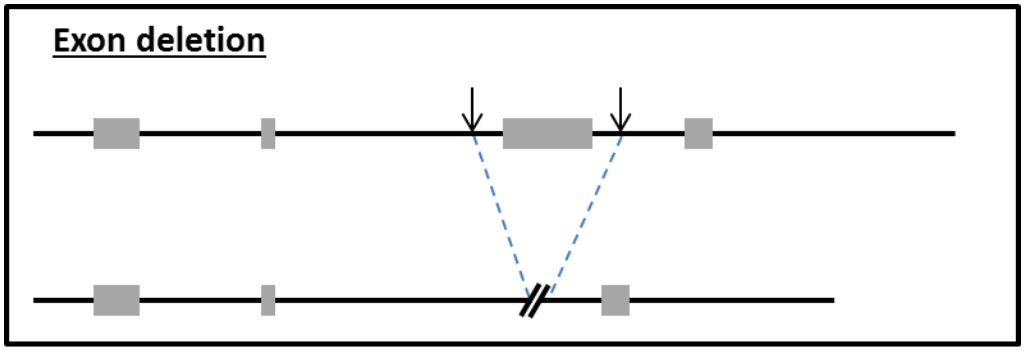
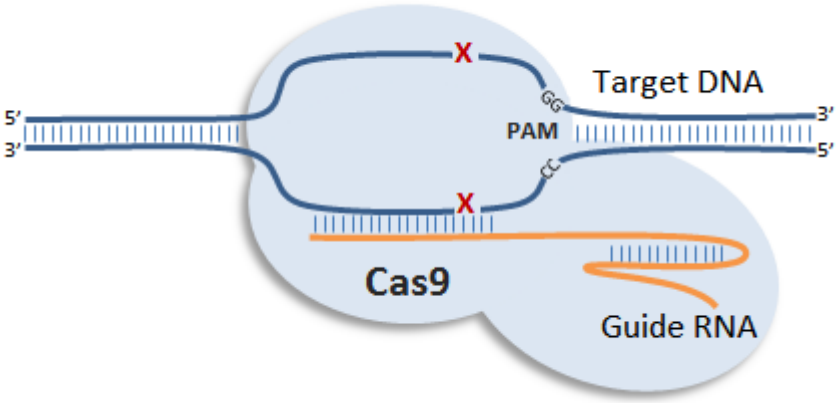
Mpeg1-CRE Genotyping Strategy

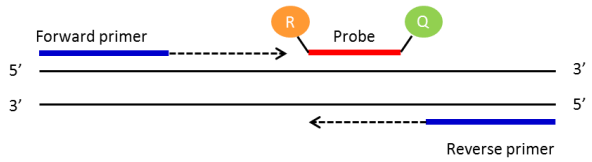
Animals have been engineered using the CRISPR/Cas9 technology. Most of the knockout alleles generated through this method will be obtained by deletion of a critical exon or by introduction of an indel (insertion/deletion) within the coding sequence of a critical exon (see picture below).



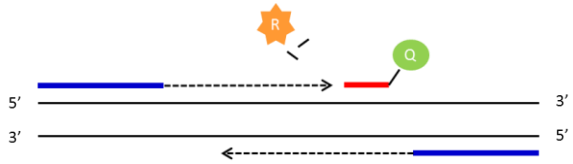


qPCR genotyping strategy

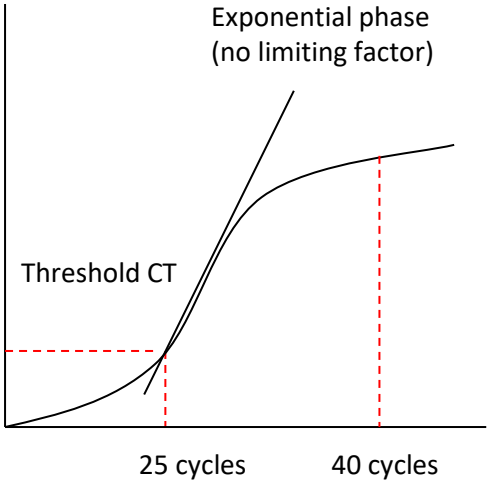
Standard PCR is the amplification of DNA between a pair of primers. Quantitative PCR employs the same principal as standard PCR, although it actually monitors the progress of the DNA synthesis as it occurs. The progress of the reaction is measured by using a Taqman probe. This is a short DNA oligo that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



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.

