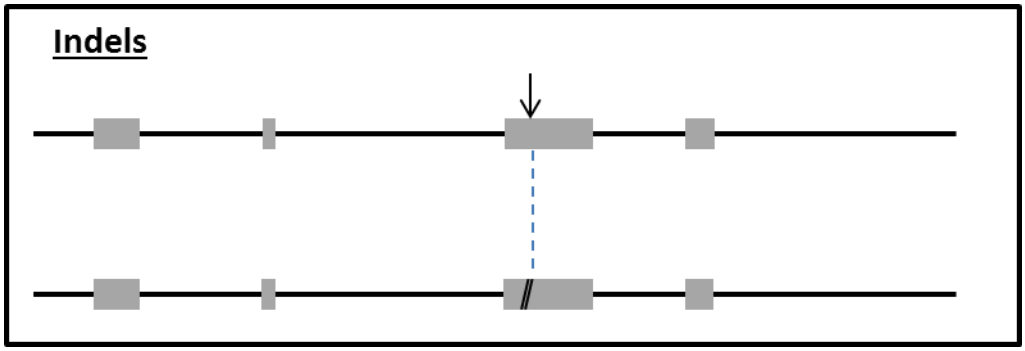
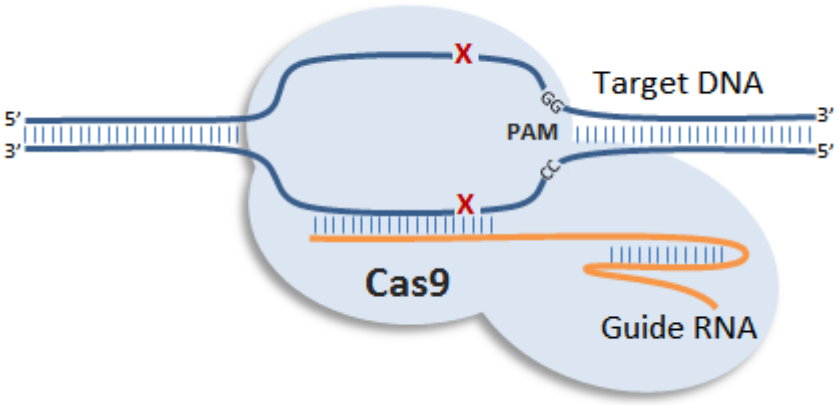
Prune1-D106N Genotyping Strategy

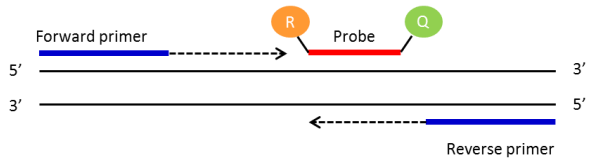
Animals have been engineered using the CRISPR/Cas9 technology. Most of the knockout alleles generated through this method will be obtained by deletion of a critical exon or by introduction of an indel (insertion/deletion) within the coding sequence of a critical exon (see picture below).



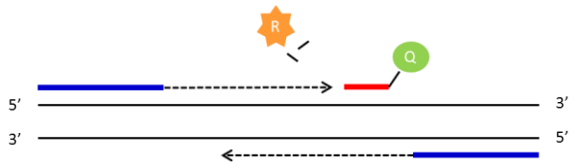


qPCR genotyping strategy

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.

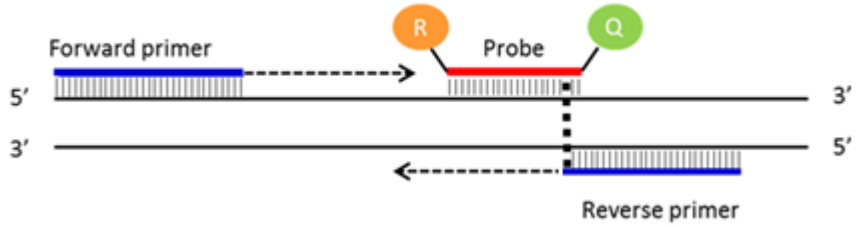
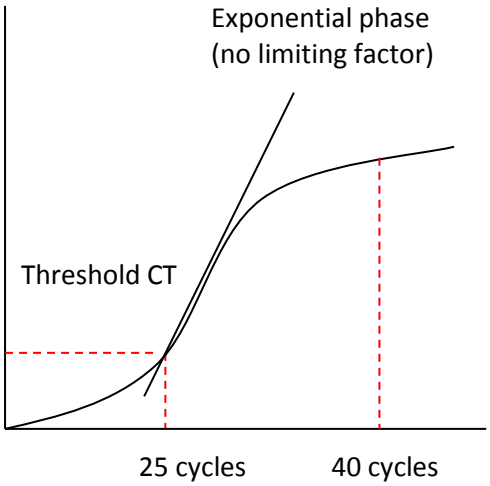


Figure1: Figure showing principle of ASPPAA PCR. The dotted line indicates the position of the SNP.

