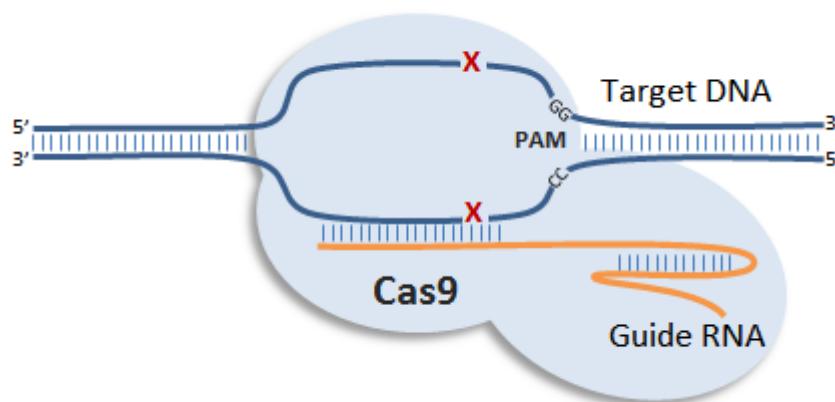
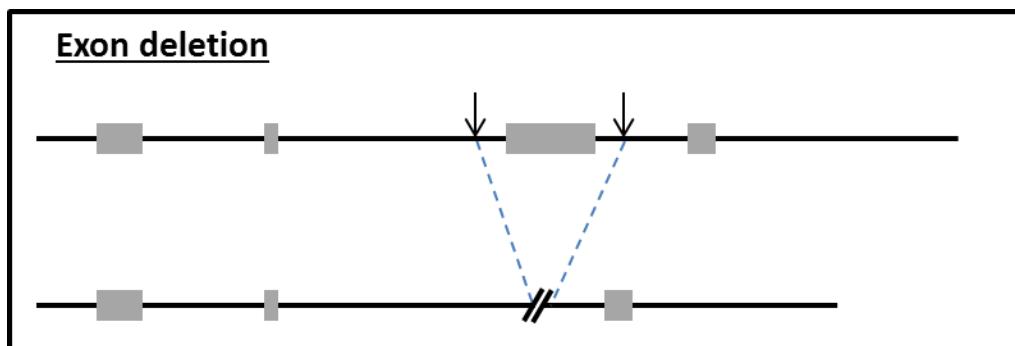


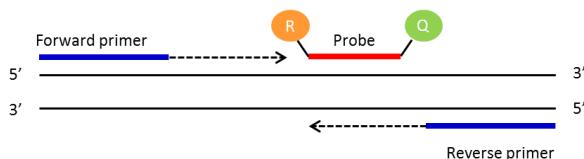
Mpeg1-CRE Genotyping Strategy

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).

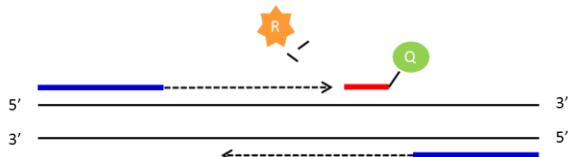


qPCR genotyping strategy

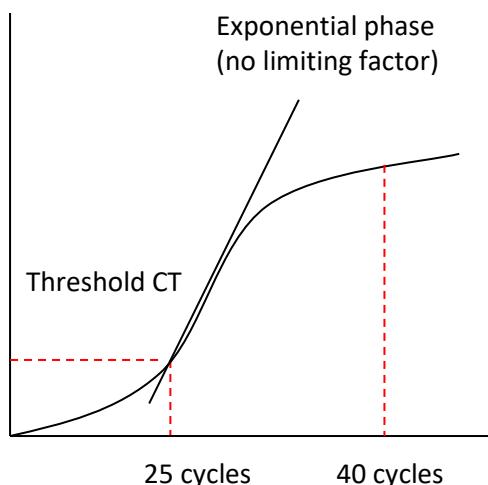
Standard PCR is the amplification of DNA between a pair of primers. Quantitative PCR employs the same principal as standard PCR, although it actually monitors the progress of the DNA synthesis as it occurs. The progress of the reaction is measured by using a Taqman probe. This is a short DNA oligo that 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.