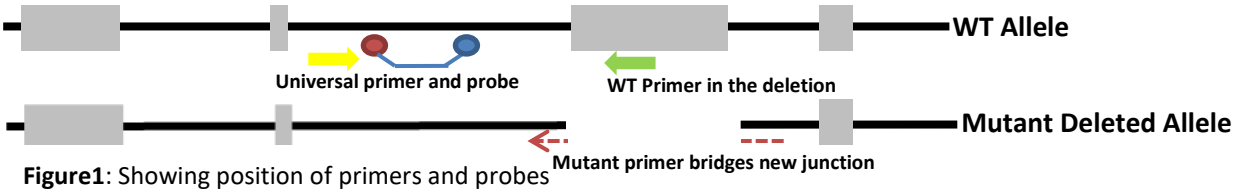


Figure1: Showing position of primers and probes

Mpeg1-CRE WT1 assay (FAM labelled)

TCGGAAGTACAAGAAGAAGGAATACCAGGAAATTGAGGAGCAGGAGAGTTTGGTTGGAAGCTTAGCAACAGATGCAACAGTCC
TTAATGGAGAAGAGGATCCAAGTCCAGCTTAA [iCRE Insertion]TTGTCTCCAAAGGAAACAGTTTCCAGCCACAGCT

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

Mpeg1-CRE MUT1 assay (FAM labelled)

TCGGAAGTACAAGAAGAAGGAATACCAGGAAATTGAGGAGCAGGAGAGTTTGGTTGGAAGCTTAGCAACAGATGCAACAGTCC
TTAATGGAGAAGAGGATCCAAGTCCAGCTTAAcctGAATTCCGCCCCTCTCCCTCCCCCCCCCCTAACGTTACTGGCCGAAGC
CGCTTGGAATAAGGCCGGTGTGCGTTTGTCTATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAA
ACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGA
AGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGC
GACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTG
GATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTA
TGGGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTCTAGGCCCCCCGAACCACG
GGGACGTGGTTTTCCTTTGAAAAACACGATGATAAGCTTGCCACAACCATGTCCAATTTACTGACCGTACACCAAAATTTGCC
TGCATTACCGGTCGATGCAACGAGTGATGAGGTTCGCAAGAACCTGATGGACATGTTCAGGGATCGCCAGGCGTTTTCTGAGC
ATACCTGGAAAATGCTTCTGTCCGTTTGCCGGTCGTGGGCGGCATGGTGCAAGTTGAATAACCGGAAATGGTTTCCCGCAGAA
CCTGAAGATGTTCGCGATTATCTTCTATATCTTCAGGCGCGCGGTCTGGCAGTAAAAACTATCCAGCAACATTTGGGCCAGCT
AAACATGCTTCATCGTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCTGTTTCACTGGTTATGCGGCGGATCCGAAAAG
AAAACGTTGATGCCGGTGAACGTGCAAAACAGGCTCTAGCGTTCGAACGCACTGATTTCGACCAGGTTCGTTCACTCATGGAA
AATAGCGATCGCTGCCAGGATATACGTAATCTGGCATTTCTGGGGATTGCTTATAACACCCTGTTACGTATAGCCGAAATTGC
CAGGATCAGGGTTAAAGATATCTCACGTACTGACGGTGGGAGAATGTTAATCCATATTGGCAGAACGAAAACGCTGGTTAGCA
CCGCAGGTGTAGAGAAGGCACTTAGCCTGGGGGTAACTAAACTGGTCGAGCGATGGATTTCCGTCTCTGGTGTAGCTGATGAT
CCGAATAACTACCTGTTTTGCCGGGTCAGAAAAAATGGTGTTGCCGCGCCATCTGCCACCAGCCAGCTATCAACTCGCGCCCT
GGAAGGGATTTTTGAAGCAACTCATCGATTGATTTACGGCGCTAAGGATGACTCTGGTCAGAGATACCTGGCCTGGTCTGGAC
ACAGTGCCCGTGTCGGAGCCGCGCGAGATATGGCCCGCGCTGGAGTTTCAATACCGGAGATCATGCAAGCTGGTGGCTGGACC
AATGTAAATATTGTCATGAACTATATCCGTAACCTGGATAGTGAAACAGGGGCAATGGTGCGCCTGCTGGAAGATGGCGATTG
ATTGTCTCCAAAGGAAACAGTTTCCAGCCACAGCT

