Name of Mouse model or mutation:
ATP13A3-P452Lfs-EM2-B6N

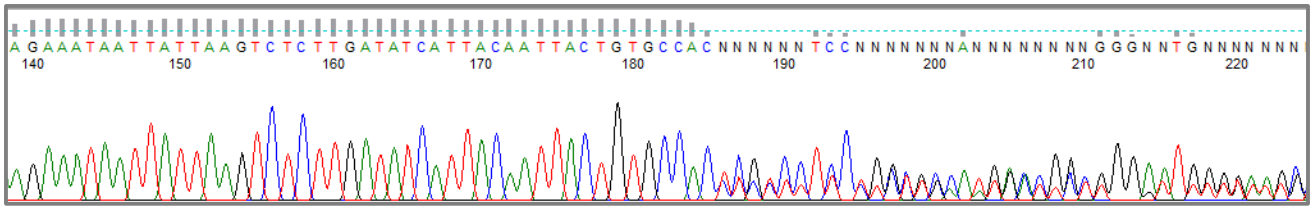
Description:
Point mutation model made using CRISPR/Cas9.

Type of mutation:
SNP: 1 nt deletion to introduce a frame shift

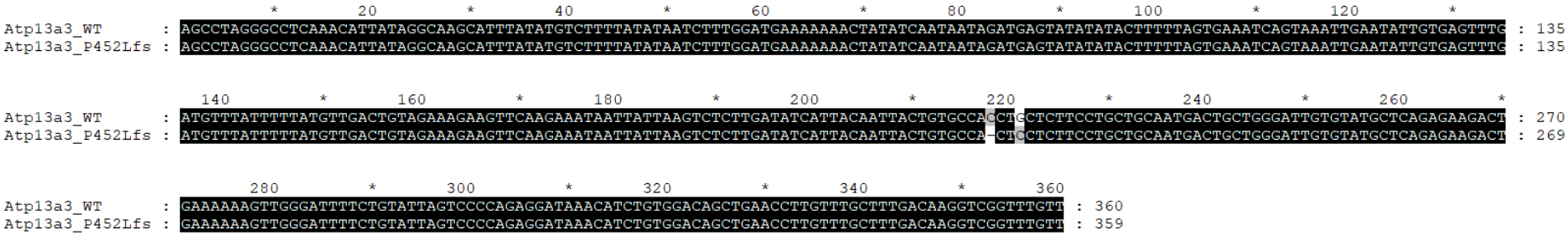
Sequence details
WT
AGCCTAGGGCCTCAAACATTATAGGCAAGCATTTATATGTCTTTTATATAATCTTTGGATGAAAAAAA
CTATATCAATAATAGATGAGTATATATACTTTTTAGTGAAATCAGTAAATTGAATATTGTGAGTTTGA
TGTTTATTTTTATGTTGACTGTAGAAAGAAGTTCAAGAAATAATTATTAAGTCTCTTGATATCATTACA
ATTACTGTGCCACCTGCTCTTCCTGCTGCAATGACTGCTGGGATTGTGTATGCTCAGAGAAGACTGA
AAAAAGTTGGGATTTTCTGTATTAGTCCCCAGAGGATAAACATCTGTGGACAGCTGAACCTTGTTTG
CTTTGACAAGGTCGGTTTGTT

Mutant
AGCCTAGGGCCTCAAACATTATAGGCAAGCATTTATATGTCTTTTATATAATCTTTGGATGAAAAAAA
CTATATCAATAATAGATGAGTATATATACTTTTTAGTGAAATCAGTAAATTGAATATTGTGAGTTTGA
TGTTTATTTTTATGTTGACTGTAGAAAGAAGTTCAAGAAATAATTATTAAGTCTCTTGATATCATTACA
ATTACTGTGCCA**[1_nt_del]**CTCCTCTTCCTGCTGCAATGACTGCTGGGATTGTGTATGCTCAGAGAA
GACTGAAAAAAGTTGGGATTTTCTGTATTAGTCCCCAGAGGATAAACATCTGTGGACAGCTGAACCT
TGTTTGCTTTGACAAGGTCGGTTTGTT