PCR reaction plot



Exponential phase (no limiting factor)

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.

Mpeg1-CRE Genotyping Strategy

Samples are genotyped with both WT loss of allele (WT-LOA) and Mutant assays. Samples for this line are genotyped using the following primers and probe (see Figure1)

- Universal probe and Universal primer designed near the CRISPR deletion for both alleles.
- Wildtype specific primer in the deletion designed for the WT allele.
- Mutant specific primer that bridges the junction designed for the CRISPR mutant allele.

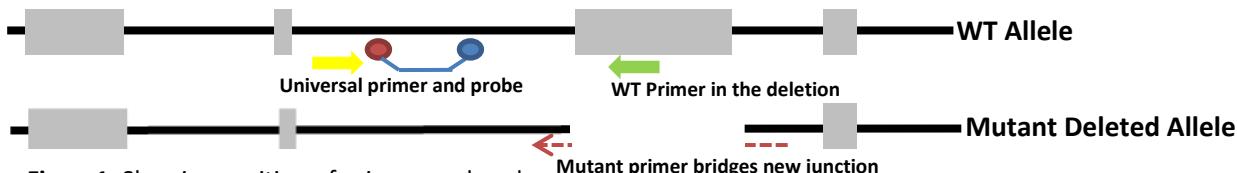


Figure1: Showing position of primers and probes

Mpeg1-CRE WT1 assay (FAM labelled)

TCGGAAGTACAAGAAGAAGGAATACCAGGAAATT GAGGAGCAGGAGAGTTGGT TCGAAGCTTAGCAACAGATGCAACAGTCC
TTAATGGAGAAGAGGATCCAAGTCCA GCTTAA [iCRE Insertion] TTGTCTCCAAAGGAAACAGTT TCCAGGCCACAGCT

Mpeg1-CRE-Univ-Probe (5nmol)
Mpeg1-CRE-WT-R (15nmol)
Mpeg1-CRE-Univ-F (15nmol)

TGGAAGCTTAGCAACAGATGCAACA
AACTGTTCTTGGAGACAATTAAGC
GAGGAGCAGGAGAGTTGGT

Mpeg1-CRE MUT1 assay (FAM labelled)