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

Dot1l internal control (VIC labelled)

TGTTTCCTTGTTCTTTTCCCCTCTAGTCGTTTTCTGTTAGTAGTTGGCATCCTTATGCTTCATCTTACAGTCGACTTGAGAGC
TGGCCCTGAATGGTCGTGCTGGGGCAAGGCTTTATTTCAGGCGTAGCACACATGGTGGCCAATGGGACTCTGTAGGATCTGCC

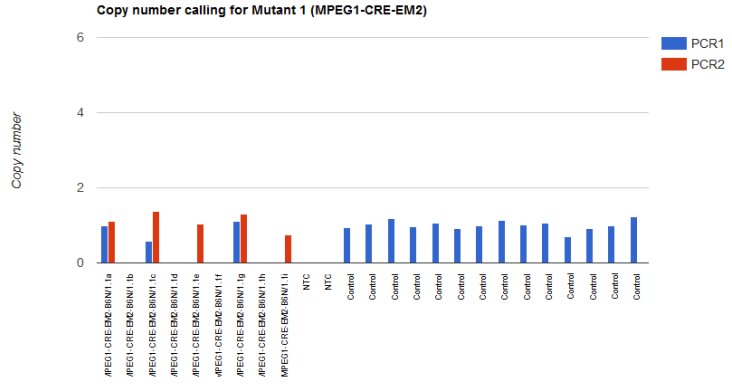
Primer 1 = GCCCCAGCACGACCATT
Primer 2 = TAGTTGGCATCCTTATGCTTCATC
Probe = CCAGCTCTCAAGTCG

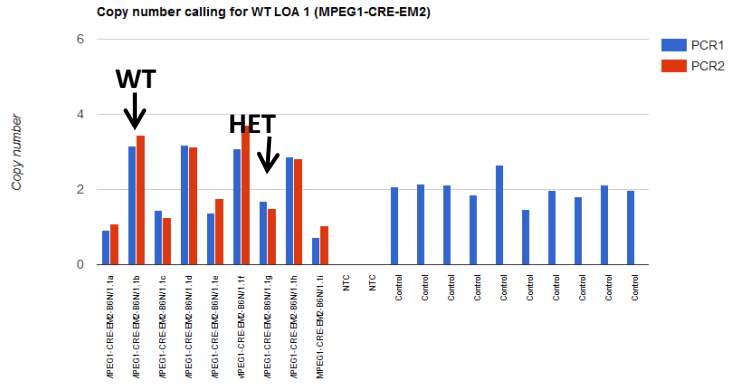
qPCR master mix

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

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
TCCAGCTTAATTGTCTCCAA	AGG
GGAGACAATTAAGCTGGACT	TGG

Long single-stranded donor sequence:
CTAGGAGTTGTCATTACCTTGGCCATCTATGGTACTCGGAAGTACAAGAAGAAGGAATACCAGGAAATTGAG
GAGCAGGAGAGTTTGGTTGGAAGCTTAGCAACAGATGCAACAGTCCTTAATGGAGAAGAGGATCCAAGTCCA
GCTTAAcctGAATTCCGCCCCTCTCCCTCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGG
TGTGCGTTTGTCTATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCC
TGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGA
AGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACC
CCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAAC
CCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGG
GCTGAAGGATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGT
GTTTAGTCGAGGTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATG
ATAAGCTTGCCACAACCATGTCCAATTTACTGACCGTACACCAAAATTTGCCTGCATTACCGGTCGATGCAACG
AGTGATGAGGTTCGCAAGAACCTGATGGACATGTTCAGGGATCGCCAGGCGTTTTCTGAGCATACCTGGAAA
ATGCTTCTGTCCGTTTGCCGGTCGTGGGCGGCATGGTGCAAGTTGAATAACCGGAAATGGTTTCCCGCAGAAC
CTGAAGATGTTCGCGATTATCTTCTATATCTTCAGGCGCGCGGTCTGGCAGTAAAAACTATCCAGCAACATTTG
GGCCAGCTAAACATGCTTCATCGTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCTGTTTCACTGGTTA
TGCGGCGGATCCGAAAAGAAAACGTTGATGCCGGTGAACGTGCAAAACAGGCTCTAGCGTTCGAACGCACTG
ATTTCGACCAGGTTCGTTCACTCATGGAAAATAGCGATCGCTGCCAGGATATACGTAATCTGGCATTTCTGGG
GATTGCTTATAACACCCTGTTACGTATAGCCGAAATTGCCAGGATCAGGGTTAAAGATATCTCACGTACTGAC

