Name of Mouse model or mutation:
KCNMA1-D434G-EM3-B6

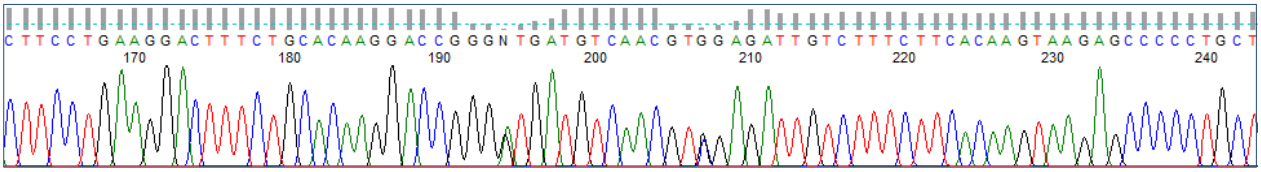
Description:
Point mutation model made using CRISPR/Cas9.

Type of mutation:
SNP: D434G

Sequence details
WT
GGATTTTGTGCAGCATCAGGTGAGCAGCCATATGCCTTTTTTTTTCTGTCCAGGAGCCTCTGAGCAAC
TGTTAGTGATATCGCTTTGCCTCATGACCCTCTGGGGATGGGTGCTGGGTACTGGGGCTGAGTGGG
GAGATGTATTGCTTCCTCTGCTGATCAGAGATCAGGGTACGGGGTCATGTTGCTGAGTCTGGTTGGC
TCTAACTGCTTTCTCCTGTACAGGCACATTGTAGTCTGTGGACACATTACTCTGGAGAGTGTCTCTAA
CTTCCTGAAGGACTTTCTGCACAAGGACCGGGATGATGTCAACGTGGAGATTGTCTTTCTTCACAAG
TAAGAGCCCCCTGCTGCCACCAGACCCTGCCACCTAAACTCATGCTTCTCTCTGAGTGCCTAGAGGTG
GCTGGGAATTAGGACAGGGTTAAATAAGCCATGATTGGTGCTGGCTCCTTAGGTAAGAGGTAGTTT
TTCTGAAAGAATGGGGAGGTTACTTTAGAACCCTTGGATCATGAAGCAGCACCCTTGTTCAGAGCTG
AAAGCAAACTCCAGTGAGGCAGTCTAGGA

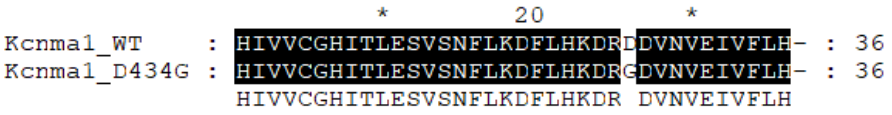
KCNMA1-D434G-EM3-B6
GGATTTTGTGCAGCATCAGGTGAGCAGCCATATGCCTTTTTTTTTCTGTCCAGGAGCCTCTGAGCAAC
TGTTAGTGATATCGCTTTGCCTCATGACCCTCTGGGGATGGGTGCTGGGTACTGGGGCTGAGTGGG
GAGATGTATTGCTTCCTCTGCTGATCAGAGATCAGGGTACGGGGTCATGTTGCTGAGTCTGGTTGGC
TCTAACTGCTTTCTCCTGTACAGGCACATTGTAGTCTGTGGACACATTACTCTGGAGAGTGTCTCTAA
CTTCCTGAAGGACTTTCTGCACAAGGACCGGG**G**TGATGTCAACGT**C**GAGATTGTCTTTCTTCACAAG
TAAGAGCCCCCTGCTGCCACCAGACCCTGCCACCTAAACTCATGCTTCTCTCTGAGTGCCTAGAGGTG
GCTGGGAATTAGGACAGGGTTAAATAAGCCATGATTGGTGCTGGCTCCTTAGGTAAGAGGTAGTTT
TTCTGAAAGAATGGGGAGGTTACTTTAGAACCCTTGGATCATGAAGCAGCACCCTTGTTCAGAGCTG
AAAGCAAACTCCAGTGAGGCAGTCTAGGA

KCNMA1-D434G-EM3-B6 Heterozygous F1 animal sequence trace:



Nucleotide Alignment:

Predicted Protein Alignment of ENSMUSE00000503015:



QC strategy employed at Harwell to check the edited allele:

Genomic DNA was extracted from ear clip biopsies and amplified in a PCR reaction using the following conditions/primer sequences:

Geno_ Kcnma1_F1 (5'-3')	GGATTTTGTGCAGCATCAGGT
Geno_Kcnma1 _R1 (5'-3')	TCCTAGACTGCCTCACTGGAG
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	63
Elongation time (min)	1
WT product size (bp)	566
Mutant product size (bp)	566
Notes	

All amplicons were sent for Sanger sequencing to check for integration of the donor oligo sequence at the target site. F1 sequences should be heterozygous unless on Y chromosome.

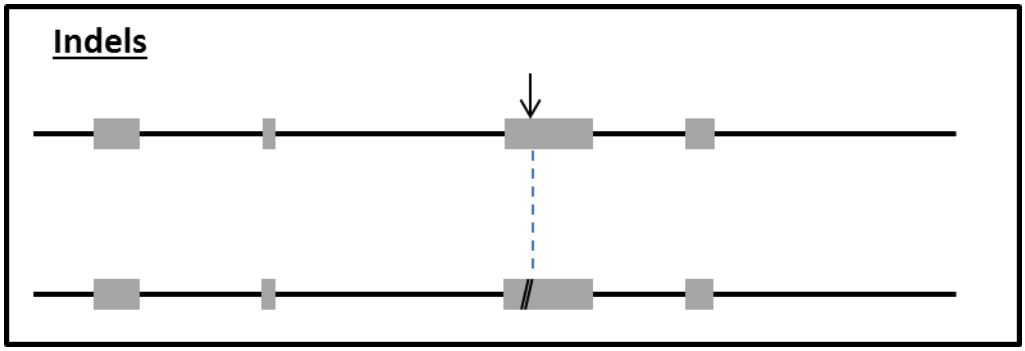
Copy counting of the donor sequence was carried out by ddPCR at the F1 stage to confirm donor oligos were inserted once on target into the genome. The following Taqman assay was used to copy count the donor sequence compared against a VIC-labelled reference assay for Dot1l:

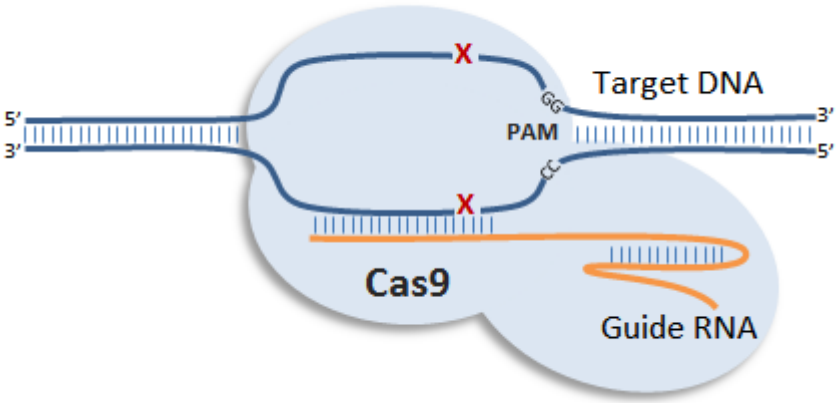
Assay name	KCNMA1-D434G-UNIV1
Forward Primer	AGGTGGCAGGGTCTGGTG
Reverse Primer	GGAGAGTGTCTCTAACTTCCTGAAG
Probe	CAGGGGGCTCTTACTTGTGAAGAAAGAC
Label	FAM-BHQ1

The ddPCR assay is universal and both WT and MUT alleles are recognised by this assay. WT controls are expected to call at 2 copies and a single integration for a correct mutation is expected to call at 2 copies for F1 (HET) animals.

Kcnma1-D434G Genotyping Strategy

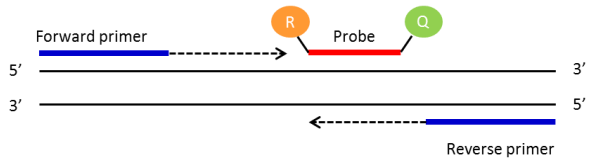
Animals have been engineered using the CRISPR/Cas9 technology. Most of the knockout alleles generated through this method will be obtained by deletion of a critical exon or by introduction of an indel (insertion/deletion) within the coding sequence of a critical exon (see picture below).