TCGGAAGTACAAGAAGAAGGAATACCAGGAAATT GAGGAGCAGGAGAGTTGGT TCGAAGCTTAGCAACAGATGCAACAGTCC
TTAATGGAGAAGAGGATCCAAGTCCAAGCTTAA cct GAATTCCGCCCCCTCCCTCCCCCCCCCTAAC CTTACTGGGGAAAGC
CGCT TCGAATAAGGCCGGTGTGCTTGTCTATGTTATTCCACCATATTGCCCTTTGGCAATGTGAGGGCCGGAA
ACCTGGCCCTGTCTTGTGACGAGCATTCCCTAGGGTCTTCCCCTCTGCCCAAAGGAATGCAAGGTCTGTTGAATGTCGTG
AGGAAGCAGTCCTCTGGAGACAAACACGTTCTGAAGACAAACACGTTCTGTAGCGACCCCTTGCAAGGCAGCGGAACCCCCCACCTGGC
GACAGGTGCCCTTGCGGCCAAAAGCCACGTGTATAAGATAACACCTGCAAAGGCGCACAACCCAGTGCCACGTTGTGAGTTG
GATAGTTGTGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGCTGAAGGATGCCAGAAGGTACCCATTGTA
TGGGATCTGATCTGGGGCCTCGGTGACATGCTTACATGTTAGTCAGGTTAAAAACGCTTAGGCCACACCATGTCCTAATTTACTGACCGTACAC
GGGACGTGGTTTCCTTGAAAAACACGATGATAAGCTGCAAGAACCTGATGGACATGTTCAAGGGATGCCAGGGCTTTCTGAGC
TGCATTACCGGTGATGCAACAGTGTAGGGTCTGCAAGAACCTGATGGACATGTTCAAGGGATGCCAGGGCTTTCTGAGC
ATACCTGGAAATGCTCTGCGTGTGGGGCTGGCGCATGGTCAAGTGAATAACCGGAAATGGTTCCCGCAGAA
CCTGAAGATGTTGCGATTATCTCTATCTCAGGGCGGGCTGGCAGTAAAAACTATCCAGCAACATTGGGCCAGCT
AAACATGCTTCATGTCGGTCTGGCGCAGCAAGTGAACAGCAATGCTGTTCACTGGTTATGCGGGGATCCGAAAG
AAAACGTTGATGGCGGTGAACAGTGCACAAACAGGCTCTAGCGTCAAGGACTGATTGCAAGGTTCTGTTACTCATGGAA
AATAGCGATCGCTGCCAGGATACTGTAATCTGGCATTCTGGGATTGCTTATAACACCCCTGTTACGTATAGCCGAAATTGC
CAGGATCAGGGTTAAAGGATATCTCACGTACTGACGGTGGGAGAATGTTAATCCATTGGCAGAACGAAAAGCTGGTTAGCA
CCGCAGGTGAGAGAAGGCACTTAGCCTGGGGTAACCTAAACTGGTCGAGCGATGGATTCCGCTCTGGTGTAGCTGATGAT
CCGAATAACTACCTGTTGCGGGTCAGAAAAATGGTGTGGCGCATCTGCCACCCAGCCAGCTATCAACTCGGCCCT
GGAAGGGATTGGAGACAACTCATGATTGATTTACGGCGCTAAGGATGACTCTGGTCAGAGATAACCTGGCTGGCTGGAC
ACAGTGGCCGTTGCGAGCGAGATATGGCCGCGTGGAGTTCAATACCGGAGATCATGCAAGCTGGCTGGAC
AATGTTAAATATTGTCATGAACTATATCCGTAACCTGGATAGTGAAACAGGGCAATGGTGCCTGCTGGAAGATGGCGATTG
ATTGTCCTCAAAGGAAACAGTTCCAGCCACAGCT

Mpeg1-CRE-Univ-Probe (5nmol)
Mpeg1-CRE-MUT-R (15nmol)
Mpeg1-CRE-Univ-F (15nmol)

TGGAAGCTTAGCAACAGATGCAACA
AGCGGCTTCGCCAGTAAC
GAGGAGCAGGAGAGTTGGT

Dot1l internal control (VIC labelled)