GGTGGGAGAATGTTAATCCATATTGGCAGAACGAAAACGCTGGTTAGCACCGCAGGTGTAGAGAAGGCACTT
AGCCTGGGGGTAACTAAACTGGTCGAGCGATGGATTTCCGTCTCTGGTGTAGCTGATGATCCGAATAACTACC
TGTTTTGCCGGGTCAGAAAAAATGGTGTTGCCGCGCCATCTGCCACCAGCCAGCTATCAACTCGCGCCCTGGA
AGGGATTTTTGAAGCAACTCATCGATTGATTTACGGCGCTAAGGATGACTCTGGTCAGAGATACCTGGCCTGG
TCTGGACACAGTGCCCGTGTCGGAGCCGCGCGAGATATGGCCCGCGCTGGAGTTTCAATACCGGAGATCATG
CAAGCTGGTGGCTGGACCAATGTAAATATTGTCATGAACTATATCCGTAACCTGGATAGTGAAACAGGGGCA
ATGGTGCGCCTGCTGGAAGATGGCGATTGATTGTCTCCAAAGGAAACAGTTTCCAGCCACAGCTTCAAGCACA
TCTTTTGCTTTGTTTTCTCTACTTCTGCCTTCCTAAGTGACTGAAGTGACAGTCGCCATAGGAAGAAAGCAGCT
ATTACAACCCTGGCAGTATTTTCTTAGGC

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 F_0 progeny.

Sequence details

WT

GTGGAAGCTGGGAGAACCTCTGGAGCTTCGTAGGGCCATGACAGTCATCCATGGGGACAGTAATG
GAATGTCAGGAGGGGAAGCTGCTGGAATCACTTTGGGAGTCACCATAGCACTAGGAGTTGTCATTA
CCTTGGCCATCTATGGTACTCGGAAGTACAAGAAGAAGGAATACCAGGAAATTGAGGAGCAGGAG
AGTTTGGTTGGAAGCTTAGCAACAGATGCAACAGTCCTTAATGGAGAAGAGGATCCAAGTCCAGCT
TAA [iCRE Insertion]
TTGTCTCCAAAGGAAACAGTTTCCAGCCACAGCTTCAAGCACATCTTTTGCTTTGTTTTCTCTACTTCT
GCCTTCCTAAGTGACTGAAGTGACAGTCGCCATAGGAAGAAAGCAGCTATTACAACCCTGGCAGTAT
TTTCTTAGGCCTCTGCACCAGGAAATTAAAGGAGCCATTTGAAGTGAGGTGTGGGGAGGGGTATGA
TTCATTTGGAAAAAGCTGGACTGGGACTAAGCCTCGGGGTGTATGCGTCCATTCTCATAGTTGTCTG
TACCTGAATGCATGTGTGTGGAACGAGGGGTGGGGGAGAAATTGGATTTGGTGCATTTAGTCTGAA
TTTGAAAGGCTTTCCAGTTTGTAAGCTGAAGAGGTCTGTGCTTTAAGTACATTACTTCTTTTCTTGTGT
TTTCAAAGAGGCCCTCAGGGTTTCAGTAAACAGTGCCCTGAAATGCCACAGGAGGCCTACATGCCCC
CTACTCTTTAGCTTTAAGGACTTGGTGTTGATTGACAGTGCCCTCCC