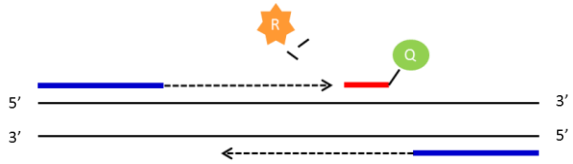


qPCR genotyping strategy

Standard PCR is the amplification of DNA between a pair of primers. Quantitative PCR employs the same principal as standard PCR, although it actually monitors the progress of the DNA synthesis as it occurs. The progress of the reaction is measured by using a Taqman probe. This is a short DNA oligo that is complimentary to part of the DNA sequence between the forward and reverse primers. At the 5' end of the probe there is a fluorescent reporter (R) and at the 3' end a quencher (Q). Whilst they are in close contact with each other there is no fluorescent signal.



As the forward primer is extended the reporter is cleaved from the probe resulting in a fluorescent signal being detected. Once the primer extends enough to release the quencher this signal is blocked. By using probes with different fluorescent signals multiple PCR assays can be multiplexed and run together.



Allele specific primer and probe amplification assay (ASPPAA) PCR

This is a new real-time PCR method (Billard *etal.,* 2012) in which an allele specific primer and an allele specific probe designed specific to the SNPs. The primer is designed such a way that its 3' end ends with a specific SNP. The probe is also designed specific to the SNPs at its 3' end giving a primer probe overlap. A maximum of 3nt overlap between a primer and probe is allowed.

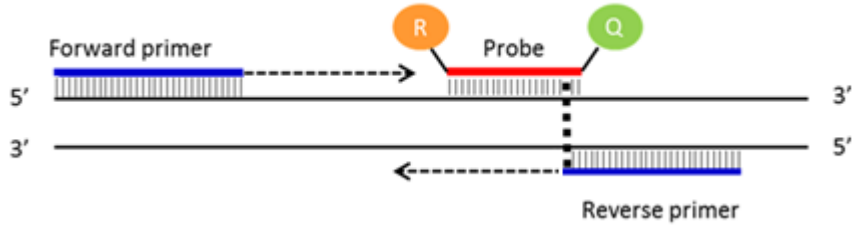
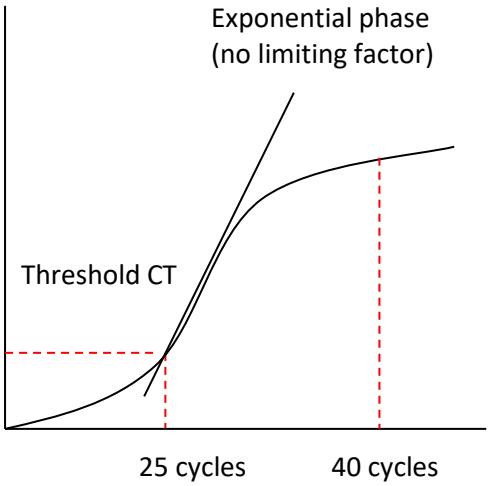


Figure1: Figure showing principle of ASPPAA PCR. The dotted line indicates the position of the SNP.

PCR reaction plot



The number of cycles the PCR takes to reach a set threshold is known as the CT value. This is inversely correlated to the amount of template DNA in the sample.

e.g. CT 25 = 2 x template DNA
CT 26 = 1 x template DNA
CT above 30 = no template represented in the sample

CT value can be used to determine how many copies of a particular allele samples have.

All our qPCR are run in duplicate. A FAM labelled genotyping assay is run in multiplex with a VIC labelled internal control Dot1l.

