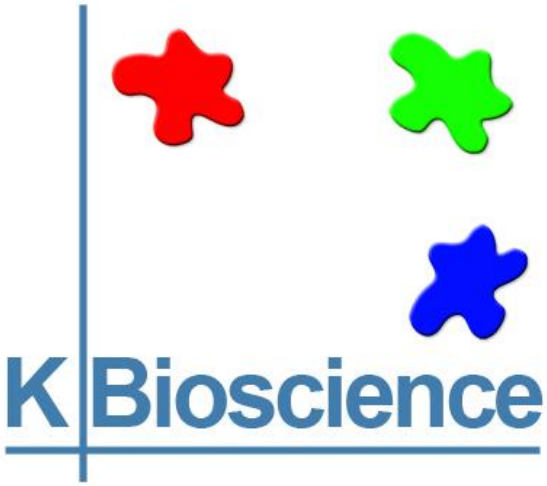


KASPar SNP Genotyping System

Reagent Manual

KBioscience

KASPar SNP Genotyping System

Introduction

The KBiosciences Competitive Allele-Specific PCR SNP genotyping system (KASPar) is a novel homogeneous fluorescent genotyping system. It has been developed at KBiosciences and is currently in use on a daily basis. KASPar offers the simplest, most cost-effective and flexible way to determine SNP genotypes. Analysis can be carried out in a variety of formats and the chemistry has been shown to function well in 96-, 384- & 1536-well plate formats. The system is comprised of two components, the Assay Mix (three unlabelled primers; this is the SNP-specific component of the system) and the Reaction Mix (all other components required including the universal fluorescent reporting system).

Sample Pack Contents

Reaction Mix (4x concentration, containing ROX as a passive reference)
$MgCl_2$ (50 mM; for A/T-rich primer designs)
DMSO (100%; for G/C-rich primer designs)
KTaq polymerase
KTaq dilution buffer
Pack leaflet

Store all KASPar kit reagents at -20°C.

Customer Requirements

1. FRET-capable plate reader[1]
2. Microtitre plate
3. ≥ 24 DNA samples @ ≥ 5ng/ l dissolved in Tris-HCl buffer (10mM; pH8.3) or PCR grade H_20
4. 10mM Tris-HCl pH8.3 or PCR grade H_20
5. Optical plate seal

The Genotyping Process

KBiosciences

[1] KASPar™ has been shown to work with a number of plate readers such as ABI7700, ABI7900, BMG Pherastar, Tecan Saffire & PerkinElmer Envision.

1. Assay design by PrimerPicker

Primer design for the KASPar chemistry is achieved using the PrimerPicker software (a free-of-charge online service and can be found at http://www.kbioscience.co.uk/primer-picker/ To use, simply navigate to this URL and paste in your SNP ID and corresponding DNA sequence (prepared according to Table 1).

SNP ID	DNA Sequence
MARKER_SLASH	ATAACTACTTTTAAAGGCACATTATTCAACCTCACTGTGCATTTCATCCTC[A/T]TGCATAATGACAGTTATTCTCTCCCAAGTCTCCTTCTGGCTCAACAGAGA
MARKER_IUPAC	ATAACTACTTTTAAAGGCACATTATTCAACCTCACTGTGCATTTCATCCTC[W]TGCATAATGACAGTTATTCTCTCCCAAGTCTCCTTCTGGCTCAACAGAGA
AVOID_BASES	ATAACTACTTTTAAAGGCACAMTATTCAACCTCACTGTGCATTTNATCCNN[W]TGCATAATGACAGTTATTCTCTCCCAAGTCTCCTTCTGGCTCAACAGAGA
SUSPICIOUS_BASES	ATAACTACTTTTAAAGGCACATTATTCAACCTCActgtgcatttcatcctC[W]TGCATAATGACAGTTATTCTCTCCCAAGTCTCCTTCTGGCTCAACAGAGA
ALSO_OK_1	ATAACTACTTTTAAAGGCACATTATSCAAACCTCAYYTGCATTTCATCCTC[W]TGCATAATGACAGTTRTTCTCTCCCAAGTCTCCWWCTGGCTCAACAGAGA
ALSO_OK_2	ATAACTACTTTTAAAGGCACATTATTCAACCTCACTGTGCATTTCATCCTCA/TTGCATAATGACAGTTATTCTCTCCCAAGTCTCCTTCTGGCTCAACAGAGA
NOT_OK	ATAACTACTTTTAAAGGCACATTATSCAAACCTCAYYTGCATTTCATCCTCWTGCATAATGACAGTTRTTCTCTCCCAAGTCTCCWWCTGGCTCAACAGAGA
BAD SNP/ID	ATAACTACTTTTAAAGGCACATTATTCAACCTCACTGTGCATTTCATCCTC[A/T]TGCATAATGACAGTTATTCTCTCCCAAGTCTCCTTCTGGCTCAACAGAGA

SNP ID	Explanation
MARKER_SLASH	The slash character "/" is used to mark the SNP
MARKER_IUPAC	A IUPAC character "W" is used to mark the SNP
AVOID_BASES	The N characters are used to completely avoid bases.
SUSPICIOUS_BASES	The lower case characters "ctgtgcatttcatcct" are used bias the primer design algorithm to favour the uppercase bases.
ALSO_OK_1	Although other SNPs are marked, only the SNP in square brackets will be targeted.
ALSO_OK_2	Although SNP is not in brackets, it is the only one and will be targeted.
NOT_OK	Even though the W SNP in the middle is coloured blue, the design algorithm does not know which SNP to design to. This sequence will be rejected.
BAD SNP/ID	The sequence is OK but the ID has spaces and a slash character. Non-alphanumeric letters will be removed from the ID.

IUPAC Code	Meaning
A	A
C	C
G	G
T/U	T
M	A/C
R	A/G
W	A/T
S	C/G
Y	C/T
K	G/T

Table 1. Guidelines for preparation of SNP-containing DNA sequence for entry into PrimerPicker.