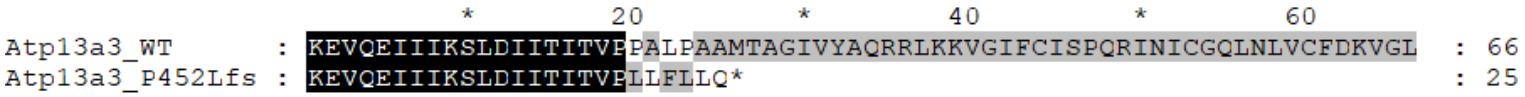
ATP13A3-P452Lfs-EM2-B6N Heterozygous F1 animal sequence trace:



Nucleotide Alignment:



Predicted Protein Alignment:



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_Atp13a3 _F1	AGCCTAGGGCCTCAAACATTA
Geno_ Atp13a3_R1	AACAAACCGACCTTGTCAAAGC
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	57
Elongation time (min)	1
WT product size (bp)	360
Mutant product size (bp)	359
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.

The following off-target sites were also checked for guide-induced changes:

OT site	Chr 10:116433781-116433803
Atp13a3_OT1_F1 primer (5'-3')	ATGGAGAACTTGATGGCGGA
Atp13a3_OT1_R1 primer (5'-3')	TGAGGTCATAGGTCACCCCG
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	60
Elongation time (min)	1
WT product size (bp)	749
Notes	Sequence with (Atp13a3_OT1_R2 primer (5'-3') AGAAGACCTGGTTAGTGCTGC)

OT site	Chr 12:3850376-3850398
Atp13a3_OT2_F1 primer (5'-3')	GGCTAGACAGAGCCTGATTCC
Atp13a3_OT2_R1 primer (5'-3')	CTGCTCCTTCACACACCTTCA
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	60
Elongation time (min)	1

WT product size (bp)	717
Notes	

OT site	Chr 1:178616246-178616268
Atp13a3_OT3_F1 primer (5'-3')	TCATTGACGAGGGTAATAGAGGG
Atp13a3_OT3_R1 primer (5'-3')	CAGGCTCCAACAGTACTTGC
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	60
Elongation time (min)	1
WT product size (bp)	708
Notes	

OT site	Chr 15:27442808-27442830
Atp13a3_OT4_F1 primer (5'-3')	TATCCGCTCATCTGCCACTC
Atp13a3_OT4_R1 primer (5'-3')	GTCTGCTTCCCTCCACTACC
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	60
Elongation time (min)	1
WT product size (bp)	710
Notes	

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/UPL assay(s) was used to copy count the donor sequence compared against a VIC-labelled reference assay for Dot1l:

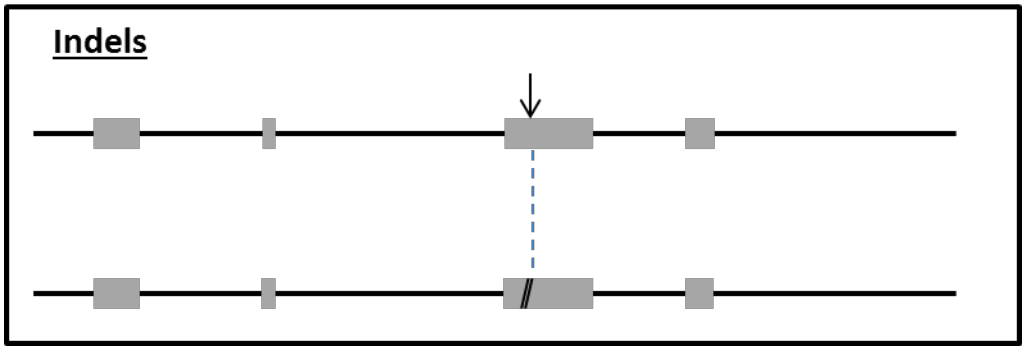
Assay name	Atp13a3-UPL18
Forward Primer	ACAATTACTGTGCCACCTGCT
Reverse Primer	GTCTTCTCTGAGCATACACAATCC
Probe	Roche UPL probe UPL18
Label	FAM
Notes	The ddPCR assay is designed against the WT allele of the Atp13a3 gene as the forward primer sits within the region of the deleted nucleotide and the UPL

