samples.

A dilution test involves setting up a range of dilutions for a subset of your samples (minimum 22), and then running each of these with the same KASP Assay Mix. For example, you could prepare neat, 2x, 4x and 8x dilutions of your DNA. When plotting the genotyping results, you will be able to see which dilution gives the clearest results. If the DNA has been diluted too far, amplification will be very slow or not occur at all. If inhibitors are present, you may find that more dilute samples work more efficiently than neat samples.

TECH TIP: Spend time optimising the DNA concentration that you use in your KASP reactions. This can make a big difference to the results obtained, and ensures you are using your DNA as efficiently as possible.



4.7.4 Drying down DNA

A useful technique when performing large-scale genotyping is the drying down of DNA in reaction plates. This 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. After arraying samples, plates should be briefly centrifuged and placed in a drying oven at approximately 55 °C for one hour.

It is important to remember to adjust the KASP reaction volume with water if dried down DNA is used; see [table 4.7](#). KASP-TF Master Mix, supplied as 2X, should always be diluted to 1X in the final reaction.

Higher throughput users may consider our [RepliKator](#) platform for plate replication.

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4.7.5 Ordering KASP Assays

When purchasing KASP assays to run in your own laboratory, there are two options available to choose between:

- KASP by design (KBD): the standard option for purchasing KASP assays. Assays are designed by our scientists in our proprietary Kraken™ software, but no wet-lab validation of the primers is performed before delivery of the KASP Assay Mix.
- KASP on demand (KOD): the premier option for purchasing KASP assays. Assays are designed by our scientists in our proprietary Kraken™ software. Our scientists then fully validate and optimise your assays in our laboratory, and send the genotyping results to you for confirmation before delivery of the KASP Assay Mix.

The success rate for KASP assays depends on a number of factors including DNA quality, genome complexity, homology and genome size. If you are confident in the process of assay optimisation, and do not have stringent time and resource limitations, then the KBD option will be a cost-effective choice for your project. If your time and/or resources are limited, or you are working with a complex genome, we would recommend the KOD option as this guarantees delivery of a fully validated KASP Assay Mix which can be run immediately on your valuable samples to give genotyping results.

The product codes for ordering KASP Assays are detailed in table 4.5. Each standard KBD or KOD Assay Mix provides sufficient material for 2,500 x 10 µL reactions.



Product code	Product name	Description
KBS-2300-001	KASP by Design (KBD)	Assay is designed, in silico-validated, and manufactured by Biosearch Technologies (not laboratory-validated). 2-3 weeks turnaround time.
KBS-2400-001	KASP on Demand (KOD) Standard service	Assay is designed, manufactured and laboratory-validated* by Biosearch Technologies. 4-6 weeks turnaround time.
KBS-2400-002	KASP on Demand (KOD) Rapid service	Assay is designed, manufactured and laboratory-validated* by Biosearch Technologies. 2-3 weeks turnaround time.

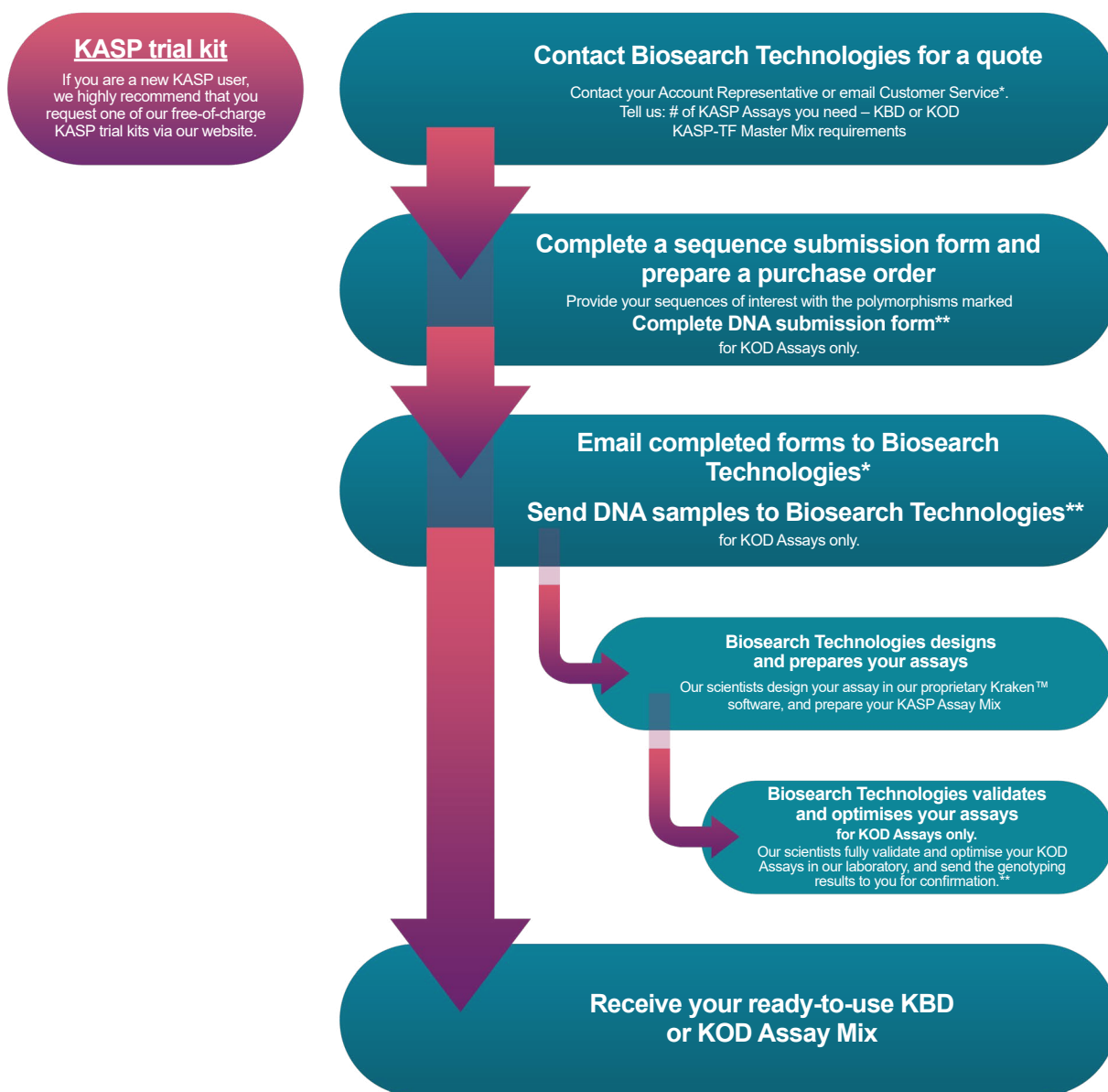
Table 4.5. KASP assay mixes available from Biosearch Technologies.

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The schematic in figure 4.9 below illustrates the process flow for ordering your KASP reagents.



* Email quote requests and completed forms to Customer Service for your region:

- Americas: Genomics.Americas@lgcgroup.com
- EMEA (Europe, Middle East and Africa): Genomics.EMEA@lgcgroup.com
- APAC (Asia, Pacific): Genomics.APAC@lgcgroup.com

** DNA submission form and sample DNA shipment required for non-human KOD Assays only. Human

*** KOD projects utilise Biosearch Technologies' in-house human DNA panel, see [Appendix A](#).

For ordering assistance, please contact our [Customer Services team](#).

Figure 4.9. Process flow for ordering of KASP end-point genotyping reagents from Biosearch Technologies.

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4.7.6 Ordering KASP-TF Master Mix

KASP-TF Master Mix contains ROX™ passive reference dye which can be used to correct any variations in pipetting across your reaction plate. The qPCR machines available on the market have differing requirements for ROX. For this reason, three formulations of KASP-TF Master Mix are available from Biosearch Technologies: low ROX, standard ROX and high ROX. These formulations only differ in the level of ROX that they contain and are otherwise identical.

To determine the correct version of KASP-TF Master Mix for your machine, please visit our [website](#) and find your instrument in the table. Instrument-specific guides for programming and running KASP are also downloadable from this location.

KASP-TF Master Mix is available in three different sizes: 2.5 mL, 25 mL and 250 mL. Part numbers are detailed in table 4.6.

