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KASP genotyping V.2

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ABSTRACT

KASP is a genotyping assay which you can use to differentiate wild types vs heterozygous vs homozygous larvae/finclips. It is well suited to genotype a SNP or a small CRISPR-generated indel. Once you confirmed it is working well, the assay is pretty fast to run so you may want to use KASP if you will routinely genotype tens of samples.

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How KASP works (in summary)

1 You can find some excellent videos on YouTube which explains the concept.

KASP is based on a PCR with three primers:

- one primer specific to the wild-type allele (WT primer)
- one primer specific to the mutant allele (MUT primer)
- one primer common to both alleles (COM primer)

The WT and MUT primers are both Forward and the COM primer is Reverse, or vice-versa.

The WT and MUT primers 5' carry tags on which a fluorophore is bound during PCR. This allows to differentiate the three genotypes when reading the plate after PCR:

- Wild-type: high signal in channel 1 only
- Homozygous: high signal in channel 2 only
- Heterozygous: high signal in both channels

Design the KASP primers

2 Eirinn Mackay, a Fish Floor alumnus (Wilson lab), created an online tool to create the KASP primer. This circumvents using the company's service, which charges ~ 60£ for a simple mix of three PCR primers.

Go to
https://kasp.eirinn.org/

We are assuming here that you already sequenced your allele (usually with Illumina MiSeq or Sanger) to know what the mutation is.

3 ***Need help obtaining the sequence of the wild-type/mutant alleles?***
Say you only know through sequencing a small window around the mutation, for example:

Wild-type:
GAGTATGAAGCCCTCCACGAAGATGAGTTG

Mutant (2-bp deletion):
GAGTATGAAGCCCTC--CGAAGATGAGTTG

We can find the genomic position of the mutation with BLAST.
https://blast.ncbi.nlm.nih.gov/Blast.cgi

Under BLAST Genomes, type *Danio rerio* > Search.
Enter the wild-type sequence from above > BLAST.

The top result should be the correct match. Check that Query cover and Percentage Identity (Per. Ident) are both 100%. Click on the result.

You will obtain the start and stop positions of the small sequence, in our case: chr20:35900655–35900684.

The mutation is the deletion of *CA* in the middle. Count from the start and you should find that the deletion is chr20:35900670–71. Record it somewhere for the future.

Now go to the UCSC Genome Browser for *Danio rerio* reference genome danRer11:
https://genome-euro.ucsc.edu/cgi-bin/hgTracks?db=danRer11
Search for the position of the mutation: chr20:35900670.

Check that you are at the right place, for example that you see your gene of interest and that this nucleotide/the surrounding ones are those you expect from sequencing (you can zoom out for this).

View > DNA.
In Position, you should have your position of interest, in our case: chr20:35,900,670-35,900,670.
Add **100** extra bases upstream (5') and **100** extra downstream (3').
Click extended case/color options > change Letters per line to a big number e.g. 999.
Submit.

You now have a 200-bp genomic window centered on the position of your mutation (i.e. ~ 100 bp on either side). This is your **wild-type sequence**, in our case:
AAAAATAGGTGGATGGAAATGTAGCTACAGATAACAGGTACTGAGATGTGTTGTTGTCTCTGCAGTTGAGGTGGT GGTGGAGTATGAGTATGAAGCCCTCCACGAAGATGAGTTGACCCTCAGGCTTGGAGACATCATCAAAAACGTAC GACGCATCGAAGAGGAGGGATGGATGGAAGGAGACCTCAACGGCAAACGAGG

To create the **mutant sequence**, simply replace the deleted CA by --. You should obtain:
AAAAATAGGTGGATGGAAATGTAGCTACAGATAACAGGTACTGAGATGTGTTGTTGTCTCTGCAGTTGAGGTGGT GGTGGAGTATGAGTATGAAGCCCTC--
CGAAGATGAGTTGACCCTCAGGCTTGGAGACATCATCAAAAACGTACGACGCATCGAAGAGGAGGGATGGATGG AAGGAGACCTCAACGGCAAACGAGG

4 Enter your wild-type and mutant sequences in https://kasp.eirinn.org/.

It creates two set of three primers:
One Forward set, where the WT and MUT primers are Forward and the COM primer is Reverse.
One Reverse set, where the WT and MUT primers are Reverse and the COM primer is Forward.