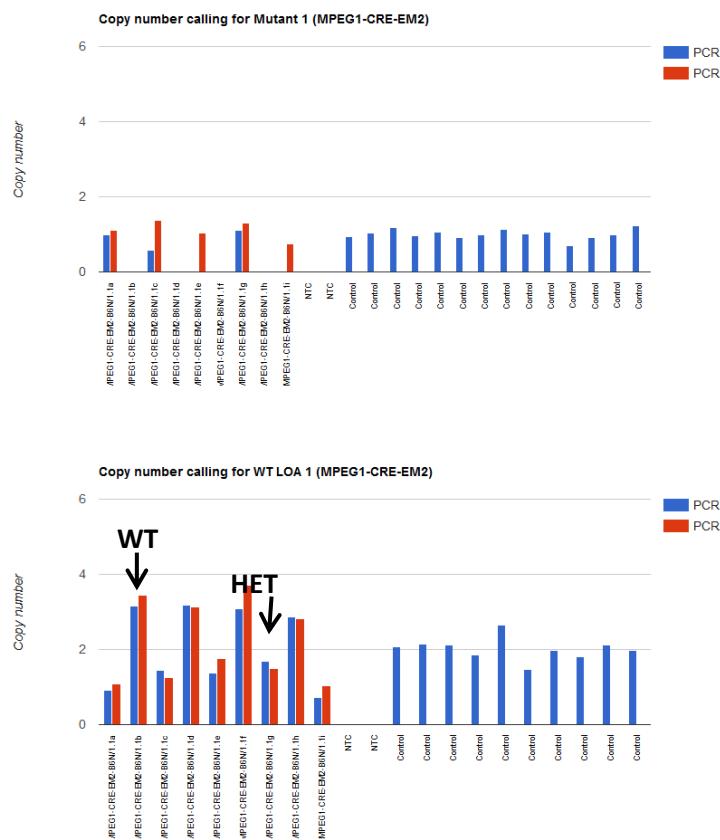
TGTTTCCTGTTCTTTCCCTCTAGTCGTTCTGTTAG TAGTTGGCATCCTTATGCTTCATCTTACAGT CGACTTGAGAGC
TGCCCTG AATGGTCGTGGGGCAAGGCTTATTCAGGGTAGCACACATGGTGGCAATGGACTCTGTAGGATCTGCC

Primer 1 = GCCCCAGCACGACCAATT
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
ddH2O	1.55µl
DNA (1/10 dilution of ABI Sample-to-SNP prep)	2.5µl

The EM3 line contains a random integration.

Mpeg1-CRE Assay copy called result, image showing both replicates and controls for WT and Mutant assays (T215656)

Version No. 1

Date: 05/09/18

Created/Updated by: Daniel Ford

Approved by: Debbie Williams

Name of Mouse model or mutation:**Mpeg1-CRE-EM1-B6N****MPEG1-CRE-EM2-B6N****Description:**

Insertion of an IRES Cre cassette at the 3' end of the Mpeg1 coding sequence

Type of mutation:

IRES Cre cassette knock-in using CRISPR/Cas9.

Delivery method:

Pronuclear injection into 1-cell stage embryo

Genetic Background:

C57BL/6NTac

Nuclease:

Cas9 mRNA

sgRNAs:

Protospacer sequence	PAM sequence
TCCAGCTTAATTGTCCTCAA	AGG
GGAGACAATTAAGCTGGACT	TGG

Long single-stranded donor sequence:

CTAGGAGTTGTCAATTACCTGGCCATCTATGGTACTCGGAAGTACAAGAAGAAAGGAATACCAGGAAATTGAG
GAGCAGGAGAGTTGGTGGAGCTTAGAACAGATGCAACAGTCCTTAATGGAGAAGAGGGATCCAAGTCCA
GCTTAAcctGAATTCCGCCCTCTCCCTCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGATAAAGGCCGG
TGTGCGTTGTCTATATGTTATTTCCACCATATTGCCGTCTTGGCAATGTGAGGGCCGGAAACCTGGCCC
TGTCTTCTTGACGAGCATTCTAGGGTCTTCCCTCTGCCAAAGGAATGCAAGGCTGTTGAATGTCGTGA
AGGAAGCAGTCCTCTGGAAGCTTCTGAAGACAAACACGCTGTAGCGACCCTTGCAGGCAGCGGAACC
CCCCACCTGGCGACAGGTGCCTCTGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCCGGCACAC
CCCACTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCAAGCGTATTCAACAAGGG
GCTGAAGGATGCCAGAAGGTACCCATTGTATGGGATCTGATCTGGGGCCTGGTCACATGCTTACATGT
GTTAGTCGAGGTAAAAAACGCTAGGCCCGAACACGGGGACGTGGTTCTTGAAAAACACGATG
ATAAGCTGCCACAACCAGTCCAATTACTGACCGTACACCAAAATTGCCATGCTGCAACGGTCATGCAAC
AGTGATGAGGTTCGCAAGAACCTGATGGACATGTTAGGGATGCCAGGCAGCTTGAGCATACTGGAAA
ATGCTTCTGTCGTTGCCGGTGTGGCGGATGGTCAAGTTGAATAACCGGAATGGTTCCCGCAGAAC
CTGAAGATGTTCGCGATTATCTTCTATCTTCAGGCCGCGGTCTGGCAGTAAAAACTATCCAGCAACATTG
GGCCAGCTAACATGCTCATGTCGGTCCGGCTGCCACGACCAAGTGACAGCAATGCTGTTACTGGTTA
TGCAGGCGGATCCGAAAAGAAAACGTTGATGCCGGTGAACGTGCAAAACAGGCTTAGCGTTGAACGCACTG
ATTTCGACCAGGTTGTTCACTCATGGAAAATAGCGATCGCTGCCAGGATATACGTAATCTGGCATTCTGGG
GATTGCTATAACACCCGTACGTAGCCGAAATTGCCAGGATCAGGGTAAAGATATCTCACGTACTGAC