Part number	Description	Format
KBS-1050-101	KASP-TF V4.0 2X Master Mix 96/384, Standard ROX (2.5 mL)	96-/384-well
KBS-1050-102	KASP-TF V4.0 2X Master Mix 96/384, Standard ROX (25 mL)	96-/384-well
KBS-1050-103	KASP-TF V4.0 2X Master Mix 96/384, Standard ROX (250 mL) (10 x 25 mL)	96-/384-well
KBS-1050-121	KASP-TF V4.0 2X Master Mix 96/384, Low ROX (2.5 mL)	96-/384-well
KBS-1050-122	KASP-TF V4.0 2X Master Mix 96/384, Low ROX (25 mL)	96-/384-well
KBS-1050-123	KASP-TF V4.0 2X Master Mix 96/384, Low ROX (250 mL) (10 x 25 mL)	96-/384-well
KBS-1050-131	KASP-TF V4.0 2X Master Mix 96/384, High ROX (2.5 mL)	96-/384-well
KBS-1050-132	KASP-TF V4.0 2X Master Mix 96/384, High ROX (25 mL)	96-/384-well
KBS-1050-133	KASP-TF V4.0 2X Master Mix 96/384, High ROX (250 mL) (10 x 25 mL)	96-/384-well
KBS-1050-111	KASP-TF V4.0 2X Master Mix 1536, Standard ROX (2.5 mL)	1536-well
KBS-1050-112	KASP-TF V4.0 2X Master Mix 1536, Standard ROX (25 mL)	1536-well
KBS-1050-113	KASP-TF V4.0 2X Master Mix 1536, Standard ROX (250 mL) (10 x 25 mL)	1536-well
KBS-1050-201	KASP-TF V5.0 2X Master Mix, Standard ROX (2.5 mL)	Array Tape™
KBS-1050-202	KASP-TF V5.0 2X Master Mix, Standard ROX (25 mL)	Array Tape
KBS-1050-204	KASP-TF V5.0 2X Master Mix, Standard ROX (250 mL)	Array Tape
KBS-1050-203	KASP-TF V5.0 2X Master Mix, Standard ROX (250 mL) (10 x 25 mL)	Array Tape

Table 4.6. KASP-TF Master Mixes available from Biosearch Technologies.

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To help calculate the volume of KASP-TF Master Mix needed for your experiments, table 4.7 details the reagent volumes required for preparing KASP genotyping mix for both 96-well and 384-well plates. Note that if the DNA samples are dried down, 2X KASP-TF Master Mix must be diluted with water to adjust the concentration to 1X.

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



4.7.7 Storage of KASP reagents

Table 4.8 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 4.8. 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.

KASP-TF Master Mix is shipped frozen, and may arrive frozen or thawed. KASP-TF Master Mix that arrives at or below 20 °C will be stable and should be frozen upon receipt. We have shown that thawing during transit will not adversely affect performance.

KASP Assay Mix is shipped at ambient temperature, unless shipped alongside KASP-TF Master Mix. As KASP Assay Mix consists of unlabelled oligonucleotides, these will be stable at room temperature for a number of days if shipments are delayed for any reason. Upon receipt, KASP Assay Mix should be frozen.

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4.7.8 Additional consumables

KASP can be performed in PCR-suitable plates that are appropriate for your machine. It is important to ensure that the plate seal used is PCR-suitable and optically clear to enable fluorescent signal to be read properly at the end of the reaction.

Higher throughput users may wish to consider our [Kube](#) heat-based plate sealer or, if using black 384-well or 1536-well plates, our [Fusion³](#) laser welding system.

TECH TIP: When sealing your reaction plate, ensure that the plate seal used is PCR-suitable and optically-clear, otherwise fluorescence at the end of the reaction will not be read properly.



4.7.9 Dispensing KASP reagents

For ease and accuracy of pipetting, we recommend preparing a KASP genotyping mix for each KASP assay on your reaction plate. This involves combining sufficient volumes of KASP Assay Mix and KASP-TF Master Mix, and including an excess to account for volume lost during pipetting. Table 4.7 details how to prepare sufficient KASP genotyping mix for 96-well and 384-well plates, when using either wet or dry DNA.

KASP reagents can be dispensed into your reaction plate(s) using manual pipetting or using robotic platforms. Our [Meridian²](#) system for liquid dispensing may be of interest to high throughput users.



4.7.10 Thermal cycling

KASP reactions can be thermal cycled on any standard thermal cycler. Similar results have been obtained on Peltier block-based thermal cyclers and our [Hydrocycler²](#) water bath-based thermal cyclers. Many users run the thermal cycle on their qPCR machine, which can then also be used to read the completed reactions. KASP is compatible with most standard qPCR machines, and detailed guides for running KASP on common qPCR machines can be accessed on our [website](#).

KASP chemistry utilises a two-step touchdown PCR method, with the extension and annealing steps incorporated into a single step. A touchdown PCR involves starting with a high annealing temperature and incrementally decreasing the annealing temperature each PCR cycle. The higher annealing temperatures in the early cycles of a touchdown ensure that only very specific base pairing will occur between the DNA and the primer, hence the first sequence to be amplified is most likely to be the sequence of interest. The annealing temperature is gradually decreased to increase the efficiency of the reaction. The regions that were originally amplified during the highly specific early touchdown cycles will be further amplified and outcompete any non-specific amplification that may occur at the lower temperatures.

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In the KASP thermal cycle, a fifteen minute activation is required (94 °C) followed by 10 cycles of touchdown PCR and 26 cycles of standard 2-step PCR (table 4.9.).

Protocol stage	Temperature	Duration	Number of cycles for each stage
Stage 1 Hot-start <i>Taq</i> 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 4.9. KASP thermal cycle protocol

* Please note that Stage 4 of the above 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, 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.



4.7.11 Plate reading

KASP uses the fluorophores FAM and HEX for distinguishing genotypes. The passive reference dye ROX is also used (optional) to allow normalisation of variations in signal caused by differences in well-to-well liquid volume. The relevant excitation and emission wavelengths are shown in table 4.10.

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

Table 4.10. Excitation and emission values for the fluorophores used in KASP.

TECH TIP: If your plate reader or qPCR machine is optimised for the fluorophore VIC, this can be used in place of HEX; excitation and emission values for VIC and HEX are extremely similar. All of the ABI qPCR machines have the ability to read VIC by default instead of HEX.

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Most FRET-capable plate readers with the relevant filter sets, and standard qPCR machines, can be used in conjunction with KASP. Do not forget to request a [KASP trial kit](#) for your instrument so that you can ensure it is set up correctly before starting your own experiments.

Please note that KASP reaction plates must be cooled to 40 °C or below before plate reads are performed. Fluorescence values read above 40 °C will not provide meaningful data.

TECH TIP: Only read KASP reactions at the end of the thermal cycle. KASP is an end-point chemistry and reading every cycle will not provide any useful information. Cooling the plate every cycle will unnecessarily affect the reaction efficiency too.

TECH TIP: Always read completed KASP reactions at 40 °C or below – fluorescence cannot be read above this temperature. Add a step at the end of the thermal cycle to cool the plate down before reading.



4.7.12 Recycling of KASP reaction plates

Following completion of the standard KASP thermal cycle, it is possible that 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 has not undergone a sufficient number of cycles to reach completion. At this stage, we recommend further cycling of the reaction plate – termed ‘recycling’.

One KASP recycling step comprises of three additional PCR cycles, as outlined in table 4.11.

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 4.11. Conditions for further cycling (recycling) of KASP reactions.

Each KASP assay will differ in the optimal number of cycles required to reach reaction completion. We recommend optimising your assay by performing several individual recycle steps with subsequent plate reads, rather than combining all of the additional cycles together and reading once at the end. 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 (see [section 6](#)).

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More detail about recycling of KASP reactions can be found in our [KASP recycling document](#).

TECH TIP: *Following assay optimisation you can increase the number of cycles in the initial KASP thermal cycle by the number of additional cycles that are required to take the reaction to completion. The assay can then be run on the remainder of your DNA sample plates without the need for additional recycling.*



4.7.13 Storage of completed reaction plates

Once you have performed the KASP thermal cycle for your plate(s) we highly recommend storing plates in a dark fridge (~4 °C for a maximum of 1 week) until the data has been analysed. This will allow you to perform additional read(s) or recycle steps if required, to ensure that you have obtained the best possible data.

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5. Data analysis

Once end-point genotyping reactions are complete, and the resulting final fluorescence values have been measured, the raw data must be interpreted to enable genotypes to be assigned to the DNA samples. This is typically achieved by viewing the fluorescence values for each sample on a Cartesian plot. These plots are often referred to as cluster plots or scatter plots. The analysis of KASP end-point genotyping is explained in this [short video](#).



5.1 Data points and axis nomenclature

The fluorescent signal from each individual DNA sample is represented as an independent data point on a cluster plot. For KASP, one axis is used to plot the FAM™ fluorescence value (typically the x-axis) and the second axis is used to plot the HEX fluorescence value (typically the y-axis) for each sample.

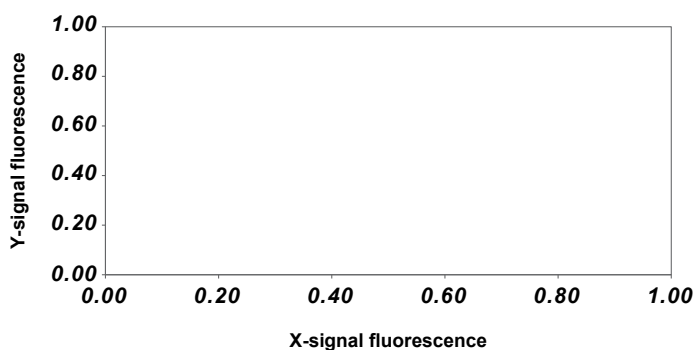


Figure 5.1. The axes used on a typical genotyping cluster plot

5.2 Homozygous samples

A sample that is homozygous for the allele reported by the X-signal oligonucleotide will only generate X-signal fluorescence during the end-point genotyping reaction. This data point will be plotted close to the x-axis, representing high X-signal and no Y-signal (blue data points in figure 5.2).

