Name of Mouse model or mutation:
TRA2B-FLOX-EM1-B6

Description:
Introduction of floxed exon by CRISPR/Cas9.

Type of mutation:
Floxed poisoned exon of Tra2b gene.

Sequence details
Tra2b WT
GTTCCAACAGTGTTCCAGTTTGTATTAAAAATTCACAAATATGCTAAAGTGTGGGCCTTTTTAATAAT
TTGAGAAGATTAACTATAACTTTCTCATAACCAACTTTTGAACTAGGATTTCTCCTTTGGACATCGAG
AAGATTAATATTTTGTTAATAGAGGGGGAAACTTACAGAATTCTTTGGACATAACCACTGTTGGAAT
CAAGTTTGCTCTGTTTTTCTTTATAGCTGTTGGCATGTAATAGTTGATTAACTTTATTTAGTATCAGGA
TTTTAGTTCTAAATTTAGTAATTAAATTAAACTCTTAGTTTCACTAATGCAGATTTCCCAAGGGTGGGT
TGAACAGATCTATAGGTTTGCTGACATTGTCTAACAATGCTGTTTTTAAATACACAGTAATGTGGCAT
TAAATCCATTTCCTTATCTAGACAAGTCACAGTATTGTAAACCTCTTCAACCCCAGCCTTTGTCATCAT
TGTGCAGATGAGTTGTCCGTGCTTTTCTTTCTTGCACTTTTGTACTAAAATGTGTCATTTTTTTGACTA
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GTAATTACTAGTCTGTTTATGTGTCCCTGTATATTTTGCTAAACATGCATGCATTATTTGTTTAATAGA
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TGCCAAATGAGGATGATTCTTTTATATTGTTTCAGTTTAACGCCTGTTCCAACTGAACTA

TRA2B-FLOX-EM1-B6

GTTCCAACAGTGTTCCAGTTTGTATTAAAAATTCACAAATATGCTAAAGTGTGGGCCTTTTTAATAAT
TTGAGAAGATTAACTATAACTTTCTCATAACCAACTTTTGAACTAGGATTTCTCCTTTGGACATCGAG
AAGATTAATATTTTGTTAATAGAGGGGGAAACTTACAGAATTCTTTGGACATAACCACTGTTGGAAT
CAAGTTTGCTCTGTTTTTCTTTATAGCTGTTGGCATGTAATAGTTGATTAACTTTATTTAGTATCAGGA
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gagGCGATCGCATAACTTCGTATAGCATACATTATACGAAGTTATTAAATACACAGTAATGTGGCATT
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AAAATGGGACTAAACCCCCCAAATCTAAGCAAAGCATCTAACACCATTATTTATATTGTGATGAAAG
CACTAGGTTTTCACAAATTTGTGGAAAGCTATACCGCTTAAGTCCAAATGTTGAGATCTACAAGTGCC
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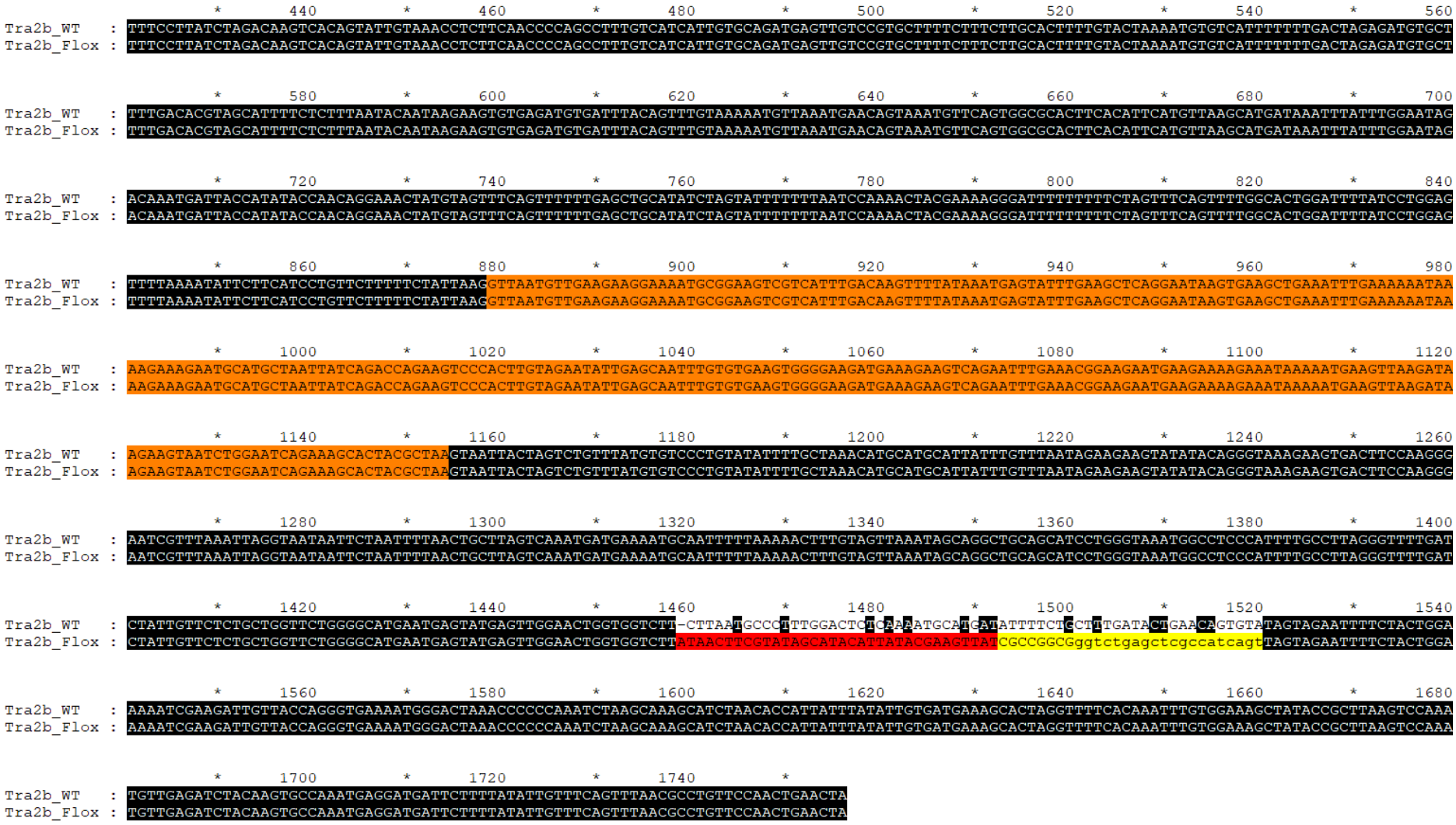
Nucleotide Alignment: Orange is poisoned exon, Red is LoxP sites, Yellow are universal primer and RE sites