2. Sample Arraying

DNA samples may be arrayed in any microtitre PCR plate; typically 96- or 384-well plates are used. The recommended amounts of DNA to use are: 4μl of DNA at ≥ 5ng/μl for 96-well and 2μl of DNA at ≥ 5ng/μl for 384-well. Genotyping should be carried out on at least 24 samples to ensure there are sufficient genotypes to show clustering. It is also strongly recommended that at least one water sample be included per 96-well plate to act as a negative control.

After arraying, samples can be dried on the 96- or 384-well plates. Where this is the case, the reaction mix must be diluted to 1x (rather than 2x for non-dried samples) to compensate for the absence of liquid in the well. Drying the samples in the plate is often useful when performing large-scale genotyping, as it allows many plates to be prepared in advance, without the concern of sample evaporation altering the reagent concentrations.

3. *Assay Mix*

The KASPar SNP genotyping chemistry allows the user to design and develop genotyping assays. Assays are comprised of three unlabelled oligonucleotides (see section 1), combined in certain proportions (Table 2). The three constituent primers are stored together in one SNP-specific ***Assay Mix*** for ease of use. The ***Assay Mix*** is then combined with the ***Reaction Mix*** (see section 4) and added to the DNA samples to be genotyped.

	Concentration in Assay Mix (M)	Volume in Assay Mix (l)
Allele Specific Primer 1 (100 M)	12	12
Allele Specific Primer 2 (100 M)	12	12
Common (reverse) Primer (100 M)	30	30
H_20 / Tris-HCl (10mM, pH8.3)	-	46
TOTAL		**100**

Table 2. Preparation of an *Assay Mix* from the constituent allele-specific and common primers.

100μl of ***Assay Mix*** is sufficient to carry out at least 650 genotypes in 96-well format or at least 1300 genotypes in 384-well format (based on 8ul and 4 l reaction volumes, respectively with plate type). ***Assay Mix*** can be safely stored at 4°C for 1-2 weeks, approximately 1 year at -20°C or indefinitely at -80°C.

4. *Reaction Mix* (4x)

KBiosciences recommends carrying out SNP genotyping using total reaction volumes of 4μl for 384-well or 8μl for 96-well genotyping (see Table 3; these total volumes can be reduced but the overall data quality may be reduced). Volumes must be scaled-up depending on the number of reactions required. The optimal $MgCl_2$ concentration in the reaction has been found to be 2.2mM, however the ***Reaction Mix*** is supplied at 1.8mM as some assays must be run at this concentration (see troubleshooting guide), therefore for most assays, $MgCl_2$ must be added to a final concentration of 2.2mM before use (i.e. an increase of 0.4mM, to increase the concentration from 1.8mM to 2.2mM).

	96-well (l)	384-well (l)	Minimum vol (l)
DNA (5ng/ml)	4	2	500
4X Reaction Mix	2	1	250
Assay Mix	0.11	0.055	13.75
KTaq Polymerase	0.026	0.013	3.25
$MgCl_2$ (50mM)	0.064	0.032	8
H_20	1.8	0.9	225
TOTAL	**8**	**4**	**1000**

Table 3. Constituent reagent volumes for 96- and 384-well genotyping using KASPar.

Nb. KBiosciences recommends a minimum total of 1000ul. This may be a greater volume than is required for the genotyping experiment but the mix can be stored at 4°C overnight or at –20°C for up to 6 months.

5. Dispensing *Assay Mix* / *Reaction Mix* Over Samples

The combined ***Assay Mix*** and ***Reaction Mix*** can now be dispensed over DNA samples. This can be achieved manually with any pipette or robotically, depending on plate type / sample number. KBiosciences is happy to advise on liquid dispensing systems.

6. Plate Sealing

Plates can be sealed with any optically clear seal. KBioscience recommends use of the Fusion Laser welding system (see Related Products) but any optically clear seal is adequate.

7. Thermo-cycling conditions

Optimal results are generally obtained using the 2-step cycling program detailed below. Cycling conditions can be adapted as required (see Assay Troubleshooting).

94°C for 15 minutes **Hot-start Activation**

94°C for 10 seconds
57°C for 5 seconds } **20 cycles**
72°C for 10 seconds

94°C for 10 seconds
57°C for 20 seconds } **18 cycles**
72°C for 40 seconds

PCR cycling can be performed on any PCR thermal cycler. Similar results have been obtained on MJ Peltier thermal cyclers and KBiosystems “Duncan” water bath cycler (see Related Products section).

8. Plate Reading

KASPar data can be obtained from any FRET-capable plate reader with the relevant filters. KASPar uses the fluors FAM and VIC for distinguishing between genotypes and ROX as a passive reference (Figure 1). The excitation and emission wavelengths for these fluors are shown in Table 4.

Table 4. Excitation and Emission values for the fluors used in KASPar

	Excitation (nm)	Emission (nm)
FAM	485	520
VIC	520	570
ROX	575	610

If required, markers can be made with most fluorescent dyes to customer specification.

9. Plotting of Data.

The FAM and VIC data are plotted on the *x*- and *y*- axes, respectively. Inclusion of a passive reference dye (ROX) allows data to be normalised by dividing FAM and VIC values by the passive reference value for that particular well, thus removing the variable of liquid volume. Genotypes can then be determined according to sample clusters (Figure 1). The inclusion of a passive reference leads to tighter clustering and, as a result, more accurate calling of data.