Mutant

GTGGAAGCTGGGAGAACCTCTGGAGCTTCGTAGGGCCATGACAGTCATCCATGGGGACAGTAATG
GAATGTCAGGAGGGGAAGCTGCTGGAATCACTTTGGGAGTCACCATAGCACTAGGAGTTGTCATTA
CCTTGGCCATCTATGGTACTCGGAAGTACAAGAAGAAGGAATACCAGGAAATTGAGGAGCAGGAG
AGTTTGGTTGGAAGCTTAGCAACAGATGCAACAGTCCTTAATGGAGAAGAGGATCCAAGTCCAGCT
TAAcctGAATTCCGCCCCTCTCCCTCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGC
CGGTGTGCGTTTGTCTATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAA
ACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTC
TGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGAC
CCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATA

AGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGT
CAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATG
GGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTCTAGG
CCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAAGCTTGCCACAACCATGTC
CAATTTACTGACCGTACACCAAAATTTGCCTGCATTACCGGTCGATGCAACGAGTGATGAGGTTCGC
AAGAACCTGATGGACATGTTCAGGGATCGCCAGGCGTTTTCTGAGCATACCTGGAAAATGCTTCTGT
CCGTTTGCCGGTCGTGGGCGGCATGGTGCAAGTTGAATAACCGGAAATGGTTTCCCGCAGAACCTG
AAGATGTTCGCGATTATCTTCTATATCTTCAGGCGCGCGGTCTGGCAGTAAAAACTATCCAGCAACA
TTTGGGCCAGCTAAACATGCTTCATCGTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCTGTT
TCACTGGTTATGCGGCGGATCCGAAAAGAAAACGTTGATGCCGGTGAACGTGCAAAACAGGCTCTA
GCGTTCGAACGCACTGATTTCGACCAGGTTCGTTCACTCATGGAAAATAGCGATCGCTGCCAGGATA
TACGTAATCTGGCATTTCTGGGGATTGCTTATAACACCCTGTTACGTATAGCCGAAATTGCCAGGATC
AGGGTTAAAGATATCTCACGTACTGACGGTGGGAGAATGTTAATCCATATTGGCAGAACGAAAACG
CTGGTTAGCACCGCAGGTGTAGAGAAGGCACTTAGCCTGGGGGTAACTAAACTGGTCGAGCGATG
GATTTCCGTCTCTGGTGTAGCTGATGATCCGAATAACTACCTGTTTTGCCGGGTCAGAAAAAATGGT
GTTGCCGCGCCATCTGCCACCAGCCAGCTATCAACTCGCGCCCTGGAAGGGATTTTTGAAGCAACTC
ATCGATTGATTTACGGCGCTAAGGATGACTCTGGTCAGAGATACCTGGCCTGGTCTGGACACAGTGC
CCGTGTCGGAGCCGCGCGAGATATGGCCCGCGCTGGAGTTTCAATACCGGAGATCATGCAAGCTGG
TGGCTGGACCAATGTAAATATTGTCATGAACTATATCCGTAACCTGGATAGTGAAACAGGGGCAATG
GTGCGCCTGCTGGAAGATGGCGATTGATTGTCTCCAAAGGAAACAGTTTCCAGCCACAGCTTCAAGC
ACATCTTTTGCTTTGTTTTCTCTACTTCTGCCTTCCTAAGTGACTGAAGTGACAGTCGCCATAGGAAGA
AAGCAGCTATTACAACCCTGGCAGTATTTTCTTAGGCCTCTGCACCAGGAAATTAAAGGAGCCATTT
GAAGTGAGGTGTGGGGAGGGGTATGATTCATTTGGAAAAAGCTGGACTGGGACTAAGCCTCGGGG
TGTATGCGTCCATTCTCATAGTTGTCTGTACCTGAATGCATGTGTGTGGAACGAGGGGTGGGGGAG
AAATTGGATTTGGTGCATTTAGTCTGAATTTGAAAGGCTTTCCAGTTTGTAAGCTGAAGAGGTCTGT
GCTTTAAGTACATTACTTCTTTTCTTGTGTTTTCAAAGAGGCCCTCAGGGTTTCAGTAAACAGTGCCCT
GAAATGCCACAGGAGGCCTACATGCCCCCTACTCTTTAGCTTTAAGGACTTGGTGTTGATTGACAGT
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’)	GTGGAAGCTGGGAGAACCTC
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’)	CGTTTTCTGAGCATACCTGGA
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-sepcifc 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	TTCAGGGATCGCCAGGCGTTT
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.