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_Tra2b _F1 (5'-3')	GTTCCAACAGTGTTCCAGTTTGT
Geno_Tra2b _R1 (5'-3')	TAGTTCAGTTGGAACAGGCGTT
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	63
Elongation time (min)	2
WT product size (bp)	1752
Mutant product size (bp)	1756
Notes	

LoxP PCR:

LoxPF	ATCCGGGGGTACCGCGTCGAG
LoxPR	ACTGATGGCGAGCTCAGACC
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	61
Elongation time (min)	3
Mutant product size (bp)	1193
Notes	This is a generic LoxP PCR hence longer extension time than required for amplicon size.

We also performed PCRs to check integration of each LoxP site by conducting a PCR from the LoxP site to external primers:

LoxPF	ATCCGGGGGTACCGCGTCGAG
Geno_Tra2b _R1 (5'-3')	TAGTTCAGTTGGAACAGGCGTT
Taq Polymerase used	Roche Expand Long Range DNTPack

Annealing Temperature (°C)	63
Elongation time (min)	2
Mutant product size (bp)	1520

Geno_Tra2b _F1 (5'-3')	GTTCCAACAGTGTTCCAGTTTGT
LoxPR	ACTGATGGCGAGCTCAGACC
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	63
Elongation time (min)	2
Mutant product size (bp)	1429

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	Tra2b-FLOX-DONOR-MUT1
Forward Primer	CGATCGCATAACTTCGTATAGCATACAT
Reverse Primer	GGACAACTCATCTGCACAATGATG
Probe	ACACAGTAATGTGGCATTAAATCCATTTCC
Label	FAM-BHQ1

This ddPCR assay recognises the 5' LoxP site specific to the Tra2b FLOX modification. Therefore, WT controls are expected to call at 0 copies and correct mutants are expected to call at 1 copy for F1 (HET) animals.