A sample that is homozygous for the allele reported by the Y-signal oligonucleotide will only generate Y-signal fluorescence during the end-point genotyping reaction. This data point will be plotted close to the y-axis, representing high Y-signal and no X-signal (red data points in figure 5.2).

5.3 Heterozygous samples

A sample that is heterozygous will contain both the allele reported by the x-signal oligonucleotide and the allele reported by the y-signal oligonucleotide. This sample will generate half as much x-signal fluorescence and half as much Y-signal fluorescence as the samples that are homozygous for these alleles. This data point will be plotted in the centre of the plot, representing half x-signal and half Y-signal (green data points in figure 5.2).

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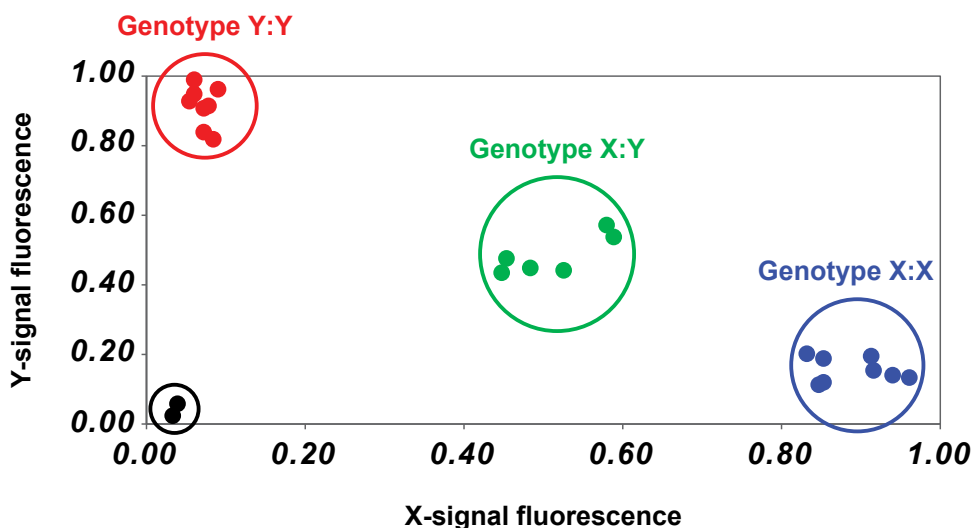
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5.4 No template controls

To ensure the reliability of the results, an end-point genotyping reaction without any template DNA must be included as a negative control. This is typically referred to as a no template control (or NTC). The NTC will not generate any fluorescence and the data point will therefore be plotted at the origin of the plot (black data points in figure 5.2).

5.5 Interpretation of genotyping clusters

All samples that have the same genotype will have generated similar levels of fluorescence and will therefore all appear close together on the plot. Based on the relative position of these clusters, it is possible to determine the genotype of all the data points. It is important that a sufficient number of individual samples are included in an end-point genotyping reaction plate to ensure that there are enough data points on the Cartesian plot to allow cluster analysis.



- Homozygous for allele X, reported by X-signal oligonucleotide
- Homozygous for allele Y, reported by Y-signal oligonucleotide
- Heterozygous, contains allele X and allele Y
- No template control (NTC)

Figure 5.2. A typical genotyping cluster plot. Each data point represents the fluorescence signal of an individual DNA sample. Samples of the same genotype will have generated similar levels of fluorescence and will therefore cluster together on the plot. Blue data points = homozygous for X-allele; red data points = homozygous for Y-allele; green data points = heterozygous; black data points = no template controls (NTCs).

TECH TIP: Always check the axis that you are plotting fluorescence on against the allele associated with that fluorophore – mistakes here can lead to big issues in interpretation of results. We have had a customer accidentally kill all the wrong fish!

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5.6 Genotyping service results from Biosearch Technologies

Data generated in our laboratories is automatically quality control checked on a per SNP basis. No template controls (NTCs) are included on each plate to enable the detection of contamination or non-specific amplification – these samples must not amplify during the reaction. The number of genotypes that are callable must be greater than 90% and minor allele frequency should be greater than 2% unless the SNP is known to be a very low frequency. A chi-squared value (X^2) is also generated that assesses distribution in multinomial datasets based on the Hardy-Weinberg equation.

Following completion of the initial 35 cycles of PCR, all genotyping reaction plates run at Biosearch Technologies are read on a BMG PHERAStar plate reader. This initial read data is visually inspected by a member of the genotyping team to assess the progression of the PCR reaction. The plates are then recycled (3 cycles per recycle step) and read after each recycle step. The laboratory operator visually inspects the read data after each recycle step and, once they are satisfied that the PCR reaction has reached end-point, identifies plates as completed. At this stage, our in-house Kraken software will automatically call genotypes for your samples. Your project manager will access the plate read data in Kraken and perform a detailed analysis of the data. This may require them to correct the automatically called genotypes that Kraken has given. Version one of your genotyping results is then exported within the Kraken system. A second project manager will then second check these results, and verify or change them in collaboration with your project manager. Your project manager will then send you the results.



5.6.1 Data format

Genotyping results files are provided in .csv (comma separated value) format. This file is designed to be viewed with our SNPViewer application, which is available as a free download from our website.

The .csv file can also be opened with most spreadsheet software and programs that can read plain text documents (e.g, MS Excel or Notepad).



5.6.2 SNPviewer application

SNPviewer provides a simple means to graphically view the results as genotyping cluster plots. A Cartesian plot is generated using the FAM and HEX fluorescent values; FAM is plotted on the x-axis and HEX is plotted on the y-axis. Genotyping calls displayed in SNPviewer cannot be edited.

To download SNPviewer, you first need to set up a user account on our website ([here](#)), and download SNPViewer [here](#). We also have a detailed [user manual for SNPviewer](#).


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5.6.3 Opening a results file in SNPviewer

To open a results file in SNPviewer, first make sure that you have downloaded and installed SNPviewer, and saved the results file to your computer.

1. Open SNPviewer by clicking on the icon .
2. A new window will appear that will allow you to select your results file. Choose the correct file and click 'OK'.
3. SNPviewer will now display the results from the file. Figure 1 shows a typical genotyping project as viewed in SNPviewer. The area on the right hand side of the SNPviewer window is used to select assay and master plates, and the corresponding cluster plots are displayed on the left hand side of the SNPviewer window.
4. When an assay name is selected from the list, the context sequence of the DNA region is displayed at the top of the SNPviewer window (figure 1, yellow box). The genotyping results are reported in terms of this context sequence e.g. if the sequence states [C/T], then the C allele will be reported with FAM (X axis) and the T allele will be reported with HEX (Y axis).

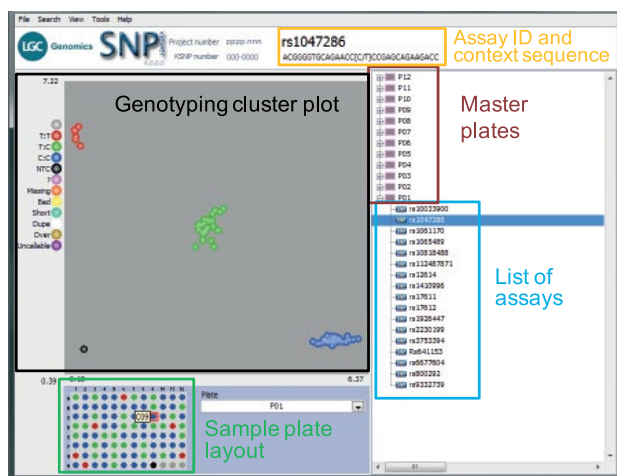


Figure 5.3. A typical genotyping project as viewed in SNPviewer. The master plates are listed in the tree on the right hand side, and can be expanded to show the assays that have been run on each plate of DNA samples. The data point colours correspond to the different genotype calls.


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5.6.3 Opening a results file in SNPviewer

To open a results file in SNPviewer, first make sure that you have downloaded and installed SNPviewer, and saved the results file to your computer.

1. Open SNPviewer by clicking on the icon .
2. A new window will appear that will allow you to select your results file. Choose the correct file and click 'OK'.
3. SNPviewer will now display the results from the file. Figure 1 shows a typical genotyping project as viewed in SNPviewer. The area on the right hand side of the SNPviewer window is used to select assay and master plates, and the corresponding cluster plots are displayed on the left hand side of the SNPviewer window.
4. When an assay name is selected from the list, the context sequence of the DNA region is displayed at the top of the SNPviewer window (figure 1, yellow box). The genotyping results are reported in terms of this context sequence e.g. if the sequence states [C/T], then the C allele will be reported with FAM (X axis) and the T allele will be reported with HEX (Y axis).