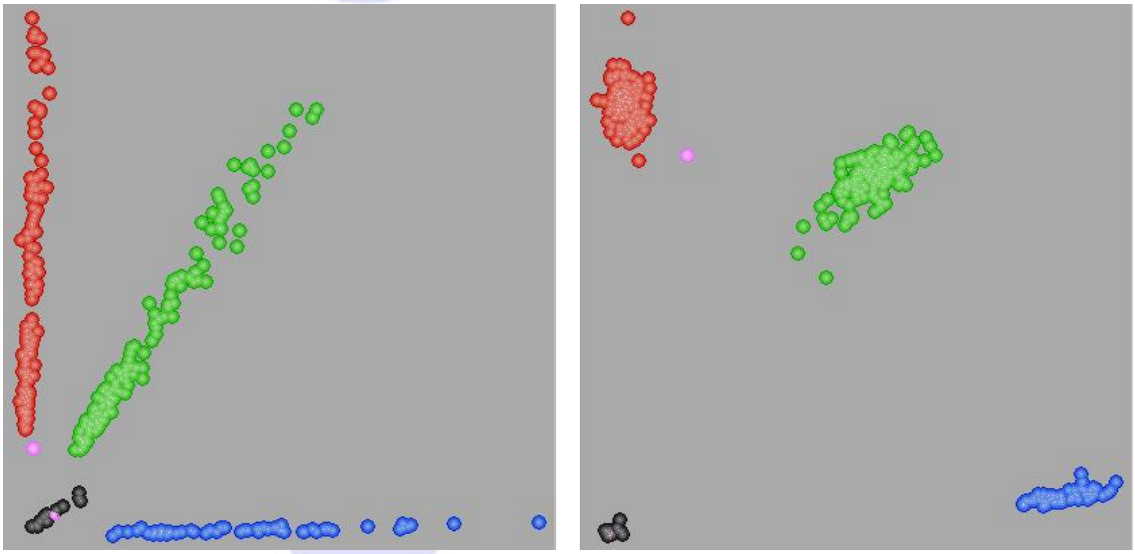


Figure 1. Genotyping data plotted using KBiosciences Klustercaller software. The same data can be viewed without normalisation with ROX (A) or with normalisation (B).

From Figure 1: genotyped samples marked red are homozygous for the VIC allele, those marked blue are homozygous for the FAM allele; those marked green are heterozygous. Refer back to PrimerPicker to convert fluor calls to allele calls.

TROUBLESHOOTING / OPTIMISING

The majority of assays will function under the standard conditions described above, however occasionally assay optimisation may be required. The sections below (A-G) describe problems that can occur, their possible causes the solutions.

A. LITTLE OR NO AMPLIFICATION OF GENOTYPING CLUSTERS

Poor amplification is characterised by groups moving more slowly than expected away from the origin, before resolving into separate clusters.

A.1 PCR cycle number

If the signature genotyping groups have not yet formed (i.e. amplification is incomplete), it is advisable to cycle the samples further and re-read on the fluorescent plate reader (see Figure 2). The recommended cycling protocol is that of the last 18 cycles in section 7 i.e. 94°C for 10 seconds; 57°C for 20 seconds and 72°C for 40 seconds.

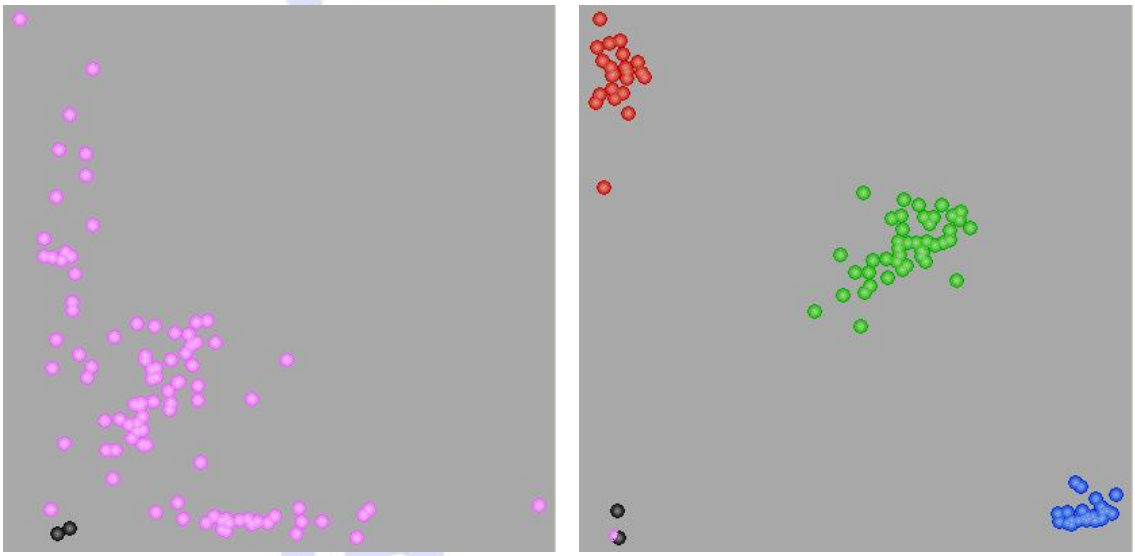


Figure 2. Example of an assay after insufficient number of PCR cycles (A) and with three extra PCR cycles (B).

A.2 G/C percentage of the primers in the assay

A.2.1 Low G/C (G/C <30%)

Where the assay oligos are of low G/C percentage (G/C <30%) poor amplification can occur. Increasing the $MgCl_2$ concentration in the reaction mix to 2.5mM or even 2.8mM can compensate for this. From Table 2 it can be seen that 0.064ml and 0.032 l MgCl2 (50mM) are added to the reaction mix to bring the MgCl2 concentration to 2.2mM in 8 and 4ml reactions respectively. For 2.5mM mixes, these figures are 0.112 l and 0.056 l, respectively. Similarly for 2.8mM mix, the volumes are 0.16 l and 0.08 l, respectively.

A.2.2 High G/C (G/C >70%)

High G/C percentage (G/C >70%) in the assay oligos can also cause poor amplification. Where problematic high G/C percentage assays are encountered, either running the assay at a lower $MgCl_2$ concentration (1.8mM), or adding 5-10% DMSO to the final volume of the assay will usually provide a solution. When adding DMSO, there is no need for a concomitant reduction in water volume to compensate as the chemistry is sufficiently robust to allow for this.

A.3 DNA concentration

Both low and high DNA concentrations can cause problems. However, for best results the DNA concentration should be between 5-40ng/ l. It is recommended that the Picogreen dsDNA fluorescent quantification system be used in preference to spectrophotometric methods, as the latter tend to overestimate concentration in this range.

A.3.1 Low DNA concentration

DNA concentration may be lower than expected causing samples to take longer to amplify. This can sometimes be addressed by simply cycling the samples further (see section A.1), though too many extra cycles may result in amplification of the negative control samples. It is preferable to repeat the genotyping with DNA in the recommended concentration range (5-40ng/ l).