Assay name	Tra2b-FLOX-3'-MUT1
Forward Primer	GGCATGAATGAGTATGAGTTGGAAC
Reverse Primer	AGTCCCATTTTCACCCTGGTAAC

Probe	AAGTTATCGCCGGCGGGTCTGA
Label	FAM-BHQ1

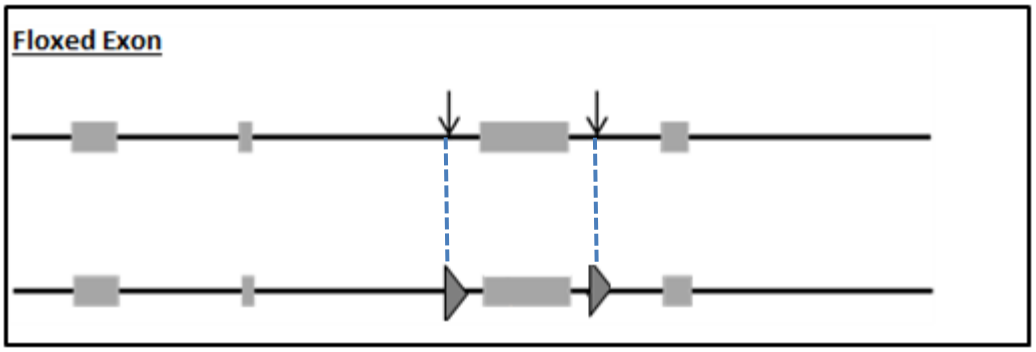
This ddPCR assay recognises the 3' LoxP site specific to the Tra2b FLOX modification. Therefore, WT controls are expected to call at 0 copies and correct mutants are expected to call at 1 copy for F1 (HET) animals.

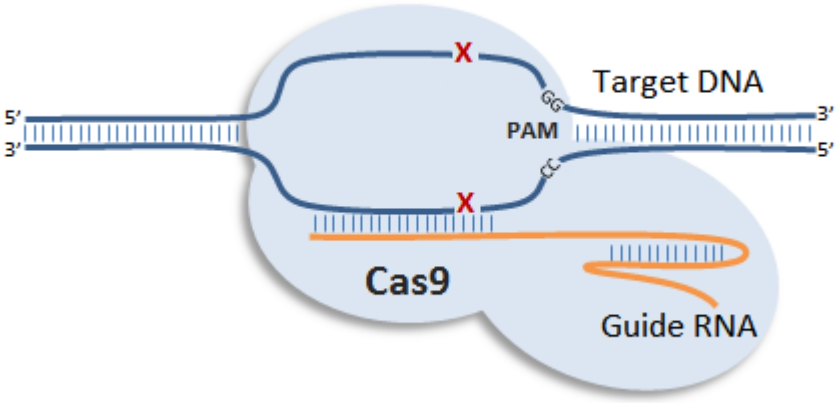
Assay name	Tra2b-CR-LOA
Forward Primer	GCGGAAGTCGTCATTTGACAAG
Reverse Primer	TCCCCACTTCACACAAATTGCT
Probe	TTGAAGCTCAGGAATAAGTGAAGCTGA
Label	FAM-BHQ1

This ddPCR assay recognises the Tra2b poisoned exon. Therefore, WT controls and correct mutants are expected to call at 2 copies for F1 (HET) animals.

Tra2b-FLOX Genotyping Strategy

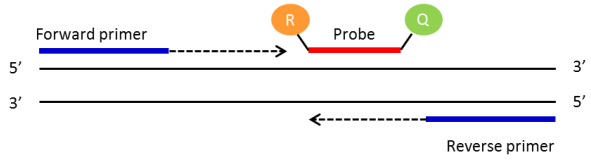
Animals have been engineered using the CRISPR/Cas9 technology. Most of the knockout alleles generated through this method will be obtained by deletion of a critical exon or by introduction of indel (insertion/deletion) within the coding sequence of a critical exon. There is also a possibility of inserting sequences like LoxP sites around critical exon to create floxed allele (see picture below).



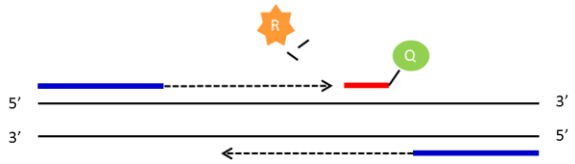


qPCR genotyping strategy

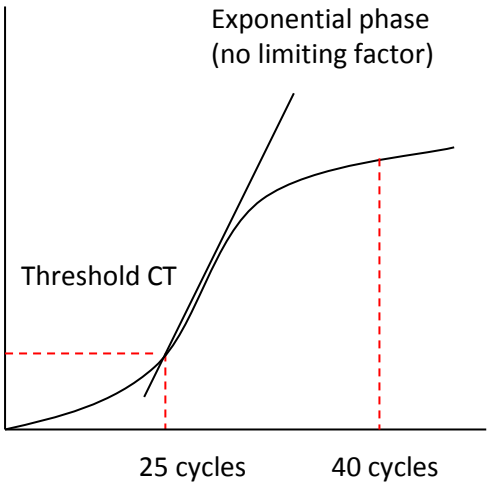
Standard PCR is the amplification of DNA between a pair of primers. Quantitative PCR employs the same principal as standard PCR, although it actually monitors the progress of the DNA synthesis as it occurs. The progress of the reaction is measured by using a Taqman probe. This is a short DNA oligo that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