GGTGGGAGAATGTTAACCATATTGGCAGAACGAAAACGCTGGTAGCACCGCAGGTGTAGAGAAGGCACCT
AGCCTGGGGTAACTAAACTGGTCAGCGATGGATTCCGTCTGGTAGCTGATGATCCGAATAACTACC
TGTTTGCCCCGGTCAGAAAAATGGTGTGCCGCCATCTGCCACCAGCCAGCTACACTCGGCCCTGGA
AGGGATTTGAAGCAACTCATCGATTACGGCGCTAAGGATGACTCTGGTCAGAGATACTGGCCTGG
TCTGGACACAGTGCCGTGCGAGCCGCGAGATATGCCCGCTGGAGTTCAATACCGGAGATCATG
CAAGCTGGTGGCTGGACCAATGTAATATTGTATGAACATATCCTAACCTGGATAGTGAACACAGGGCA
ATGGTGCCTGCTGGAGATGGCGATTGATTGTCTCAAAGGAAACAGTTCCAGGCCACAGCTTCAAGCACA
TCTTTGCTTGTCTACTCTGCCTCTAAGTGACTGAAGTGACAGTGCCTAGGAAGAAAGCAGCT
ATTACAACCTGGCAGTATTTCTTAGGC

Microinjection mixes:

Microinjection buffer (MIB; 10 mM Tris–HCl, 0.1 mM EDTA, 100 mM NaCl, pH7.5) was prepared and filtered through a 2 nm filter and autoclaved. Cas9 mRNA, sgRNAs and lssDNA donors were diluted and mixed in MIB to the working concentrations of 100 ng/µl, 50 ng/µl each and 50 ng/µl, respectively. Injected embryos were re-implanted in CD1 pseudo-pregnant females. Host females were allowed to litter and rear F₀ progeny.

Sequence details

WT

GTGGAAGCTGGAGAACCTCTGGAGCTTCGTAGGCCATGACAGTCATCCATGGGACAGTAATG
GAATGTCAGGAGGGAGCTGCTGGAATCACTTGGAGTCACCATAGCACTAGGAGTTGTCATTA
CCTTGGCCATCTATGGTACTCGGAAGTACAAGAAGAAGGAATACCAGGAAATTGAGGAGCAGGAG
AGTTTGGTTGGAAGCTTAGCAACAGATGCAACAGTCCTTAATGGAGAAGAGGATCCAAGTCCAGCT
TAA [iCRE Insertion]