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

Prune1-D106N Genotyping Strategy

Samples are genotyped with both WT and Mutant assays. These are FAM labelled assays that are designed to detect the critical exon that has been modified. If the animal contains the modified allele the copy number of the WT assay should drop by 1 and the mutant assay should raise by 1. For autosomal genes that have been targeted this means the following

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

Prune1-D106N CRISPR/Cas9 mutant in which SNPs are as highlighted

WT ATCCTTGTTGACCACCATATTTTACCCAAGTAAGT

Mutant ATCCTTGTTAACCACCATATTACTTCCCAAGTAAGT

Prune1-D106N-WT1 assay (FAM labelled probe)

TTATACCAGTTTTAAATATAAAGCGTTCTGAGCTTCCTCTGCGCGGGGACAATGTCTTCTTTCTCCAG
GAGGTTAAGATTCCAGAGCCAGCCCTGATCTTCCGGGACGAGATTGACCTCCTAGCCCTGCACCAGG
CTGGCCAGCTCACCCTCATCCTTGTTGACCACCATATTTTACCCAAGTAAGTCAGTGAAGGACACTCG
GCCTGCACGGGGAGAAAGGAGGAAGGAGAGCCGTCCACTAGAGTCTCAAAGGCTCATTTGTTGTCA
TTTTTTCTCGTTTGTTTGTTTGCTTGCTTCTCGTGTGAGATAGGGCCTCACTATGCAGCCTTGGCTG

Prune1-D106N-WT1 primers and probe

Primer 1 = CAGAGCCAGCCCTGATCTTC
Primer 2 = ACTGACTTACTTGGGTAAAATATGGTGGTC
Probe = CGGGACGAGATTGACCTCCTAGCC

Allele specific primer and probes

PRUNE1-D106N-MUT1 assay (FAM labelled probe)

TTATACCAGTTTTAAATATAAAGCGTTCTGAGCTTCCTCTGCGCGGGGACAATGTCTTCTTTCTCCAG
GAGGTTAAGATTCCAGAGCCAGCCCTGATCTTCCGGGACGAGATTGACCTCCTAGCCCTGCACCAGG
CTGGCCAGCTCACCCTCATCCTTGTTAACCACCATATTACTTCCCAAGTAAGTCAGTGAAGGACACTC
GGCCTGCACGGGGAGAAAGGAGGAAGGAGAGCCGTCCACTAGAGTCTCAAAGGCTCATTTGTTGT
CATTTTTTCTCGTTTGTTTGTTTGCTTGCTTCTCGTGTGAGATAGGGCCTCACTATGCAGCCTTGGCTG

PRUNE1-D106N- MUT1 primers and probe

Primer 1 = CAGAGCCAGCCCTGATCTTC
Primer 2 = AACCACCATATTACTTCCCAAGTAAGTC
Probe = CGGGACGAGATTGACCTCCTAGCC

Dot1l internal control (VIC labelled)

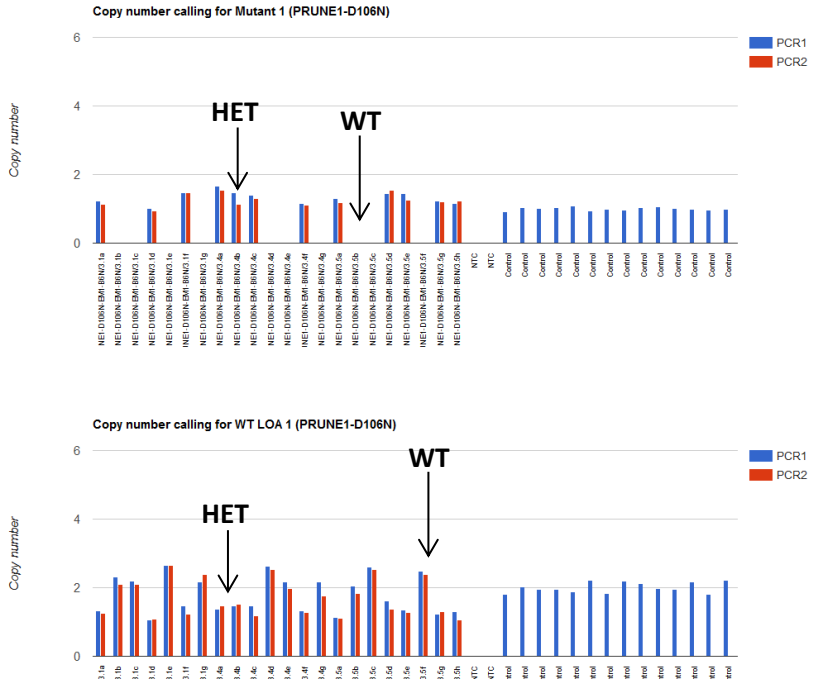
CCCCTCTAGTCGTTTTCTGTTAGTAGTTGGCATCCTTATGCTTCATCTTACAGTCGACTTGAGAGCTGGCCCTGA
ATGGTCGTGCTGGGGCAAGGCTTTATTTCAGGCGTAGCACACATGGTGGCCAATGGGACTCTGTAGGATCTGCCC