A.3.2 High DNA concentration

DNA concentration may be higher than expected, causing samples to amplify more quickly than expected. To resolve this, dilute the DNA samples to the recommended 5ng/ul (as determined by Picogreen). Conversely, samples of very high DNA concentration can also cause poor / no amplification. Where this is the case, it is usually because there is also a high concentration of a PCR-inhibiting contaminants present (which would normally have been diluted down to non-problematic levels).

A.4 DNA samples dissolved in a buffer that contains EDTA

After purification, DNA samples are often eluted in / diluted in TE buffer, which contains EDTA. EDTA chelates Mg^{2+} ions leading to insufficient Mg^{2+} for the reaction to proceed. However this can easily be overcome by increasing the $MgCl_2$ to compensate. For example, if the DNA samples contain 1mM EDTA, in a 4 l reaction, where the DNA samples account for 50% of the reaction volume, addition of extra Mg^{2+} to the amount of 0.5mM will resolve the issue.

A.5 *Assay Mix* storage conditions

When preparing an ***Assay Mix*** (see section 3), it is advisable to divide it into aliquots to avoid multiple freeze/thaw cycles. Aliquots of ***Assay Mix*** can be safely stored at 4°C for 1-2 weeks, approximately 1 year at -20°C or indefinitely at -80°C. Storage beyond these limits, or multiple free/thaw cycles will increasingly affect the performance of the assay (see Figure 3).

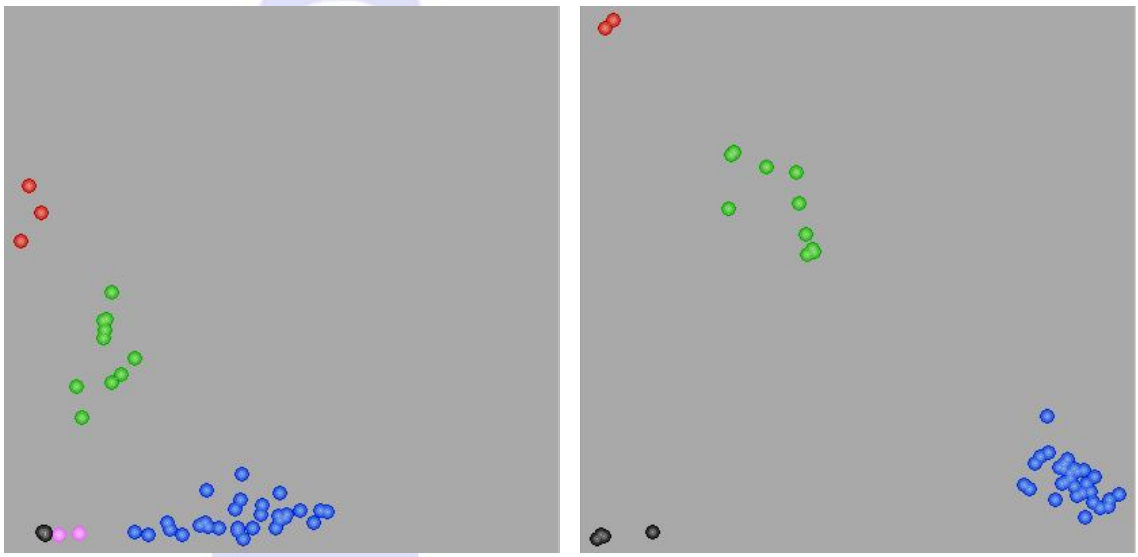


Figure 3. ***Assay Mixes*** **that have been subjected to multiple freeze/thaw cycles or otherwise stored incorrectly can lead to poor amplification in the subsequent reaction (A). Where a fresh aliquot of the same assay is used, results were much improved (B).**

B. AMPLIFICATION TOO FAST

If the reaction is coming to completion too quickly, it may be an indication of too much DNA in the reaction. To remedy this, either reduce the amount of DNA in the reaction or reduce the number of PCR cycles in the second phase from 18 to 15 (see section 7), such that the total number of cycles is now 35.

C. SCATTERED GROUPING OF GENOTYPING CALLS

This is generally caused by non-specific amplification in the PCR and can be remedied by increasing the annealing temperature in the thermal cycling protocol from 57°C to 63°C. In extreme cases, a touch-down step can be added to the protocol, as shown below.

94°C for 15 minutes	**Hot-start Activation**
94°C for 10 seconds 68-63°C for 5 seconds 72°C for 10 seconds	**6 cycles (annealing step dropping 1°C per cycle)**
94°C for 10 seconds 63°C for 5 seconds 72°C for 10 seconds	**14 cycles**
94°C for 10 seconds 63°C for 20 seconds 72°C for 40 seconds	**18 cycles**

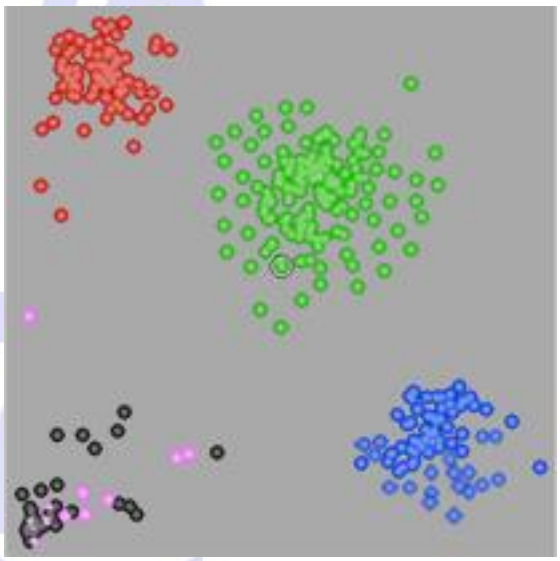


Figure 4. Scattered grouping caused by poor specificity (A). Hot-start cycling conditions lead to greater specificity and better clustering of the genotypes.

D. LITTLE / NO SEPARATION OF THE HETERO- AND A HOMOZYGOTE GROUP

An imbalance in the allele-specific primers can result in the heterozygous group migrating towards one of the homozygote groups, making genotype calling difficult.