TTGTCCTCAAAGGAAACAGTTCCAGGCCACAGCTCAAGCACATCTTGCTTGTCTACTTCT
GCCTTCCTAAAGTGACTGAAGTGACAGTCGCCATAGGAAGAAAGCAGCTATTACAACCTGGCAGTAT
TTCTTAGGCCTCTGCACCAGGAAATTAAAGGAGCCATTGAAGTGAGGTGTGGGAGGGGTATGA
TTCATTGGAAAAAGCTGGACTGGACTAAGCCTGGGTGTATGCGTCCATTCTCATAGTTGTCG
TACCTGAATGCATGTTGTGGAACGAGGGTGGGGAGAAATTGGATTGGCATTAGTCTGAA
TTGAAAGGCTTCCAGTTGTAAGCTGAAGAGGTCTGTGCTTAAAGTACATTACTTCTTCTGTG
TTCAAAGAGGCCCTCAGGGTTCAAGTAAACAGTGCCTGAAATGCCACAGGAGGCATGCC
CTACTCTTAGCTTAAGGACTTGGTGTGATTGACAGTGCCTCCCC

Mutant

GTGGAAGCTGGAGAACCTCTGGAGCTTCGTAGGCCATGACAGTCATCCATGGGACAGTAATG
GAATGTCAGGAGGGAGCTGCTGGAATCACTTGGAGTCACCATAGCACTAGGAGTTGTCATTA
CCTTGGCCATCTATGGTACTCGGAAGTACAAGAAGAAGGAATACCAGGAAATTGAGGAGCAGGAG
AGTTTGGTTGGAAGCTTAGCAACAGATGCAACAGTCCTTAATGGAGAAGAGGATCCAAGTCCAGCT
TAAcctGAATTCCGCCCTCCCTCCCCCCCCCTAACGTTACTGGCGAAGCCGCTTGAATAAGGC
CGGTGTGCGTTGTCTATATGTTATTTCCACCATATTGCCGTCTTGGCAATGTGAGGGCCGGAA
ACCTGGCCCTGTCTTGTGACGAGCATTCTAGGGTCTTCCCTCGCCAAAGGAATGCAAGGTC
TGTGAATGCGTGAAGGAAGCAGTTCTCTGGAGCTTCTGAAGACAAACAACGTCTGTAGCGAC
CCTTGAGGCAGCGAACCCCCCACCTGGCGACAGGTGCCTTGCGGCAAAAGGCCACGTGTATA

AGATACACCTGCAAAGGCAGACAACCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGT
 CAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCAGAAGGTACCCATTGTATG
 GGATCTGATCTGGGGCCTCGGTGCACATGTTACATGTGTTAGTCGAGGTTAAAAAACGTCAGG
 CCCCCGAACCACGGGACGTGGTTCTTGAACACGATGATAAGCTGCCACAACCAGTC
 CAATTACTGACCGTACACAAAATTGCCTGCATTACCGGTGATGCAACGAGTGTGAGGTTCGC
 AAGAACCTGATGGACATGTTAGGGATGCCAGGCAGGTTCTGAGGCATACTGGAAAATGCTCTGT
 CGTTGCCGGTGTGGCGCATGGTCAAGTTGAATAACCGGAAATGGTCCCGCAGAACCTG
 AAGATGTCGCGATTATCTTATATCTCAGGCAGCGGTCTGGCAGTAAAAACTATCCAGCAACA
 TTGGGCCAGCTAAACATGCTCATCGTCGGTCCGGCTGCCACGACCAAGTGACAGCAATGCTGT
 TCACTGGTTATGCCGGATCGAAAAGAAAAGTTGATGCCGGTGAACGTGCAAAACAGGCTCTA
 GCGTCGAACGCACTGATTCGACCAAGGTTGTTCACTCATGGAAAATAGCGATCGCTGCCAGGATA
 TACGTAATCTGGCATTCTGGGATTGCTTAAACACCTGTTACGTATAGCCAAATTGCCAGGATC
 AGGGTAAAGATATCTCACGTACTGACGGTGGAGAATGTTAATCCATTGGCAGAACGAAAAGC
 CTGGTAGCACCGCAGGTGTAGAGAAGGCACCTAGCCTGGGGTAACAAACTGGTCAGCGATG
 GATTCCGCTCTGGTAGCTGATGCCGAATAACTACCTGTTGCCGGTCAGAAAAATGGT
 GTGCCCGCCATCTGCCACCAAGCCAGCTATCAACTCGGCCCTGGAAGGGATTTGAAGCAACTC
 ATCGATTGATTACGGCGCTAAGGATGACTCTGGTAGAGAGATACTGGCTGGACACAGTC
 CCGTGTGGAGCCGAGATGGCCGAGATGGCCGCTGGAGTTCAATACCGGAGATCATGCAAGCTGG
 TGGCTGGACCAATGTAATATTGTCATGAACATATCCGTAACCTGGATAGTGAACACAGGGCAATG
 GTGCGCCTGCTGGAGATGGCGATTGATTGTCTCCAAGGAAACAGTTCCAGCCACAGCTCAAGC
 ACATCTTGCTTGTCTACTTCTGCCCTCTAAGTGAATGACTGAAGTGACAGTCGCCATAGGAAGA
 AAGCAGCTATTACAACCTGGCAGTATTTCTAGGCCTCTGCACCAGGAATTAAAGGAGGCCATT
 GAAGTGAGGTGTGGGAGGGGTATGATTGATTCATTGGAAAAAGCTGGACTGGACTAACGCTCGGG
 TGTATGCGCATTCTCATAGTTGTCTGTACCTGAATGCATGTGTGGAACGAGGGTGGGGAG
 AAATTGGATTGGTGCATTAGTCTGAATTGAAAGGCTTCAAGAGGCCCTCAGGGTTCAAGCTGAAGAGGTCTGT
 GCTTAAGTACATTACTCTTCTGTGTTCAAAGAGGCCCTCAGGGTTCAAGCTGAACACAGTC
 GAAATGCCACAGGAGGCCTACATGCCCTACTCTTAGCTTAAGGACTTGGTGTGATTGACAGT
 GCCCTCCC

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_Mpeg1_F1 (5'-3')	GTGGAAGCTGGAGAACCTC
Geno_Mpeg1_R1 (5'-3')	GGGAGGGCACTGTCAATCAA
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	63
Elongation time (min)	2.5
WT product size (bp)	873
Mutant product size (bp)	2412

Notes	
Cre_F1 (5'-3')	CGTTTCTGAGCATACTGGAA
Cre_R1 (5'-3')	ATTCTCCCACCGTCAGTACG
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	58
Elongation time (min)	1
Mutant product size (bp)	472
Notes	

Cre positive animals (those animals that showed amplification of both a mutant sized band (2412 bp) in the external PCR and in the Cre-specific PCR (472 bp)) were then interrogated further. We carried out two overlapping PCRs that gave shorter amplicons that were more amenable to getting full sequence coverage by Sanger sequencing:

Geno_Mpeg1_F1 (5'-3')	GTGGAAGCTGGGAGAACCTC
Cre_R1 (5'-3')	ATTCTCCCACCGTCAGTACG
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	58
Elongation time (min)	1.5
Mutant product size (bp)	1440

Cre_F2 (5'-3')	AAACGTTGATGCCGGTGAAC
Geno_Mpeg1_R1 (5'-3')	GGGAGGGCACTGTCAATCAA
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	58
Elongation time (min)	1.5
Mutant product size (bp)	1182

All amplicons were sent for Sanger sequencing to check for integration of the donor sequence at the target site.

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	Cre Assay
Forward Primer	CGCAAGAACCTGATGGACATG
Reverse Primer	ACCGGCAAACGGACAGAA
Probe	TTCAGGGATGCCAGGCAGTTT
Label	FAM-BHQ1

The ddPCR assay is a specific to the Cre recombinase sequence. WT controls are expected to call at 0 copies and correct mutants are expected to call at 1 copy for F1 (HET) animals.