Order the three primers of the Forward set, as you would for any PCR primers.

The three primers of the Reverse set serve as "second try" if the Forward set does not work. You can buy them now too (short DNA oligos are cheap) or wait to try the Forward set first.

Prepare the primer mix

5 When you receive the lyophilised primers, you can prepare 100 µM stocks in nuclease-free H2O. For example, if one primer is 16.4 nmoles, you want to add 164 µL nuclease-free H2O to make a 100 µM stock. Vortex and spin-down a couple of times to make sure that the pellet was dissolved completely.

Next, prepare a KASP primer mix (all three primers together) such as that***:

- COM primer = [M] 29 micromolar (µM)
- WT primer = [M] 14.5 micromolar (µM)
- MUT primer = [M] 14.5 micromolar (µM)

For example, to make 200 µL of KASP primer mix from the 100 µM stocks:

- 58 µL COM primer (100 µM)
- 29 µL WT primer (100 µM)
- 29 µL MUT primer (100 µM)
- 84 µL nuclease-free H2O

Note

*** My logic for these concentrations was: for a standard PCR, I usually put a total of 0.6 µL (2 × 0.3 µL) of 20 µM primer in a reaction volume of 20 µL, i.e. final primer concentration = 0.8 µM. Here, we use 0.056 µL of primer mix in a final volume of 4.056 µL (see below). Therefore, to reach the same final primer concentration, we want a primer stock of 58 µM. In the case of a heterozygous sample, the COM primer works on 100% of the templates while the WT and MUT primers only amplify 50% of the templates each, so the COM primer's concentration should be double the WT and MUT primer's concentrations, i.e. the 58 µM total primer concentration should be splitted as: 29 µM COM / 14.5 µM WT / 14.5 MUT.

Prepare the run

6 Dilute your genomic DNA.

Your genomic DNA is likely too concentrated. This has been my recurrent experience with 8-dpf larvae lysed with HotSHOT using 50 µL BASE + 50 µL NEUTRALISATION. Even if the larvae/finclips you used were smaller, the PCR will still have ample template after dilution, and it is likely that you will get more wells with clear results.

You can safely add 50–80 µL nuclease-free H2O on top of your ~ 100 µL of genomic DNA. Spin down the plate.

7 Think about the lay-out of your plate.

Make sure to include minimum 3 non-template control (NTC) wells. Include more if you have enough free wells.

When first testing the KASP assay, also include a few samples which you know are wild-type at this locus. You can also include some other samples of known genotypes, for example the samples you sequenced to confirm that you are obtaining the same results with KASP and sequencing.

8 Use a white, low-profile PCR plate.
Also get a normal seal.

9 Prepare a MasterMix.

Per well:

- 2 µL 2× KASP mix
- 0.056 µL primer mix

Prepare 15–20% extra.

For example, for 96 wells, prepare for 115 wells:

- 230 µL 2× KASP mix
- 6.44 µL primer mix

Leave On ice

10 Pipet 2 µL of genomic DNA in each well.

Try to place the drop on the wall on one side (always the same) of each well.

You can use a 8-tip multichannel pipet, in which case you fill the plate column by column; or a 12-tip multichannel pipet, in which case you fill the plate row by row. I prefer the 8-tip one.

In the NTC wells, you should ideally pipet 2 µL of the buffer in which your genomic DNA is, typically a 50:50 mix of 1× BASE and 1× NEUTRALISATION solutions from HotSHOT. Alternatively, 2 µL of nuclease-free H2O should work fine as well.