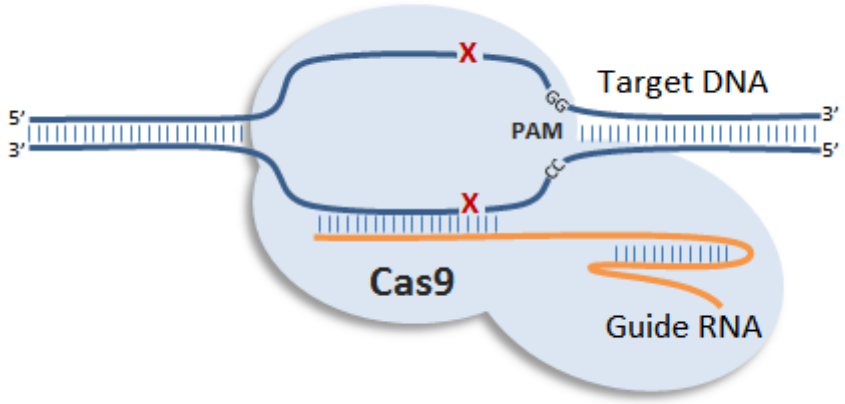
	probe sits over the SM site. Therefore WT controls are expected to call at 2 copies and a single integration for a correct mutation is expected to call at 1 copy for F1 (HET) animals.

Assay name	Atp13a3-P452Lfs-UNIV1
Forward Primer	AAGTCTCTTGATATCATTACAATTACTGTGC
Reverse Primer	GAAAATCCCAACTTTTTTCAGTCTTCTC
Probe	TGCAATGACTGCTGGGATTGTGTA
Label	FAM-BHQ1
Notes	The ddPCR assay is universal to both WT and MUT alleles. Therefore 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.

Atp13a3-P452Lfs Genotyping Strategy

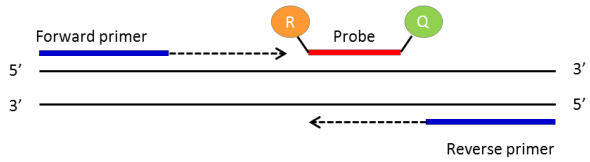
Animals have been engineered using the CRISPR/Cas9 technology. Most of the knockout alleles generated through this method will be obtained by deletion of a critical exon or by introduction of an indel (insertion/deletion) within the coding sequence of a critical exon (see picture below).



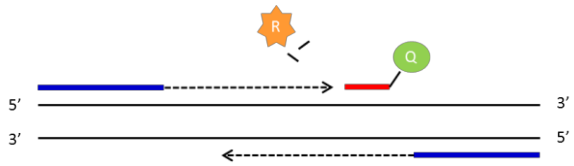


qPCR genotyping strategy

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.

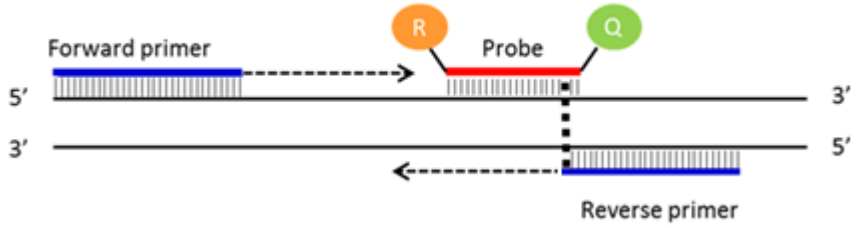
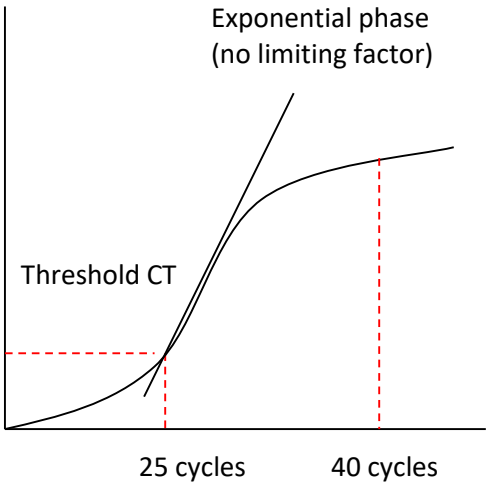


Figure1: Figure showing principle of ASPPAA PCR. The dotted line indicates the position of the SNP.