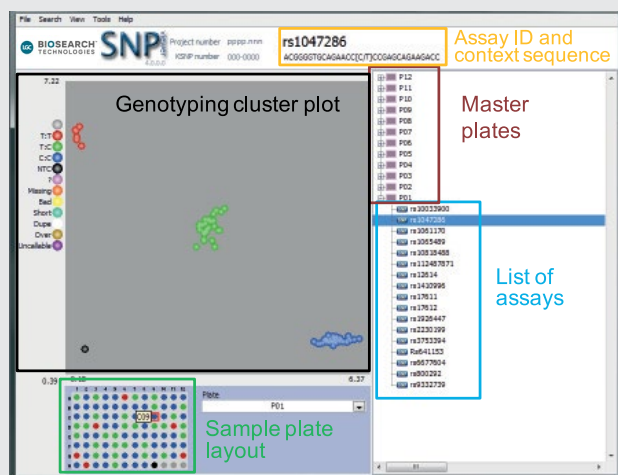


Figure 5.3. A typical genotyping project as viewed in SNPviewer. The master plates are listed in the tree on the right hand side, and can be expanded to show the assays that have been run on each plate of DNA samples. The data point colours correspond to the different genotype calls.

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5.7 Genotyping results from your own laboratory

The software provided with most qPCR machines has the capability to generate genotyping cluster plots. For detailed step-by-step instructions for generating cluster plots from your specific qPCR instrument, please visit our [website](#).



5.7.1 KlusterCaller software

If you are using a FRET-capable plate reader, or a qPCR instrument that is not listed or does not offer a cluster plot option, you may be interested in our [KlusterCaller](#) software. This is a standalone version of the genotyping and reporting module that is built into our Kraken workflow management software. When dual emission genotyping data from a fluorescent reader is imported into the KlusterCaller software, a traditional cluster graph for each assay is automatically generated. Proprietary algorithms built into the software automatically discern if the assay results are homozygous for allele 1, homozygous for allele 2, heterozygous, or inconclusive. KlusterCaller has the ability to import, display, and export data in 96-, 384-, and 1536-well plate formats. We have a comprehensive guide detailing how to import plate data into KlusterCaller which can be accessed [here](#).



5.7.2 Key factors to consider when analysing cluster plots

When viewing and analysing end-point genotyping data on a cluster plot, ensure that these guidelines are followed:

- Include a minimum of 22 data points (plus 2 NTCs) on each plot to facilitate cluster analysis.
- Check the scaling of the x and y-axes and, if required, rescale so that they are comparable. This will ensure that clusters are correctly interpreted.
- Where possible, include positive controls for each genotype as this will facilitate data analysis.
- Do not combine data from different reaction plates on the same plot, even if the results are for the same end-point assay.
- Do not view results for more than one end-point assay on an individual cluster plot – always view results assay by assay.
- Always sense-check genotypes that are automatically assigned by the instrument software as they may need to be manually adjusted.



5.7.3 Common issues with interpretation of genotyping cluster plots

This section outlines a number of issues that are common when interpreting results from genotyping.

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5.7.3.1 Too few individual data points

Biosearch Technologies recommends that a minimum of 22 DNA samples plus 2 NTCs are run per assay per plate to enable cluster analysis to be performed effectively. Running fewer samples than the recommended 22 (plus 2 NTCs) can make it difficult to score genotypes reliably, and may cause misinterpretation of genotyping results.

Figure 5.4. provides an example of how data can be misinterpreted when too few samples are run; the limited number of data points on figure 5.4.A affects the scaling of the y-axis, and hence suggests that one of the data points is heterozygous (green triangle). When sufficient data points are included (figure 5.4.B), the presence of true heterozygote data points (green circles) make it clear that this potential heterozygous sample is actually homozygous for allele X (blue triangle).

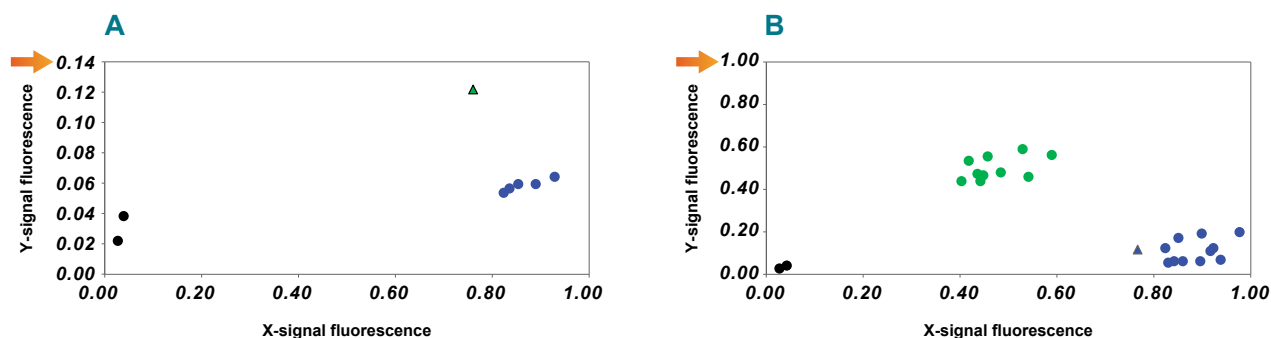


Figure 5.4. Too few individual data points.

Plot A shows a cluster plot where only six DNA samples have been genotyped. There is separation between the blue circles and the green triangle, and the results suggest that the green triangle is a heterozygote. Plot B shows the same data, but including the results for an additional sixteen DNA samples. The data point represented with a green triangle in A is identified with a blue triangle in B. By including the required number of DNA samples, the genotyping clusters are sufficiently distinct and the triangular data point now clearly belongs to the homozygous cluster (blue). The orange arrows (on A and B) indicate the effect that the number of plotted data points has on the automatic scaling of the Y axis.

5.7.3.2 Scaling of X and Y axes is not comparable

Of the two reporting fluorescent molecules used in end-point genotyping chemistry, one may produce a greater signal than the other. This difference in relative fluorescence does not negatively affect the genotyping data when viewed as a cluster plot as the x- and y-axes of the plot will be scaled independently to adjust for the different maximum signal produced by each of the fluorophores.

The software supplied with a number of widely available qPCR instruments can sometimes automatically scale the x- and y-axes so that they are not comparable. This can affect the view of the genotyping clusters and may cause misinterpretation of genotyping results. To prevent this issue, manually inspect the minimum and maximum values for the x- and y-axes. If they differ considerably, rescale the axes so that both have the same minimum and maximum value.

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Figure 5.5. provides an example of how data can be misinterpreted when the x- and y-axes are not scaled comparably; on figure 5.5.A the two clusters present (red and blue) can be interpreted as the two homozygote genotypes, yet once the x-axis is scaled correctly to match the y-axis (figure 5.5.B) it is clear that the blue homozygous cluster (genotype X:X) actually represents heterozygote samples (green, genotype X:Y).

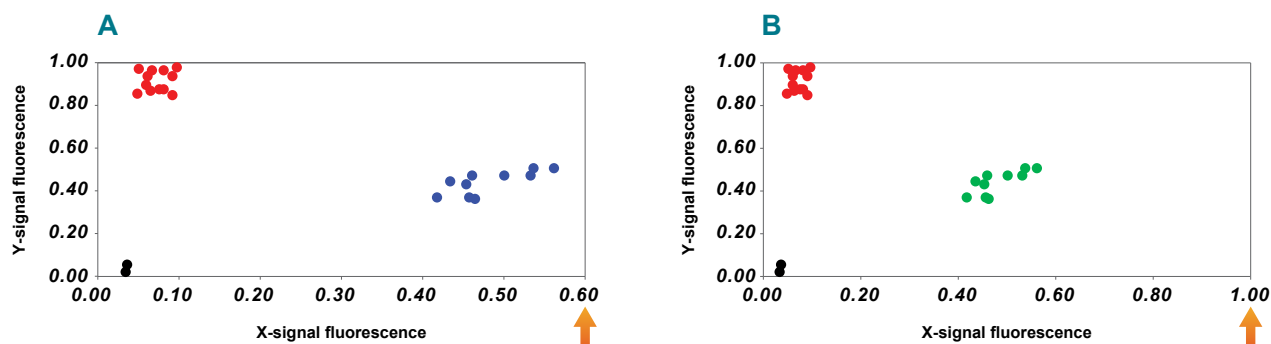


Figure 5.5. Scaling of X and Y axes is not comparable (polymorphic results).

Plot A shows a cluster plot with two clear genotyping clusters; these have been assigned as homozygous for allele X (blue data points) and homozygous for allele Y (red data points). In plot A, the X and Y axes are not scaled comparably; X has a maximum of 0.60 whilst Y has a maximum of 1.00. Plot B shows the same data as plot A, but with the X axis scaled correctly to match the Y axis. It is then clear that the blue cluster actually represents samples with a heterozygous genotype (green data points).