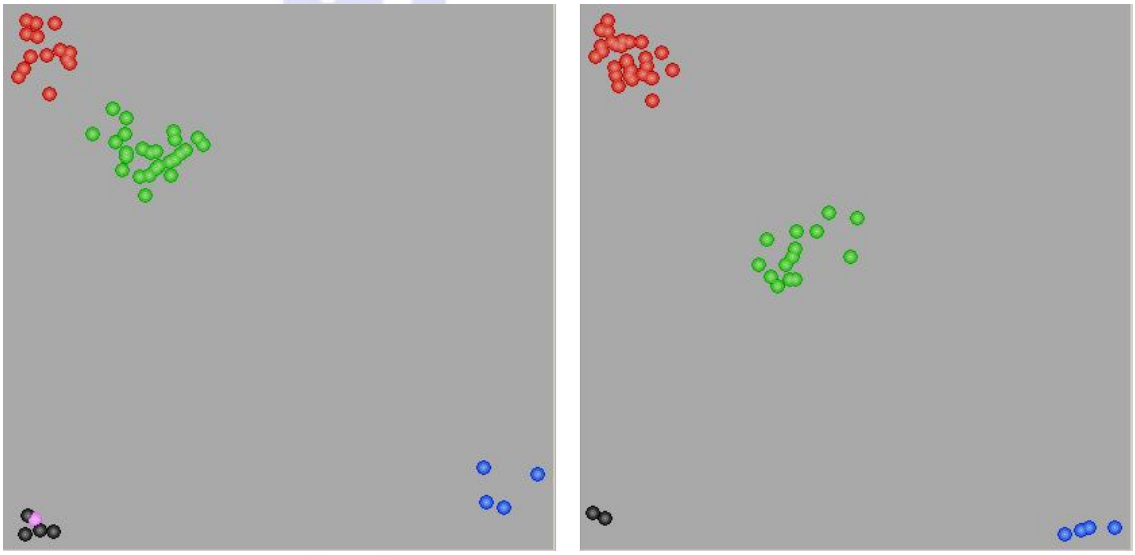


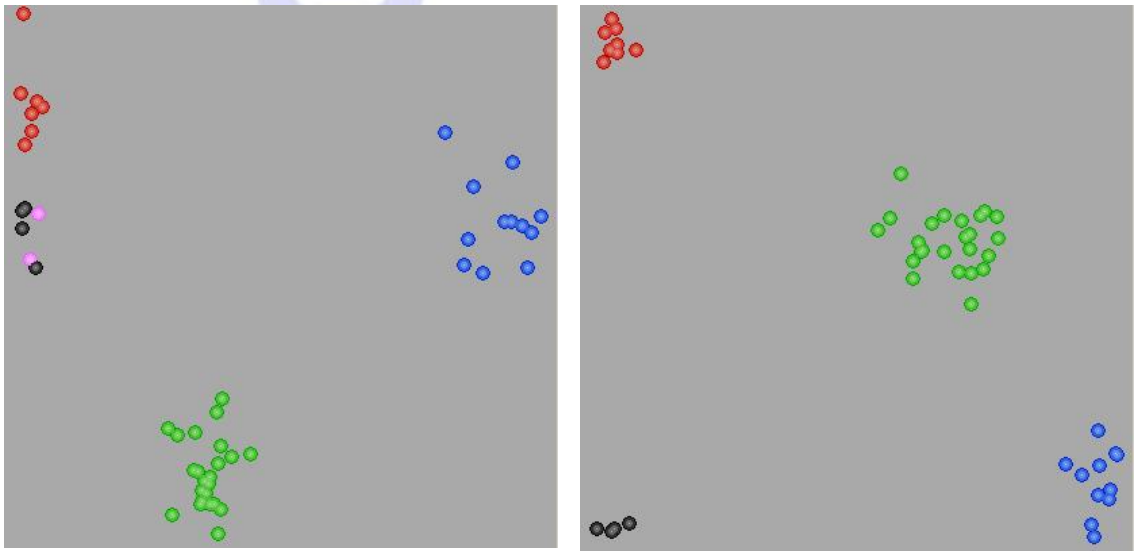
Figure 5. An imbalance in allele-specific primers leading to the heterozygous group being positioned near the red homozygous group (A). Additional allele-specific primer for allele 1 (FAM) causes the position of the group to migrate to the centre (B), allowing more reliable genotype scoring.

Such an imbalance can occur for a variety of reasons: poor synthesis of one of the allele-specific primers; accidental addition of a low quantity of the allele-specific primer when making the assay; or more efficient amplification of one allele-specific primers over the other. Regardless of cause, the problem can be ameliorated by adding more of the alternate allele-specific primer (see Figure 5).

E. GENOTYPING GROUPS AMPLIFYING IN AN UNEXPECTED PATTERN

E.1 Heterozygous group migrating to a much lower position (with respect to the homozygotes) on the *x*- or *y*-axis than expected

Figure 6 is an example of the effect that occurs when the assay aliquots are thawed without subsequent mixing (or not mixed before they are aliquoted) causing the forward primers to saturate the fluorescence quenching system. This effect is characterised by the genotyping clusters seemingly adopting incorrect



positions on the plot (despite often clustering well into groups).

Figure 6. Genotyping groups positioned in a unexpected way (A); typically the blue homozygous groups is further up the *y*-axis with respect to the heterozygotes. This is remedied by mixing the assay aliquot before dispensing (B).

Whilst mixing the assay aliquots after thawing and before use is strongly recommended for the reasons described in Figure… it is worth noting that the problem could also have occurred when making the assay if the constituent primers were hydrated and then freeze/thawed without proper mixing.

E.2 Heterozygous cluster too close to the origin

A related problem to that described in E.1 is seen when the homozygous clusters positions appear to amplify correctly but the heterozygous group amplifies less than might be expected, remaining close to the origin (Figure 7). This is caused by the forward primers saturating the quenching system and can be resolved by remaking the ***Assay Mix*** using a lower concentration of forward primers (instead of 12 M try 8 M).