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

Prune1-D106N copy called result, image showing both replicates and controls (T259490)



Version No. 1

Date: 20/08/19

Created/Updated by: Daniel Ford

Approved by: Ramki Kurapati

Name of Mouse model or mutation:
Prune1-D106N-EM1-B6N

Description:
Point mutation generated using CRISPR/Cas9 reagents.

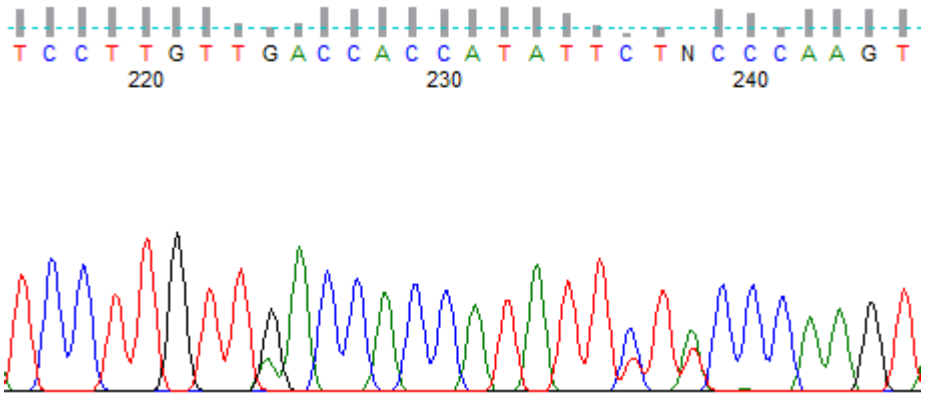
Type of mutation:
Point mutation: D106N

Sequence details
Prune1 WT:
CACTCGCTCGGCTTGCTTATCCAGTATTTAAAAAAAAAAACAAACCATCCCTTTCTTTTCTTTTTTTTTT
TTAAAATATATTTATCTATTATATGTAAGTACACTGTAGCTGTCTTCAGACACCCCAGAAGAGGACAT
CAGATCTCATTACAGATGGTTGTAAGCCACCATGTGGTTGCTGGGATTTGAACTAGGGACCTCTGGA
AGAGTAGTCAGTGCTCTTAACCACTGAGCCATCTCTCCAGCCCCATCCTTTTATTTTCATAGGCTTGAT
AATTGTGGCCTTGAGAAGTGGTAACTTACCCAAGGTCATGTCTTCTTTGGCCTTCTGTTTTGCATTTG
TGTATTAGGGAGAGAACTCAGAACCCAACCTCAGGGTCAGAGTTAGGGTGGGGGTTACTTTTTCTT
GCATTTTCCTAAGCCTTATTATGTCTTCTTTTCAATTCTGTAACCAGACGTCTGAAGCTGAAGACATCT
TTATACCAGTTTTAAATATAAAGCGTTCTGAGCTTCCTCTGCGCGGGGACAATGTCTTCTTTCTCCAG
GAGGTTAAGATTCCAGAGCCAGCCCTGATCTTCCGGGACGAGATTGACCTCCTAGCCCTGCACCAGG
CTGGCCAGCTCACCCTCATCCTTGTTGACCACCATATTTTACCCAAGTAAGTCAGTGAAGGACACTCG
GCCTGCACGGGGAGAAAGGAGGAAGGAGAGCCGTCCACTAGAGTCTCAAAGGCTCATTTGTTGTCA
TTTTTTCTCGTTTGTTTGTTTGCTTGCTTCTCGTGTGAGATAGGGCCTCACTATGCAGCCTTGGCTGGC
CTCTAAATCACTATGTATTCCAGTCTAGCCCTGAAGTCAGAAATCCACCTGCCTCGGAGGCTAACTAT
AATTGATGGGTCATATCCAATAAAAAAAAATAGTATCACTTCTCTAGTTATGTGATGTTTATGATATA
AAGAAACAAAAATATATAGTTAAATAATTATTCAAAATGTTCAAAATAACTTAAATTTGCCTTTAATG
TCAGAACTTTTGCTGCTTTAAGATACAGGG