The scaling of the axes can be a particular issue if all of the data points are the same genotype e.g. monomorphic for the x-signal. In this scenario, only x-allele signal will have been generated from each sample and hence the x-allele axis may be scaled to have a much higher maximum than the y-allele axis. This can result in monomorphic results appearing as very scattered across the plot (figure 5.6.A) – rescaling the axes can improve data visualisation and hence the ability to confidently assign a monomorphic genotype (figure 5.6.B).

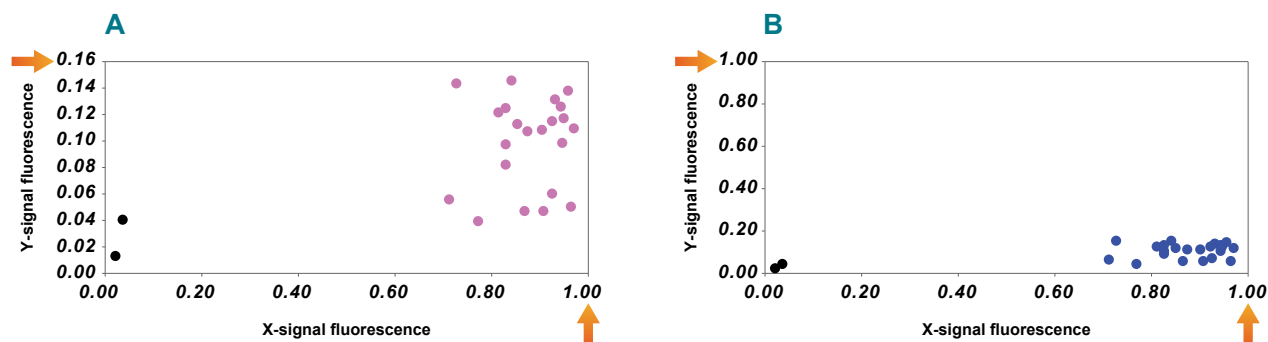


Figure 5.6. Scaling of x- and y-axes is not comparable (monomorphic results).

Plot A shows a cluster plot where the data points do not appear to be forming into clusters (pink, no genotype assigned). The orange arrows on plot A indicate the maximum fluorescence values for the x- and y-axes; in this case the x-axis has a maximum of 1.00 whilst the y-axis has much smaller maximum of 0.16. Plot B shows the same data as plot A, but with the y-axis re-scaled to have the same maximum fluorescence value as the x-axis (1.00, as indicated by the orange arrow). By re-scaling the y-axis to match the x-axis, the genotyping data points are positioned correctly and it is then clear that all of the data points belong to one cluster and represent samples with a homozygous genotype (blue cluster).

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5.7.3.3 Reliance on the software's in-built auto calling option

The software packages supplied with a number of widely available qPCR instruments typically have the ability to automatically assign genotypes to data points based on their position on the cluster plot. Please be aware that the algorithms used by these packages for data analysis will differ in robustness, and no software should be considered as 100% reliable. Reliance on the auto called genotypes with no manual review may result in misinterpretation of genotyping results.

Biosearch Technologies strongly recommends that genotyping data is always visually inspected and that results are sense-checked to ensure that they meet expectations. Ensuring that the X and Y axes on the cluster plot are scaled comparably can aid data interpretation. The inclusion of positive controls (samples of known genotype) on each reaction plate can help to provide confidence in results that are auto called by the software, and will ensure that there are always three clusters present on the cluster plot. Figures 5.7.A and 5.7.B demonstrate the effect of axes scaling, and figure 5.7.C shows the benefits of including positive controls on each reaction plate.

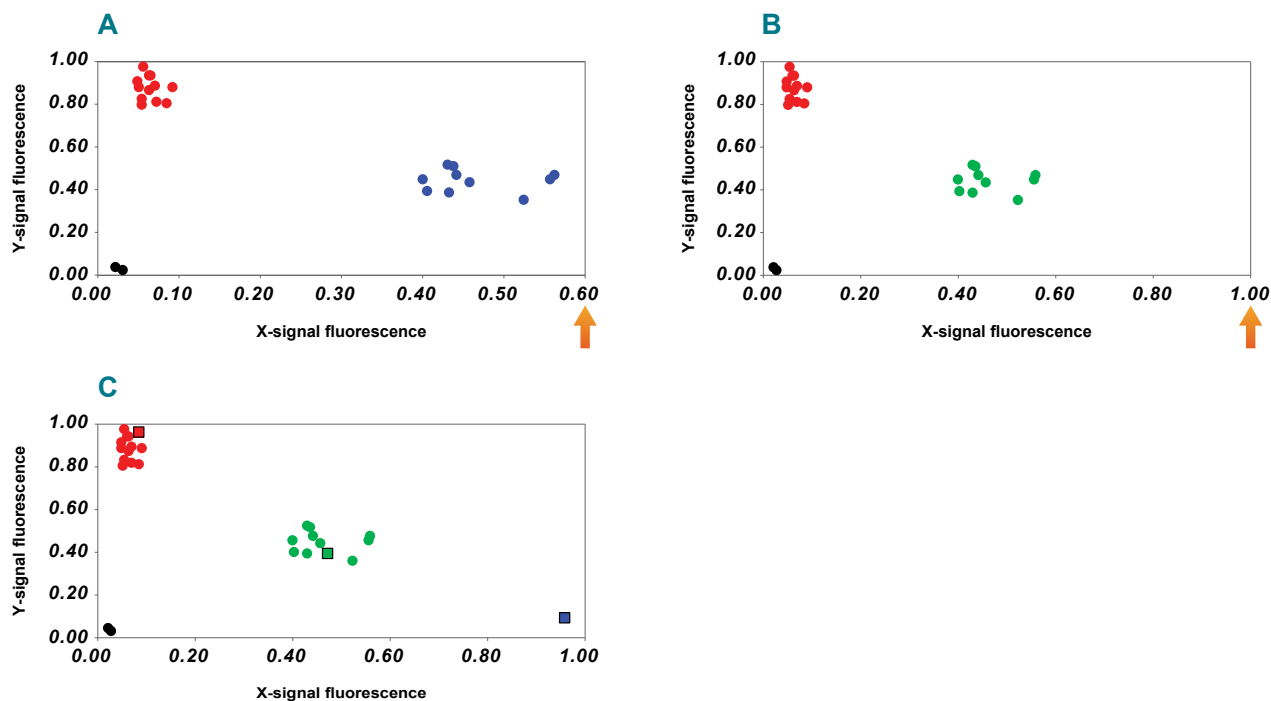


Figure 5.7. Reliance on the software's in-built auto calling option.

Plot A shows a cluster plot with two clear genotyping clusters that have been auto called using qPCR instrument software. The orange arrow on plot A indicates the maximum fluorescence value for the X axis, in this case 0.60. Plot B shows the same data as plot A, but with the x-axis re-scaled to have the same maximum fluorescence value as the x-axis (1.00, as indicated by the orange arrow). Rescaling the axes ensures that the genotyping clusters are positioned correctly – in this case, the blue cluster from plot A is now in the centre of the plot and has been manually assigned as a heterozygote cluster (green data points). Plot C shows the same data as plot A and plot B, but with three additional data points, indicated by square markers. These represent a positive control sample for each genotype. Inclusion of these positive controls ensures that the three possible genotypes are represented and can hence improve the accuracy of auto calling features that are in-built in qPCR instrument software.

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5.7.3.4 Combining data from multiple reaction plates on the same plot

Biosearch Technologies does not recommend combining data from multiple reaction plates. This is because the fluorescence generated, and the resultant scaling of the cluster plots, will differ from plate to plate. Viewing combined data on one plot is likely to make the genotyping clusters less defined, and will affect interpretation of the results.

Figure 5.8. shows data for the same end-point assay run on two separate reaction plates; viewing each set of data independently (figures 5.8.A and 5.8.B) gives three tight genotyping clusters. When the data are plotted together on the same plot, however, the clusters become difficult to interpret (figure 5.8.C).