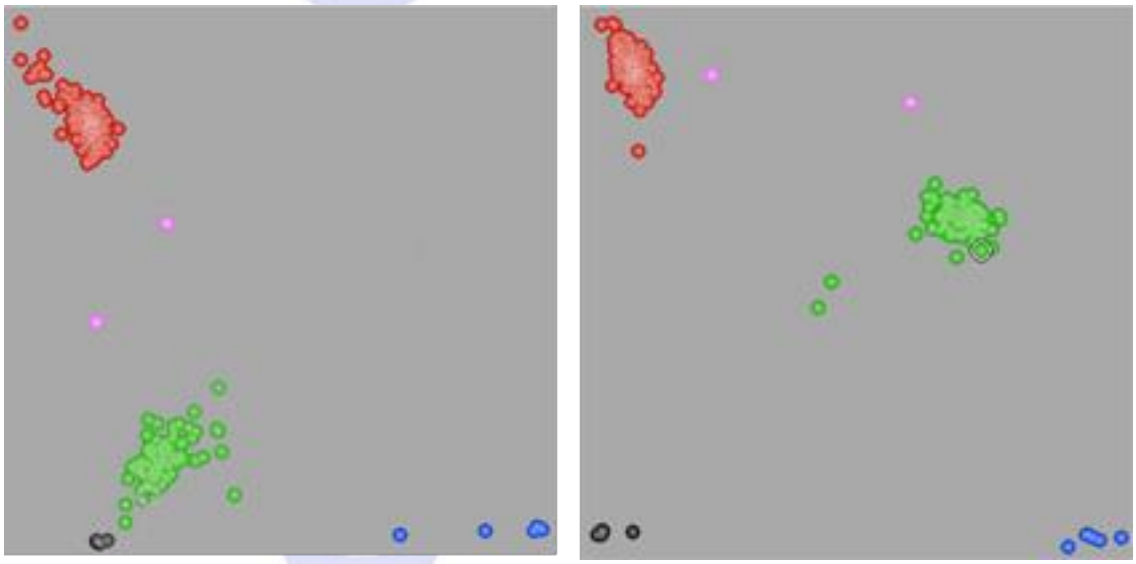


Figure 7. Heterozygous clusters too close to the origin (A).
Genotyping carried out again with the *Assay Mix* correctly mixed after thawing (B).

E.3 Too many genotyping groups

When primers (forward or reverse) are designed such that they encompass a region containing other polymorphism(s), more than three clusters can sometimes be resolved in the resulting genotyping plot. The non-target polymorphism causes differential PCR amplification efficiencies, leading to a situation where it is not possible to confidently determine which clusters represent heterozygotes and which are homozygotes.

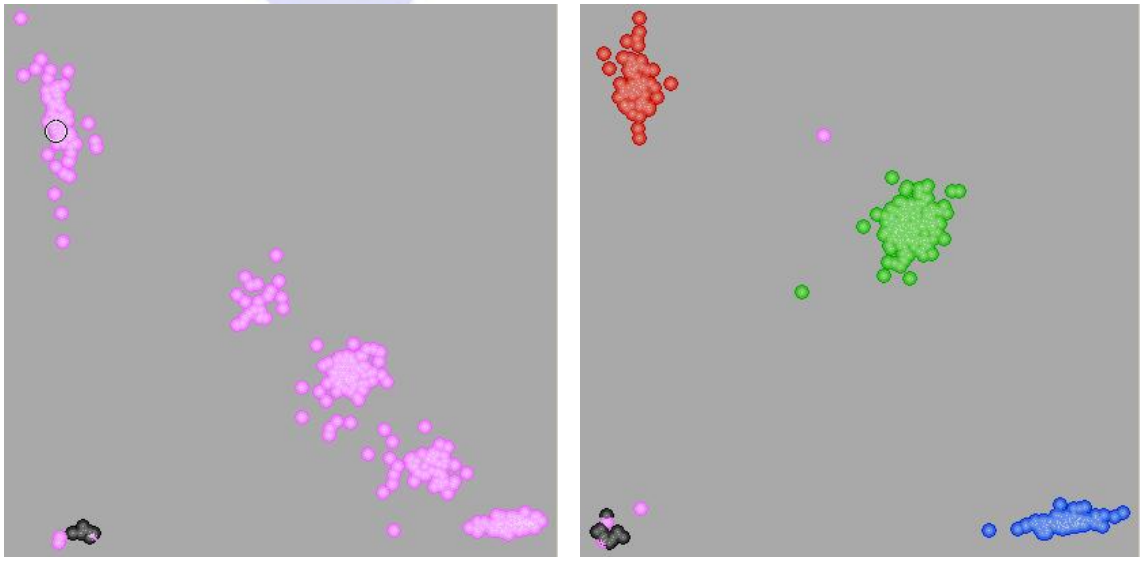


Figure 8. Multiple genotyping clusters caused by the presence of polymorphism(s) within primer binding site(s); in A, the reverse primer was designed in a region containing polymorphism. B represents the same assay with the reverse primer re-located to a non-polymorphic region.

The multiple genotyping clusters shown in Figure 8 (A) can be resolved by re-locating the primer(s) to a region containing no polymorphisms, if one is available.

E.4. Fewer groups than expected

Some assays will report just one genotyping cluster (monomorphic genotyping). This can be a genuine result if the population being analysed contains only one genotype with respect to the (low frequency) SNP being studied. However, monomorphic data can also occur because the SNP is not real, or because one of the allele-specific primers is not functioning (due to, for example, a failed oligo synthesis).