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

Atp13a3-P452Lfs Genotyping Strategy

Samples are genotyped with a WT 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

Atp13a3-P452Lfs

WT ATTACTGTGCCACCTGCTCTTCCTGCTGCAATGACTG
MUT ATTACTGTGCCA[1nt_DEL]CTCCTCTTCCTGCTGCAATGACTG

Atp13a3-P452Lfs-WT1assay (FAM labelled probe) ASPPAA PCR

Sequence in **BOLD UNDERLINED** letters is the 1nt deletion and a silent mutation from Atp13a3-P452Lfs .

ATATATACTTTTTAGTGAAATCAGTAAATTGAATATTGTGAGTTTGATGTTTATTTTTATGTTGACTG
TAGAAAGAAGTTCAAGAAATAATTATTAAGTCTCTTGATATCATTACAATTACTGTGCCA**C**CT**G**CTCT
TCCTGCTGCAATGACTGCTGGGATTGTGTATGCTCAGAGAAGACTGAAAAAAGTTGGGATTTTCTGTA

Atp13a3-P452Lfs-WT1 primers and probe

Primer 1 = ATGTTGACTGTAGAAAGAAGTTCAAGA
Primer 2 = GTCATTGCAGCAGGAAGAG**C**
Probe = CAATTACTGTGCCACCT**G**CT

Allele specific primer and probes

Dot1l internal control (VIC labelled)

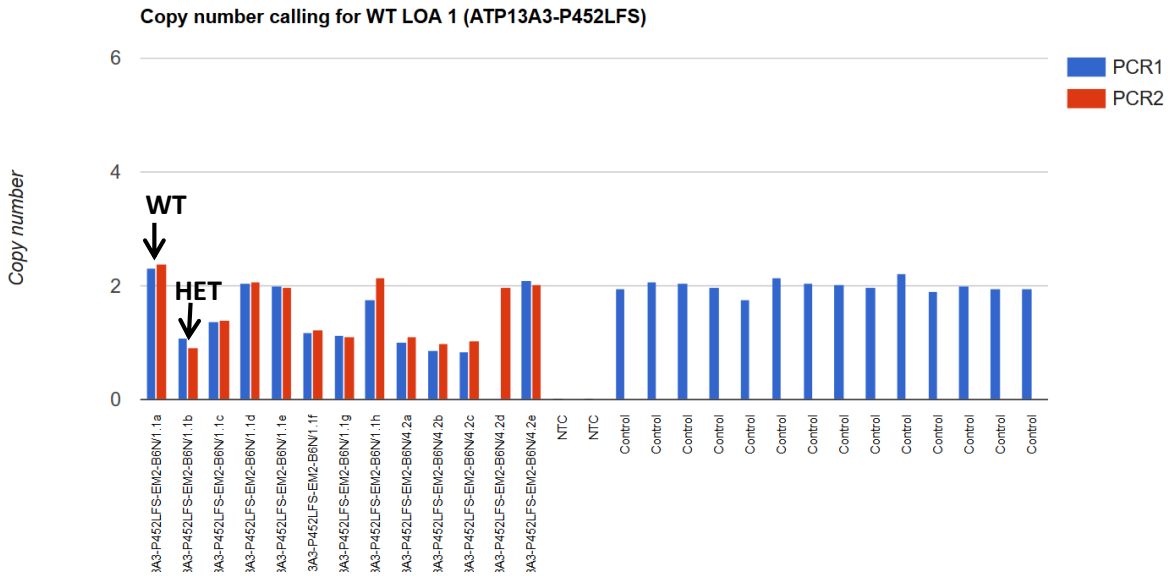
TCATAGGGTGACTGGCCAACCCAGGGAAGCCGGAGTGCTGCGTCTTCTGTTTCCTTGTTCTTTTCCCCTCTAGTC
GTTTTCTGTTAGTAGTTGGCATCCTTATGCTTCATCTTACAGTCGACTTGAGAGCTGGCCCTGAATGGTCGTGCT
GGGGCAAGGCTTTATTTCAGGCGTAGCACACATGGTGGCCAATGGGACTCTGTAGGATCTGCCCACACCCATCAG

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

Atp13a3-P452Lfs LOA copy called result, image showing both replicates and controls



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