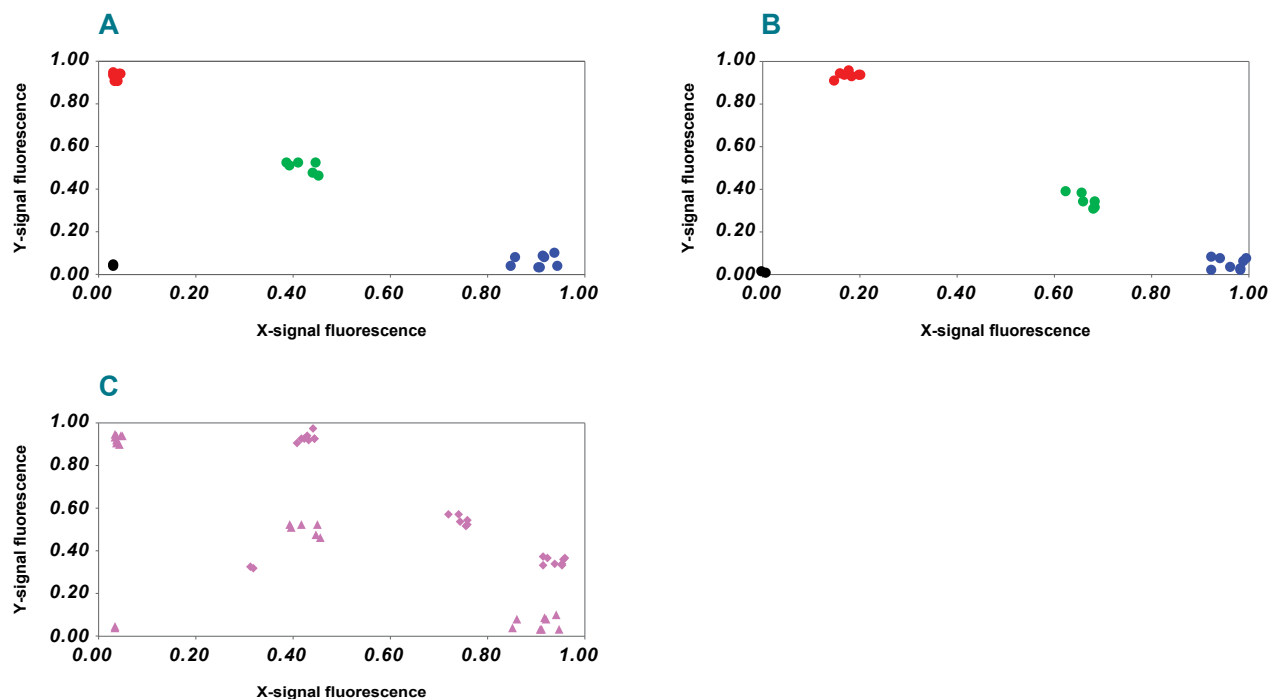


Figure 5.8. Combining data from multiple reaction plates on the same plot.

Plot A shows the data for an individual end-point assay, generated on reaction plate A. Plot B shows the data for the same end-point assay, generated on reaction plate B. In each case, the cluster plot axes have been scaled appropriately for the signal values generated on each reaction plate and, on both plots, clear clusters are visible and can be assigned genotyping calls. Plot C illustrates the effect of plotting data from two different reaction plates on the same cluster plot; the axes have been scaled based on all the fluorescent signal values generated across the two reaction plates (Triangular data points are derived from Plot A, square data points are derived from Plot B). Variations in the fluorescence generated between plates have resulted in multiple clusters being present on the plot. Consequently, it is no longer possible to confidently assign genotyping calls.

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5.7.3.4 Combining data from multiple end-point assays on one plot

Biosearch Technologies does not recommend combining data from different end-point assays on the same cluster plot. Each individual end-point assay will amplify template DNA at a slightly different rate, and will subsequently generate differing levels of fluorescent signal. Plotting data from more than one assay on the same plot does not provide any meaningful comparison, and will result in the data being very difficult to interpret (figure 5.9.).

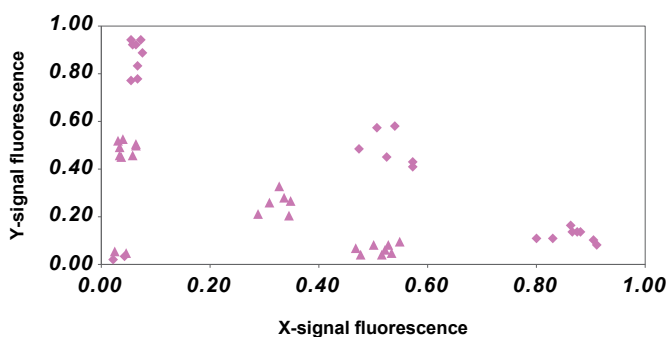


Figure 5.9. Combining data from two end-point assays on one plot.

This cluster plot contains data points from two different end-point assays. One assay has generated greater fluorescent signal values than the other assay and, as a result, there are multiple clusters present on the plot. Consequently, data interpretation and assigning of genotyping calls is much more difficult.

Figure 5.10. illustrates the effect of viewing data from multiple assays on the same cluster plot; both data quality and ease of interpretation are drastically affected. In figure 9A, the pink smear of data points represents data from four different end-point assays and the ability to confidently assign genotyping calls is reduced. Figures 5.10.B-9E identify the data points for each of the individual assays and, by viewing this data individually, the three genotyping clusters are much more apparent. All four assays have generated good genotyping data, with three clear clusters, and if the data points for each assay were viewed on an individual plot then it would be straightforward to confidently assign genotyping calls.

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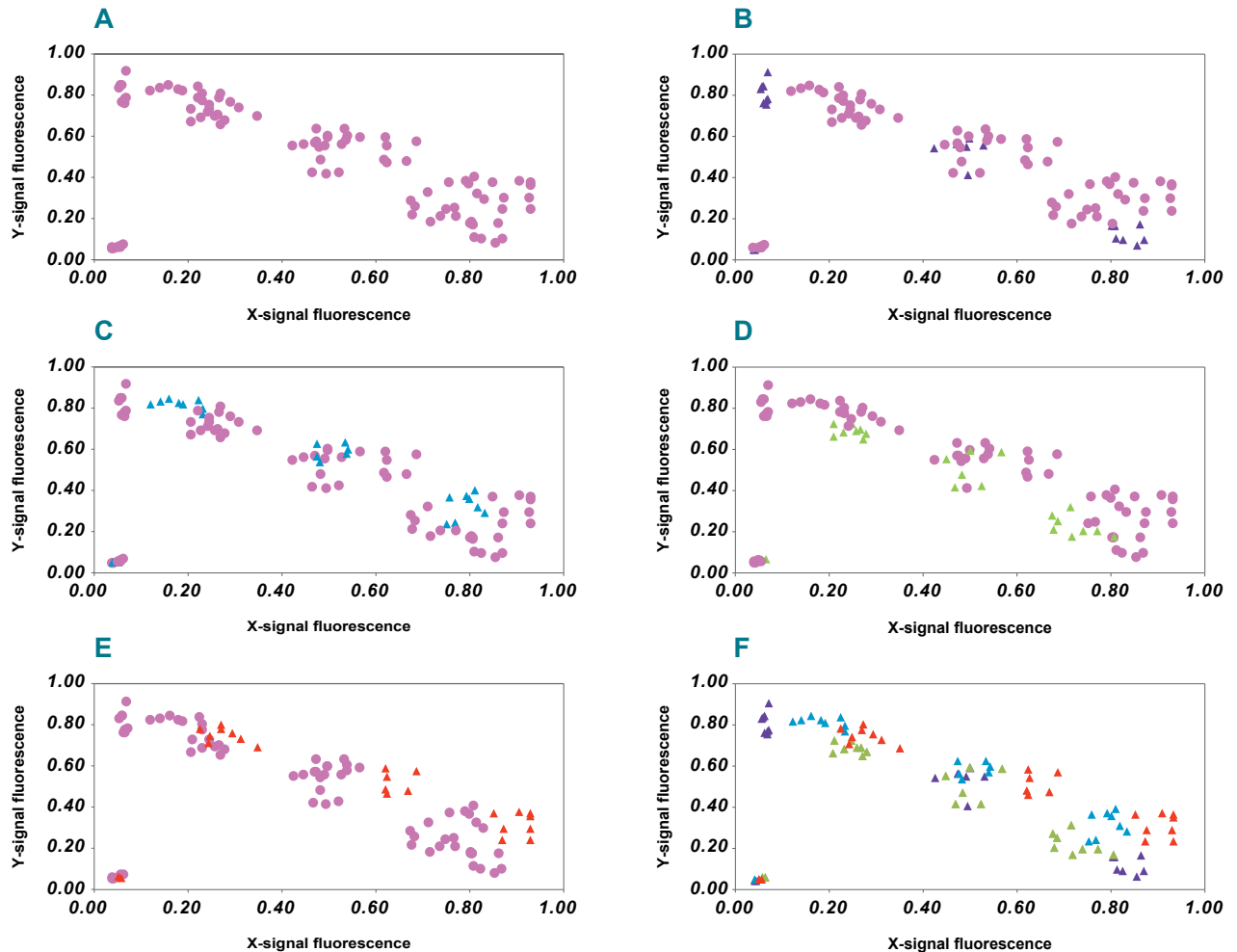


Figure 5.10. Combining data from multiple end-point assays on one plot.

Plot A shows the data for multiple end-point assays on a single cluster plot. The data points form a smear across the plot and consequently it is not possible to confidently assign genotyping calls. Plots B, C, D and E identify the data for an individual assay using a unique colour for the assay-specific data points (purple, blue, green and red respectively). In each case, three distinct clusters are clearly visible and it would be possible to confidently assign genotyping calls if these data points were viewed on their own cluster plot. Plot F shows the same data as plot A, but with the data points for each end-point assay identified by their unique colour.