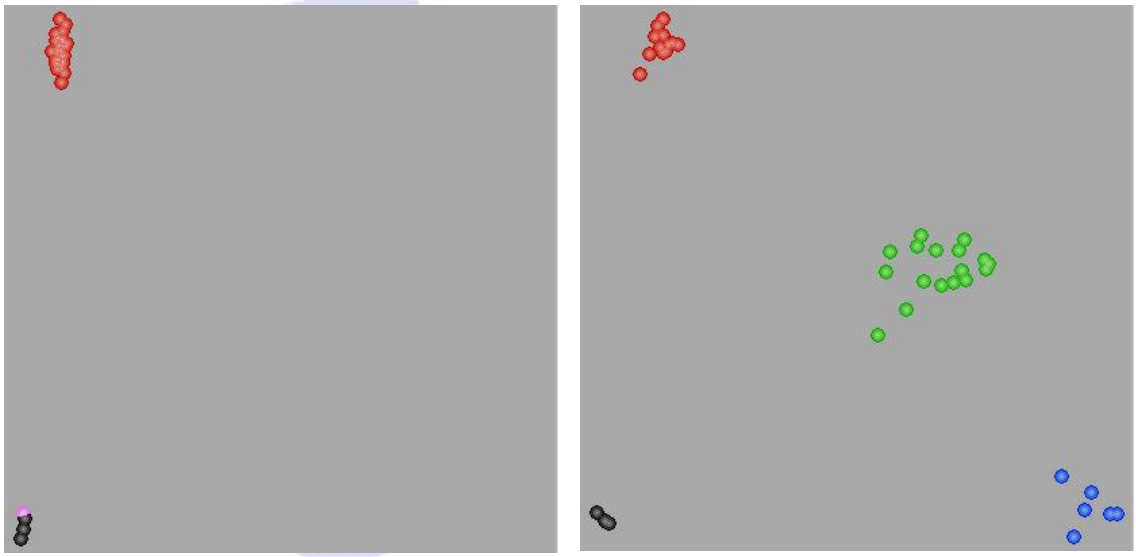


Figure 9. Monomorphic data (A). Results from the same assay re-designed (B).

F. MIXTURE OF GOOD AND BAD AMPLIFICATION OF SAMPLES ON THE SAME PCR PLATE

Sometimes, the samples can amplify correctly and cluster tightly, but some samples will not amplify well / at all, clustering instead around the origin. The problem can occur because the DNA quality of some samples is poor (see section A.3.1). Other possibilities are poor DNA arraying (such that there is little / no DNA in some wells supposed to contain it, Figure 10) or poor dispensing of reaction mix.

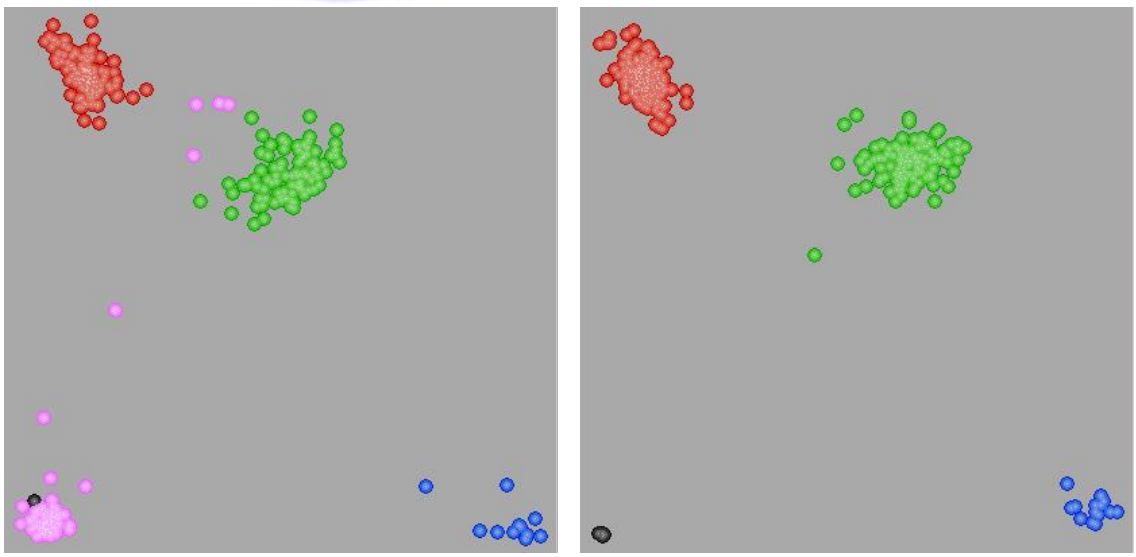


Figure 10. Little or no amplification of SOME samples whilst others have amplified well (A) can occur for a variety of reasons. In this case, the problem was poor DNA arraying resolved by correct DNA arraying (B).

Additionally, the good and bad amplification on the same plate can be caused by incomplete sealing of the plate (Figure 11).

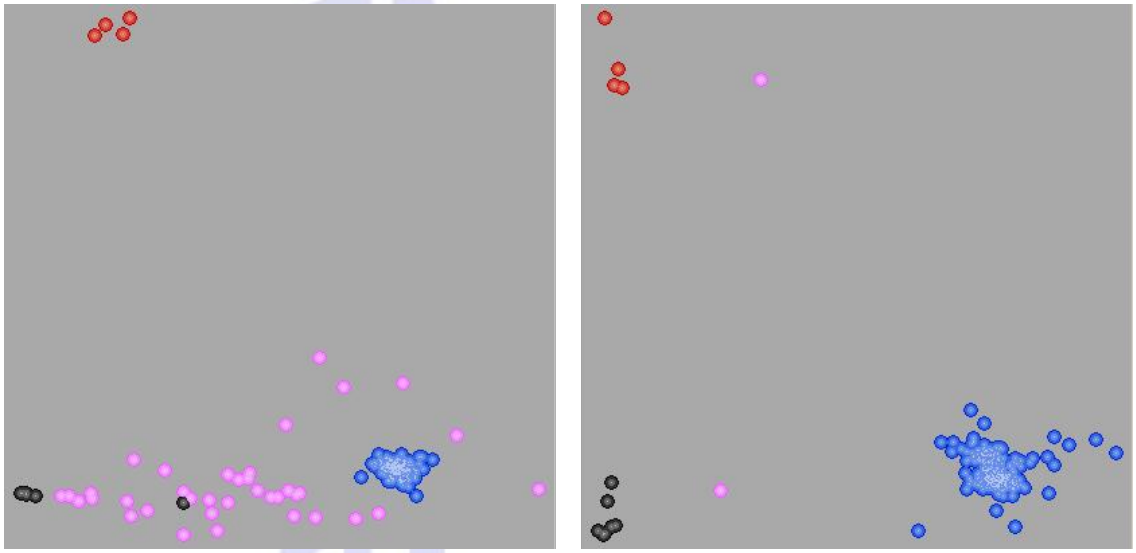


Figure 11. Poor sealing of the wells of a 384-well PCR plate prior to thermocyling in a waterbath-based PCR machine (Duncan thermocycler) (A). When the genotyping was repeated with correct plate sealing, the data improved (B).