11 Pipet 2 µL of MasterMix in each well.

Place the drop on the wall on the side opposite the genomic DNA. If you are careful about not touching the genomic DNA, you do not need to replace tip at every well. I usually change tip only once per column or row or when pipetting into the NTC wells.

12 Seal the plate.
Spin it down.

Run the PCR

13 PCR programme (from official KASP's protocol):

1. 94˚C – 15 min

2. 94˚C – 20 sec
3. 61–55˚C – 60 sec
> back to 2. for 10 more cycles (11 total).
Decreasing 0.6˚C per cycle, so: 61, 60.4, 59.8, 59.2, 58.6, 58, 57.4, 56.8, 56.2, 55.6, 55.

4. 94˚C – 20 sec
5. 55˚C – 60 sec
> back to 4. for 26 more cycles (27 total).

Read the results

14 Are you on FishFloor @UCL? The plate readers are in the Central Lab with Bill. You should book your visit on the Google Calendar. I usually use the CFX1 machine, so qpcrcfx1@gmail.com.

Open software.

Plate > Edit selected
> right click on one NTC (no template control) well, Copy Well
> select your negative control wells, Paste Wells
> any empty well, select and Clear Wells (right column)
> leave positive controls as Unknown
(so should be all Unknown or NTC)
> Save plate
> Next
> make sure CFX1 is ticked
> click on Open lid

Place your plate

> click on Close lid
> Start Run
> save results in your folder

15 Results should pop up
> Allele discrimination
> tick View call map

The scatterplot is X = first colour/channel, Y = second colour/channel.

In an ideal world:
- all the NTC wells are labelled black and are in the bottom left corner, i.e. low fluorescence in both channels
- the HET wells are labelled green and are in a cluster in top right corner, i.e. high fluorescence in both channels
- the WT wells are labelled orange and are in a bottom right cluster, i.e. high fluorescence in only one channel
- the HOM wells are labelled blue and are in a top left cluster, i.e. high fluorescence in only one channel

However, the results are rarely so perfect in my experience.

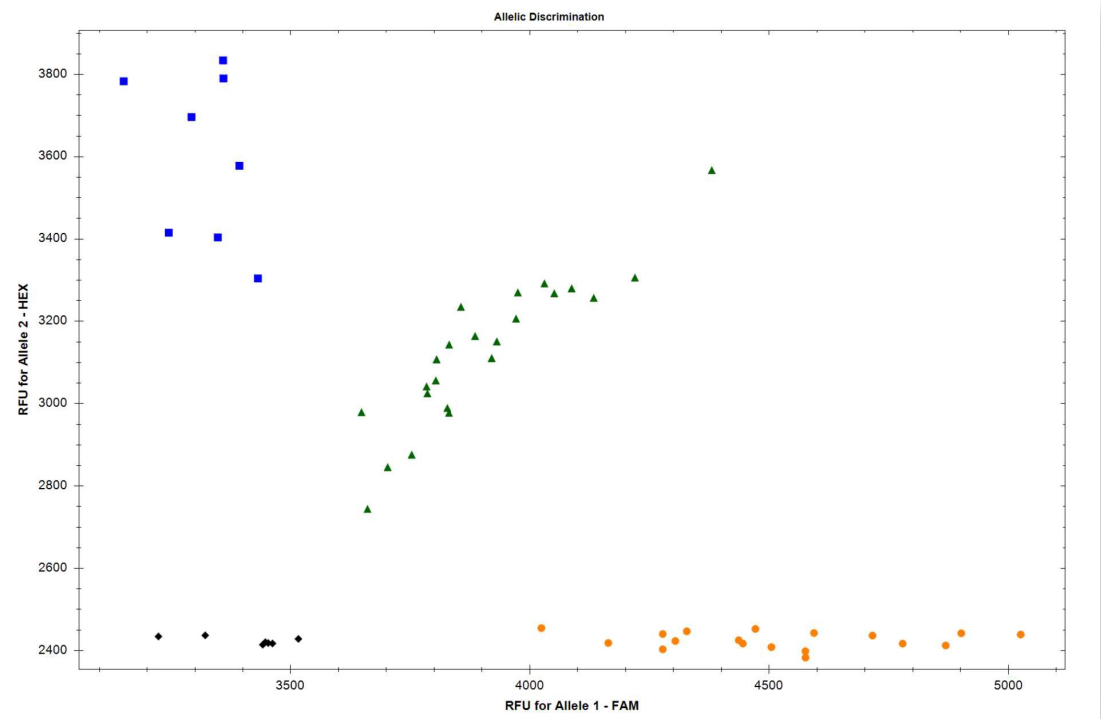
First, confirm that you are reading correctly which cluster is HOM vs WT based on the positions of your known WT wells. If you have other samples of known genotypes, also check their calls now. The HET cluster