Tra2b-FLOX Genotyping Strategy

Samples are genotyped with a break point loss of allele (BP-LOA) and mutant assays. These are FAM labelled assays that are designed to detect the critical exon that has been floxed. If the animal contains the modified allele the copy number of the BP-LOA assay should drop by 1 and mutant assay increase by 1. For autosomal genes that have been targeted this means the following

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

Tra2b-FLOX-3'-WT1 assay (FAM labelled probe)

Fragment Sequence
The following sequence is the 3' end of the Tra2b gene sequence at which the 3' loxp is inserted. Black sequence is missing in Tra2b-FLOX allele due to the insertion of loxp sites and can be used to design BP-LOA qPCR assay.

CAGCATCCTGGGTAAATGGCCTCCCATTTTGCCTTAGGGTTTTGATCTATTGTTCTCTGCTGGTTCTGGGGCATG
AATGAGTATGAGTTGGAACTGGTGGTCTTCTTAATGCCCTTTGGACTCTCAAAATGCATGATATTTTCTGCTTTG
ATACTGAACAGTGTATAGTAGAATTTTCTACTGGAAAAATCGAAGATTGTTACCAGGGTGAAAATGGGACTAAAC

Primer 1 = GAGTTGGAACTGGTGGTCTTC
Primer 2 = AGTCCCATTTTCACCCTGGTAAC
Probe = TGGACTCTCAAAATGCATGATATTTTCTGC

Tra2b-FLOX-3'-MUT1assay (FAM labelled probe)

The following sequence is the 3' end of the Tra2b-FLOX allele and sequence in lower case letters is the mutant sequence

CAGCATCCTGGGTAAATGGCCTCCCATTTTGCCTTAGGGTTTTGATCTATTGTTCTCTGCTGGTTCTGGGGCATG
AATGAGTATGAGTTGGAACTGGTGGTCTTataacttcgtatagcatacattatacgaagttatcgccggcgggtc
tgagctcgccatcagtTAGTAGAATTTTCTACTGGAAAAATCGAAGATTGTTACCAGGGTGAAAATGGGACTAAA

Primer 1 = GGCATGAATGAGTATGAGTTGGAAC
Primer 2 = AGTCCCATTTTCACCCTGGTAAC
Probe = AAGTTATCGCCGGCGGGTCTGA

Dot1l internal control (VIC labelled)

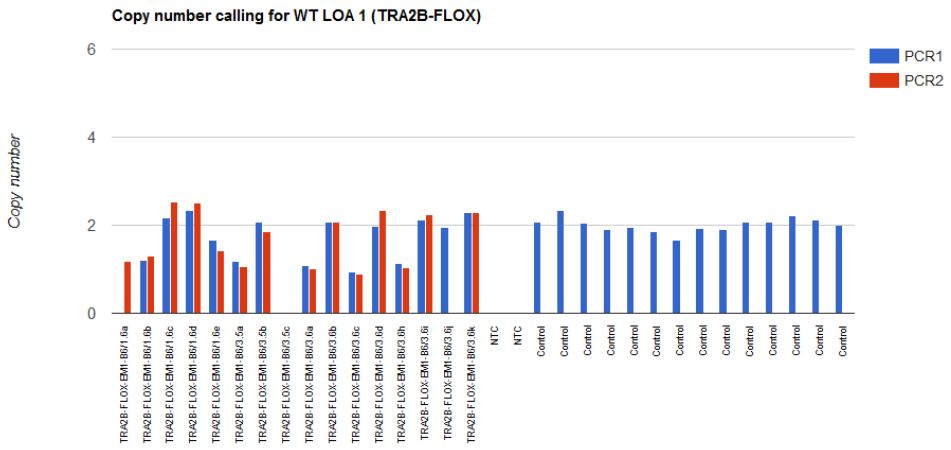
TCATAGGGTGACTGGCCAACCCAGGGAAGCCGGAGTGCTGCGTCTTCTGTTTCCTTGTTCTTTTCCCCTCTAGTC
GTTTTCTGTTAGTAGTTGGCATCCTTATGCTTCATCTTACAGTCGACTTGAGAGCTGGCCCTGAATGGTCGTGCT
GGGGCAAGGCTTTATTTCAGGCGTAGCACACATGGTGGCCAATGGGACTCTGTAGGATCTGCCCACACCCATCAG

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

Tra2b-FLOX LOA copy called result, image showing both replicates and controls



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