Prune1-D106N-EM1-B6:
CACTCGCTCGGCTTGCTTATCCAGTATTTAAAAAAAAAAACAAACCATCCCTTTCTTTTCTTTTTTTTTT
TTAAAATATATTTATCTATTATATGTAAGTACACTGTAGCTGTCTTCAGACACCCCAGAAGAGGACAT
CAGATCTCATTACAGATGGTTGTAAGCCACCATGTGGTTGCTGGGATTTGAACTAGGGACCTCTGGA
AGAGTAGTCAGTGCTCTTAACCACTGAGCCATCTCTCCAGCCCCATCCTTTTATTTTCATAGGCTTGAT
AATTGTGGCCTTGAGAAGTGGTAACTTACCCAAGGTCATGTCTTCTTTGGCCTTCTGTTTTGCATTTG
TGTATTAGGGAGAGAACTCAGAACCCAACCTCAGGGTCAGAGTTAGGGTGGGGGTTACTTTTTCTT
GCATTTTCCTAAGCCTTATTATGTCTTCTTTTCAATTCTGTAACCAGACGTCTGAAGCTGAAGACATCT
TTATACCAGTTTTAAATATAAAGCGTTCTGAGCTTCCTCTGCGCGGGGACAATGTCTTCTTTCTCCAG
GAGGTTAAGATTCCAGAGCCAGCCCTGATCTTCCGGGACGAGATTGACCTCCTAGCCCTGCACCAGG

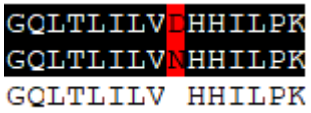
CTGGCCAGCTCACCCTCATCCTTGTT**A**ACCACCATATTA**C**T**T**CCCAAGTAAGTCAGTGAAGGACACTC
GGCCTGCACGGGGAGAAAGGAGGAAGGAGAGCCGTCCACTAGAGTCTCAAAGGCTCATTTGTTGT
CATTTTTTCTCGTTTGTTTGTTTGCTTGCTTCTCGTGTGAGATAGGGCCTCACTATGCAGCCTTGGCTG
GCCTCTAAATCACTATGTATTCCAGTCTAGCCCTGAAGTCAGAAATCCACCTGCCTCGGAGGCTAACT
ATAATTGATGGGTCATATCCAATAAAAAAAAATAGTATCACTTCTCTAGTTATGTGATGTTTATGATA
TAAAGAAACAAAAATATATAGTTAAATAATTATTCAAAATGTTCAAAATAACTTAAATTTGCCTTTAA
TGTCAGAACTTTTGCTGCTTTAAGATACAGGG

Prune1-D106N-EM1-B6 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_PRUNE1_D106N_F1	CACTCGCTCGGCTTGCTTAT
Geno_PRUNE1_D106N_R1	CCCTGTATCTTAAAGCAGCAAAAGT
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	63
Elongation time (min)	1.5
WT product size (bp)	1049
Mutant product size (bp)	1049
Notes	Sequenced with the following primers Geno_Prune1_F2 AGAGTTAGGGTGGGGGTTAC Geno_Prune1_R2 CCCATCAATTATAGTTAGCCTCC

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 sex chromosome.

Off-target site with ≤2 mismatches for the guide used were checked with the following primers:

Off-target site	Sequence	Type	Primers used (5'-3)
18:10652328-10652350	TGA**A**TTACTTGGGTAAAAT**T** GGG	Exonic	Geno_ Prune1_sgRNA2_OT1_F3 ACCATGTGACACGGCGTAA Geno_Prune1_sgRNA2_OT1_R3 AGCCCAAACATATCACCAAACAC Sequencing primers: Prune1_sgRNA2_OT1_SeqF1 GTAATCTTTCTAATGTGCGGGA Prune1_sgRNA2_OT1_SeqR1 AGTGCTGGGTTATGTAATCTAAAATG

All amplicons were sent for Sanger sequencing. No evidence of off-target cutting was observed at these sites in the correct mutants.

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	Prune1-D106N-UNI1
Forward Primer (5'-3')	ACGGCTCTCCTTCCTCCTTTC
Reverse Primer (5'-3')	CAGAGCCAGCCCTGATCTTC
Probe (5'-3')	CGTGCAGGCCGAGTGTCCTTC
Label	FAM-BHQ1

The ddPCR assay recognises sequence common to both the WT Prune1 and the D106N mutant. Therefore, WT controls and correctly targeted F1 D106N heterozygote animals will call at 2 copies.

Reference Assay Name	Dot1l
Forward primer (5'-3')	GCCCCAGCACGACCATT
Reverse primer (5'-3')	TAGTTGGCATCCTTATGCTTCATC
Probe (5'-3')	CCCAACAGGCCTGGATTCTCAATGC
Label	VIC

VIC-labelled reference assay for Dot1l gene.