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6. Troubleshooting (kit only)

This section is specifically aimed at customers who are using Biosearch Technologies products and protocols in their own laboratory for times when unexpected results are obtained.

6.1 Troubleshooting DNA isolation

Troubleshooting information for DNA extraction is split into sections, depending on the chemistry used.

6.1.1 QuickExtract DNA extraction

Plant species are notoriously diverse, which impacts sample preparation, DNA yield, and presence of debris and potential PCR inhibitors. It is important to empirically determine the best ratio of sample to QuickExtract DNA Extraction Solution to get the most DNA yet minimise the release of undesirable cellular components. A good starting point is to use one to three leaf punches (4 mm to 6 mm diameter), or 10-100 mg of seed chip material per 200 μ L of QuickExtract DNA Extraction Solution for processing. From this, determine which ratio results in the production of optimally amplifiable DNA, meaning reliable production of a strong expected PCR product with little background and minimal primer-dimer.

The most commonly reported issue with DNA extracted using QuickExtract is that it has not been possible to amplify using PCR. Typically we recommend using 5 μ L or less of the extracted DNA as template in for the PCR. If amplification not evident, try diluting the extracted DNA prior to using as template in the reaction. Test a dilution series (1 in 4, 1 in 8, 1 in 16 and 1 in 32) of the processed sample to determine the best balance between DNA concentration and PCR inhibitors. Although it may seem counterintuitive to use less starting material, dilution of the DNA will also result in dilution of any inhibitors that may be present, and consequently better results may be achieved.

6.1.2 sbeadex DNA purification

Table 6.1 outlines common problems that can be experienced when using a sbeadex kit to perform DNA purification; possible causes are detailed and corrective actions suggested.

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Problem	Possible cause	Corrective action
PCR inhibition	Incomplete buffer removal	Ensure all the buffer is removed before adding the next buffer. If necessary, adjust the liquid handling parameters for automated systems.
Low yield	Inefficient binding	Ensure that the lysate, binding buffer and sbadex particles are mixed thoroughly
	Too much DNA in sample causes slimy pellet	Re-elute the DNA from the beads using the same volume of elution buffer.
	Insufficient lysis	Increase the digestion time at the start of the protocol.
	DNA loss during third wash	The pH of the water used for the third wash must be pH≤7. A higher pH will lead to partial elution of the nucleic acid.
Coloured eluates PCR inhibition	Incomplete buffer removal	Ensure all the buffer is removed before adding the next buffer. If necessary, adjust the liquid handling parameters for automated systems.
	Heavily stained sample material	Incomplete lysis. Contact technical support for advice.
Particles present in eluates	Aspirating too fast	Reduce the speed at which supernatants are removed.
	Loose pellet	Increase contact time with the magnet to allow time for a tighter pellet to form.
	Disrupting pellet during aspiration	Position tip further away from pellet whilst removing supernatants.

Table 6.1. Troubleshooting common issues during sbadex DNA purification.

6.2 Troubleshooting KASP end-point genotyping

6.2.1 Troubleshooting the laboratory workflow

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 (6.2a-6.2d) below as a checklist to ensure that all aspects of your laboratory setup are correct.

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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 °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

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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 4.7 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 via our website .
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 program.

Table 6.2.d

Tables 6.2.a-d. Common causes of unexpected results for KASP end-point genotyping.

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6.2.2 Troubleshooting a specific KASP Assay

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, our assay-specific troubleshooting section should be used to determine the potential cause(s) and suggested solutions. Before using this section, we strongly recommend repeating your experiment using the same DNA and same KASP Assay Mix to eliminate the possibility of experimental error.

6.2.2.1 Insufficient amplification

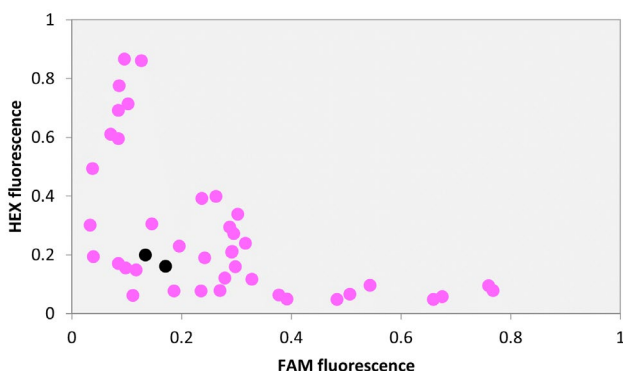


Figure 6.1. Genotyping data points move more slowly than expected away from the plot origin, and do not form distinct genotyping clusters.

Plot characteristics: genotyping clusters move more slowly than expected away from the origin. Data points do not resolve into separate clusters (figure 6.1).

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 4.7.12).

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. For high G/C assays, add DMSO to the reaction.

Cause 3 DNA concentration too low (slow to amplify).

Suggested solution: repeat the genotyping with DNA at the appropriate concentration (see Section 3.3). The reaction plate could also be thermally cycled further and re-read as this may address the issue (see Section 4.7.12)

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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 Master Mix contains 2.5 mM $MgCl_2$ and Biosearch Technologies do not recommend exceeding a final $MgCl_2$ concentration of 3.0 mM.

6.2.2.2 Troubleshooting a specific KASP Assay

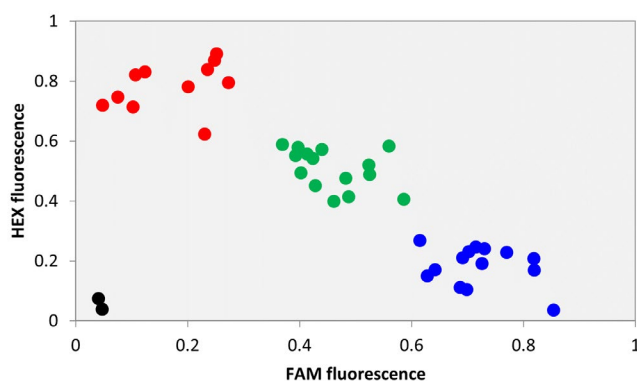


Figure 6.2. Genotyping clusters are not distinct or tight, and data points are scattered across the plot.

Plot characteristics: genotyping clusters are not distinct or tight, data points are scattered (figure 6.2).

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 4.7.12).

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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.

6.2.2.3 Scattered grouping of genotyping calls

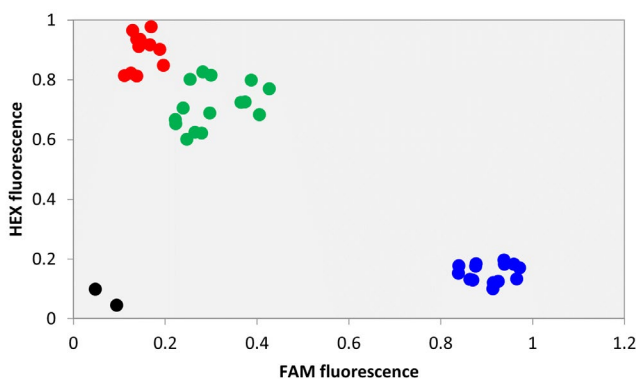


Figure 6.3. Three clusters are present on the plot, but the heterozygous cluster (green data points) has migrated towards a homozygous cluster (red data points).

Plot characteristics: the heterozygous group migrates towards one of the homozygous groups, making genotype scoring difficult (figure 6.3).

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:

- Design-only (KBD) – 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.
- Laboratory-validated (KOD) – contact technical support for assistance (techsupport@lgcgroup.com), and include screenshots of the data that you have obtained.

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6.2.2.4 Heterozygote group is too close to the origin

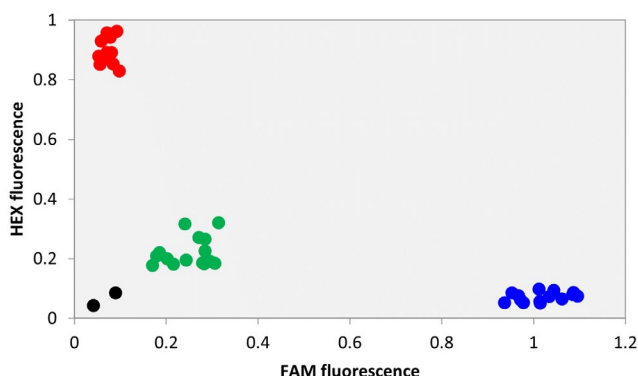


Figure 6.4. The two homozygous clusters (blue and red data points) appear to amplify correctly, but the heterozygous cluster amplifies less than expected and remains close to the plot origin.

Plot characteristics: the homozygous groups appear to amplify correctly but the heterozygous group amplifies less than expected, remaining close to the origin (figure 6.4).

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:

- Design-only (KBD) – 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.
- Laboratory-validated (KOD) – contact technical support for assistance (techsupport@lgcgroup.com), and include screenshots of the data that you have obtained.