References:

Billard A., Laval V., Fillinger S., Leroux P., Lachaise H., Beffa R., et al. (2012).
The allele-specific probe and primer amplification assay, a new real-time PCR method for fine quantification of single-nucleotide polymorphisms in pooled DNA.
Appl. Environ. Microbiol. 78 1063–1068. 10.1128/AEM.06957-11

Kcnma1-D434G Genotyping Strategy

Samples are genotyped with a WT loss of allele (WT-LOA) assay. This is a FAM labelled assay that is designed to detect the critical exon that has been targeted. If the animal contains the modified allele the copy number of this assay should drop by 1. For autosomal genes that have been targeted this means the following

WT= 2 copies of the LOA assay
HET = 1 copy of the LOA assay
HOM = 0 copies of the LOA assay

Kcnma1-D434G CRISPR/Cas9 mutant in which SNPs are as highlighted

WT ACTTTCTGCACAAGGACCGGGATGATGTCAACGTGGAGATTGTCTTTCTTCACAAG
Mutant ACTTTCTGCACAAGGACCGGGGTGATGTCAACGTCGAGATTGTCTTTCTTCACAAG

Kcnma1-D434G-WT1 assay (FAM labelled probe)

TCTGCTGATCAGAGATCAGGGTACGGGGTCATGTTGCTGAGTCTGGTTGGCTCTAACTGCTTTCTCCTGTACAGG
CACATTGTAGTCTGTGGACACATTACTCTGGAGAGTGTCTCTAACTTCCTGAAGGACTTTCTGCACAAGGACCGG
GATGATGTCAACGTGGAGATTGTCTTTCTTCACAAGTAAGAGCCCCCTGCTGCCACCAGACCCTGCCACCTAAAC

Kcnma1-D434G-WT1 primers and probe

Primer 1 = CAGGCACATTGTAGTCTGTGGAC
Primer 2 = GCTCTTACTTGTGAAGAAAGACAATCTCC

Allele specific primer and probes

Probe = CGGGATGATGTCAACGTGGA

Dot1l internal control (VIC labelled)

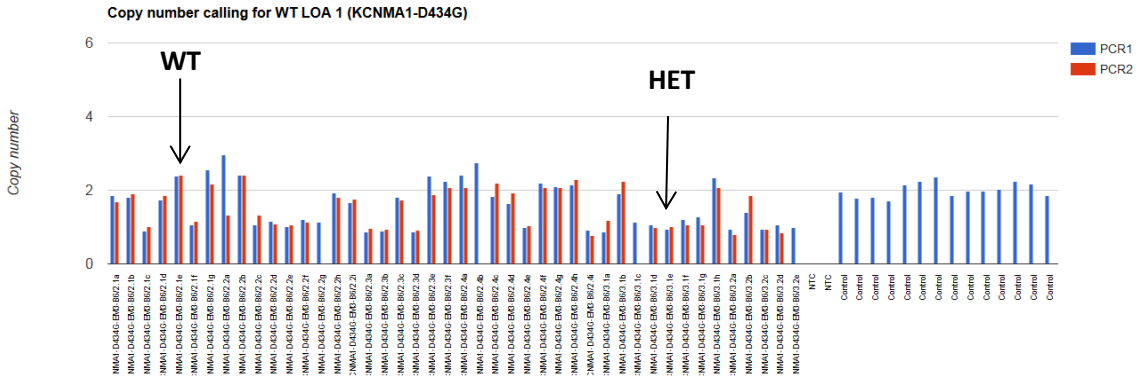
CCCCTCTAGTCGTTTTCTGTTAGTAGTTGGCATCCTTATGCTTCATCTTACAGTCGACTTGAGAGCTGGCCCTGA
ATGGTCGTGCTGGGGCAAGGCTTTATTTCAGGCGTAGCACACATGGTGGCCAATGGGACTCTGTAGGATCTGCCC

Primer 1 = GCCCCAGCACGACCATT
Primer 2 = TAGTTGGCATCCTTATGCTTCATC
Probe = CCAGCTCTCAAGTCG

qPCR master mix

ABI GTX Taqman master mix	5µl
Primers Dot1L_2F (20µM)	0.225µl
Primers Dot1L_R (20µM)	0.225µl
Probe DotL_2M (5µM)	0.2µl
FAM Assay (probe 5µM & primers 15µM each)	0.3µl
ddH20	1.55µl
DNA (1/10 dilution of ABI Sample-to-SNP prep)	2.5µl

Kcnma1-D434G copy called result, image showing both replicates and controls



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Approved by:	Debbie Williams