should always be in the middle so there is little risk of misidentification.

To polish the results, a process which seems to work well:

1. Exclude any samples which have abormally high values (right click on scatterplot > Exclude from analysis). It may seem like your run failed completely until you exclude those samples. Indeed, they force the plot to be extremely "zoomed-out", preventing to see the clusters.
2. Is there any samples (not NTC) which are coloured in black? Exclude them. Those are labelled as NTC wells because they had low fluorescence.
3. Exclude any NTC wells which are inside or close to another cluster.
4. Exclude any wells which are in-between clusters. You essentially want to create a empty spaces between clusters.

A final plot looking like this is good:



If you expect a specific ratio of genotypes (e.g. 25% WT / 25% HOM / 50% HET for a heterozygous in-cross), count your results as additional check.

I think the software calls the genotype of each well based on a clustering algorithm.

16 You can export a PDF report with:

Tools
> Report
> untick everything except sections Header and Allele discrimination
> Update report
> Save in your folder
> Email PDF report to yourself
> Close everything
  > No to save template
  > Save results in your folder

You can also export results in a CSV format.

17 Are you using a QuantStudio 6 Pro machine? Instructions for reading the plate:

Set up plate:
> Analysis (left panel) > Genotyping
> QuandStudio 6 Pro
> 96-well 0.1 mL
> Run mode Standard
> click to create Genotyping protocol

> Reaction Volume = 5 uL
Delete all steps using the Minus sign, except Post Read. Set to 30C / 1 min

> Plate Setup
> Select unknown wells. Click + on right to create new Sample. Then tick, so should all be Sample 1.
> Same with SNP Assays. So should all be Sample 1 & SNP Assay 1.

In SNP Assay, check the following:
> Allele 1 Reporter is VIC
> Allele 1 Quencher is NFQ-MGB
> Allele 2 Reporter is FAM
> Allele 2 Quencher is NFQ-MGB

> Select your negative controls, click +, change Type to Negative Control. Tick to apply. Apply same SNP assay.
So, NTC wells should be Sample 2 (marked N) & SNP Assay 1.

Make sure Passive Reference at the top is ROX.

Run Summary, scroll down, click machine then Send to Run Queue.

Browse to folder where you want to store results.
Change name.

On machine, open with button top right. Load plate. Close.

Load plate file, find your protocol in Run Queue (usually last for some reason).

Start run.

If file is not in expected folder, can Transfer file.

Also do Actions > Export to CSV.

18 Throw the plate.
Close the software.

TROUBLESHOOTING

19 Did not work?

It may be that your genomic DNA is still too concentrated. Diluting the genomic DNA has often been the solution for me. To avoid overflowing the genomic DNA plate, you can also pipet only 1 µL nuclease-free H2O + 1 µL genomic DNA in every well, instead of 2 µL genomic DNA, which will dilute by a further 2×.

If that does not help, you can also try the Reverse set of primers.

Other troubleshooting advice? Add them here or comment on this section.