G. MISREADING OF DATA

Even if the genotyping has proceeded correctly, data can still be corrupted by faults with the plate reader. Also, if the plate is not inserted correctly into the plate reader (or if not correctly placed on a plate holder), the data will not be analysed correctly (Figure.

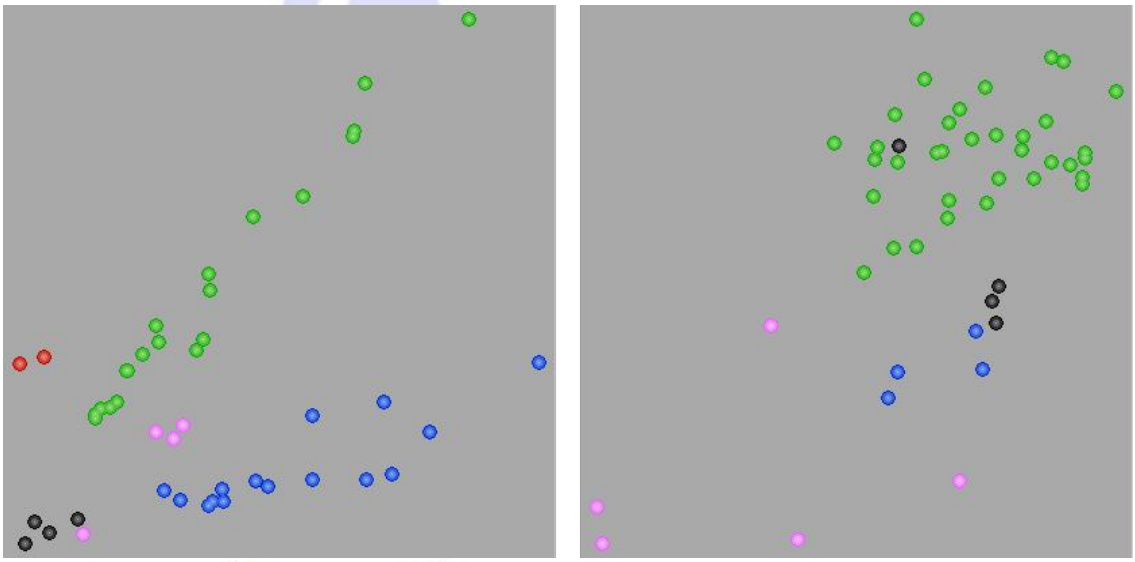


Figure 12. Good genotyping data (B) can appear poor (A) due to a plate reader mis-reading.

Plate readers can miss-read as a result of overheating (Figure 13).

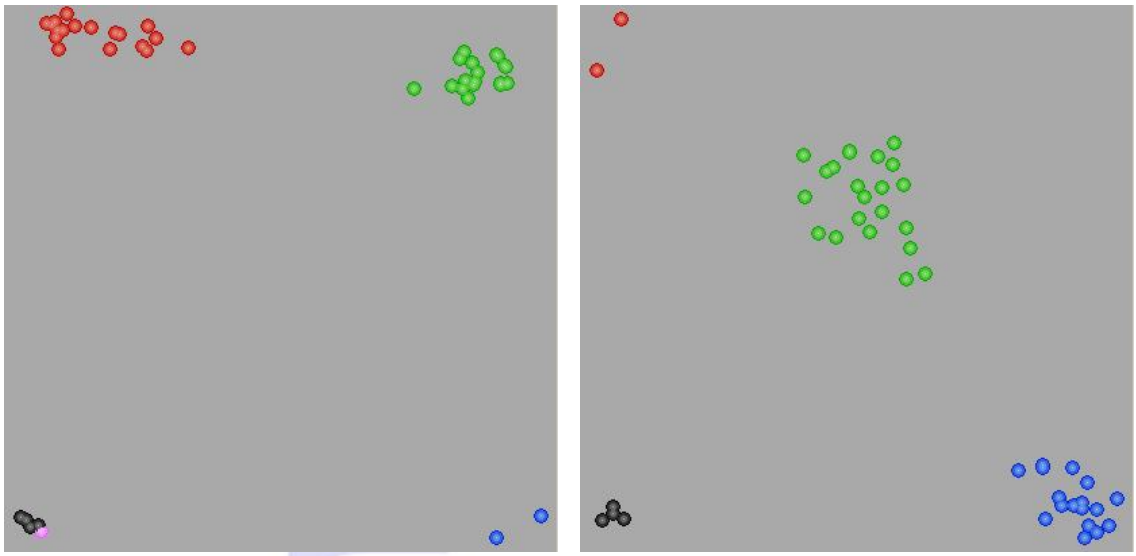


Figure 13. Good genotyping data (A) being read using a plate reader that had overheated leading to a mis-read (B)

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Related products

Klustercaller.
Klustercaller is a semi automated calling software for cluster based genotyping data. It has the functionality the calling module built in to our LIMS package. It may be used with your own genotyping projects or with KBioscience genotyping data.
www.kbioscience.co.uk\software\klustercaller\index.htm

LIMS.
KBiosciences has developed a dedicated Laboratory Information Management System (LIMS) for all genotyping, sequencing & DNA extraction work. The KBiosciences LIMS is now available to purchase.
www.kbioscience.co.uk\software\lims\index.htm

Duncan thermocyclers (DT24 and DT108).
Water bath-based thermocycler for high throughout PCR applications.
www.kbioscience.co.uk\instrumentation\duncan\index.htm

Microtitre plates.
KBioscience produces 384-and 1536-well microtitre plates.
www.kbioscience.co.uk\instrumentation\plates\index.htm

Fusion Laser Welder.
Able to seal virtually all plate densities from single tubes to 3456 plates.
www.kbioscience.co.uk\instrumentation\lasersealing\index.htm

Genotyping Service.
KBiosciences offer a very competitively-priced, in-house genotyping service based on the KASPar chemistry.
www.kbioscience.co.uk\lab_services.htm

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