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6.2.2.5 Too many genotyping groups

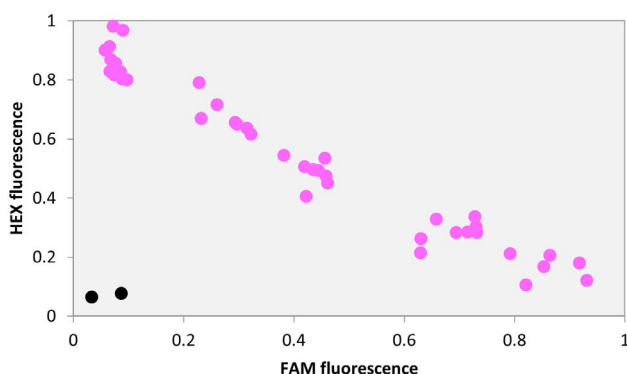


Figure 6.5. There appear to be more than three genotyping clusters visible on the plot.

Plot characteristics: more than three genotyping clusters are visible on the cluster plot (figure 6.5).

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:

- Design-only (KBD) – 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.
- Laboratory-validated (KOD) – 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.

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6.2.2.6 Fewer genotyping groups than expected

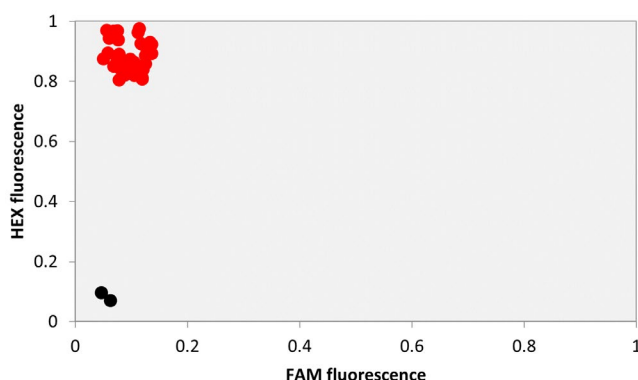


Figure 6.6. All data points cluster together in one position on the plot – in this case, samples are monomorphic for the allele detected by HEX.

Plot characteristics: monomorphic results on the genotyping plot (figure 6.6).

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.

- Design-only (KBD) – 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.
- Laboratory-validated (KOD) – 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.

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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.

6.2.2.7 Some samples do not amplify

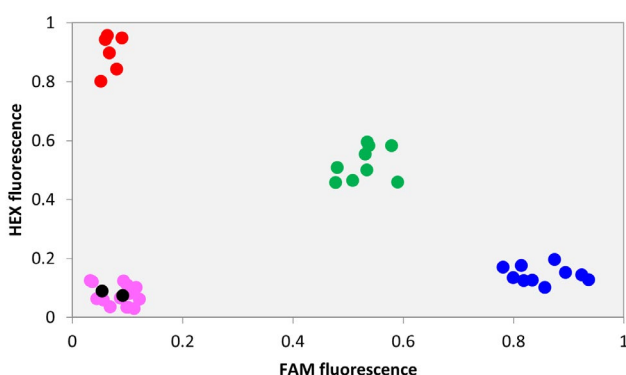


Figure 6.7. Some samples amplify and form genotyping clusters as expected (blue, green and red data points), whilst other samples fail to amplify (pink data points).

Plot characteristics: some samples amplify as expected whilst others do not amplify at all and remain clustered at the origin (figure 6.7).

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 Master Mix that was dispensed into the plate.

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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.

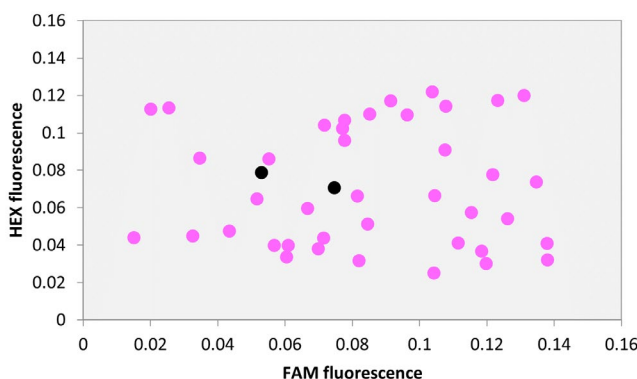


Figure 6.8.A. Data points are scattered across the plot and there is no apparent pattern to the genotyping results.

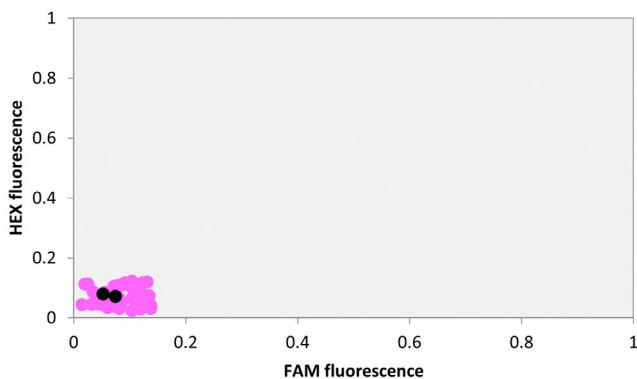


Figure 6.8.B. The x- and y-axes have been rescaled and all data points group with the NTCs, indicating that no amplification has occurred.

Plot characteristics: no pattern to the results, scattered data points (figure 6.8.A). Rescaling of the x- and y-axes can help to clarify if amplification has occurred or not (figure 6.8.B).

Cause 1: plate reader fault.

Suggested solution: investigate plate reader and re-read plate..

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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 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 3.3). The reaction plate could also be thermally cycled further and re-read as this may address the issue (see Section 4.4.12).

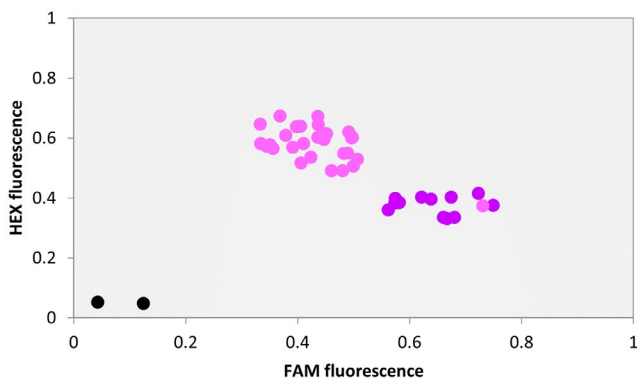


Figure 6.9A. Distinct genotyping clusters are apparent but their position on the plot is skewed.

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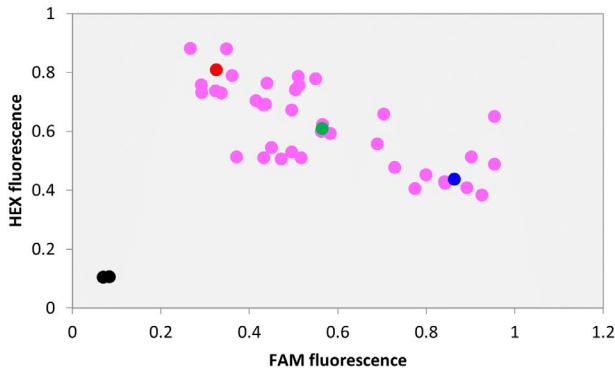


Figure 6.9B. It is difficult to discern clear genotyping groups, even when positive control samples for each genotype (blue, green and red data points) are included.

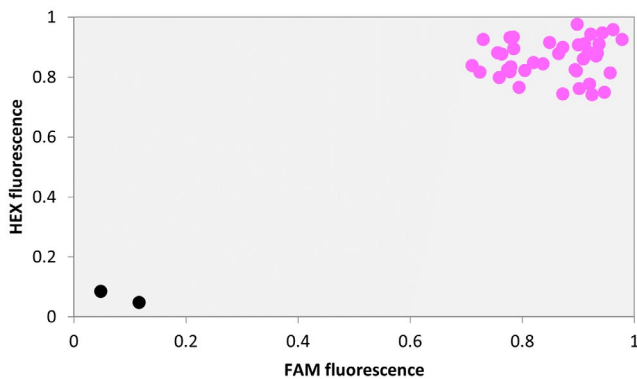


Figure 6.9C. All of the data points cluster in one position on the plot.

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 6.9A), it is difficult to discern clear genotyping groups (figure 6.9B), or all of the data points cluster in one position (figure 6.9C).

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.

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- Design-only (KBD) – 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.
- Laboratory-validated (KOD) - 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.

Appendices

Appendix A: Customer service contact information

To receive a prompt response, please contact the customer service team that corresponds to your region:

Region	Customer service email
North America, Latin America	Genomics.americas@lgcgroup.com
Europe, Middle East, and Africa	Genomics.emea@lgcgroup.com
Asia Pacific, excluding China	Genomics.apac@lgcgroup.com
China	Genomics.china@lgcgroup.com

Telephone numbers and postal addresses for our global locations can be found [here](#).

For information regarding country-specific distributors, please visit [here](#).

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