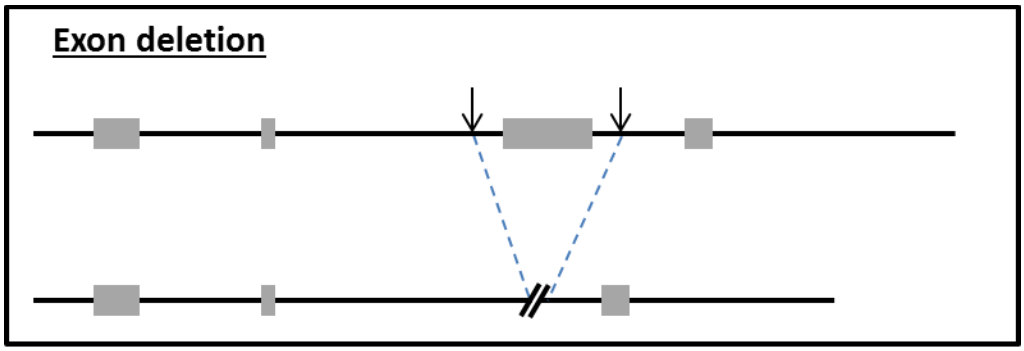
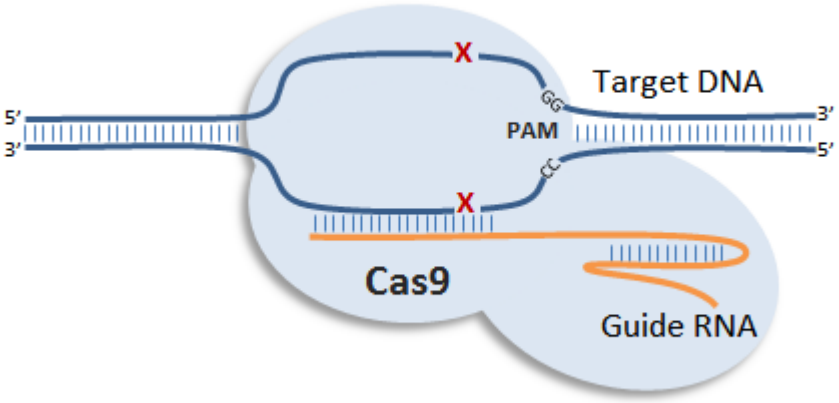
Inpp5k-DEL1152 Genotyping Strategy

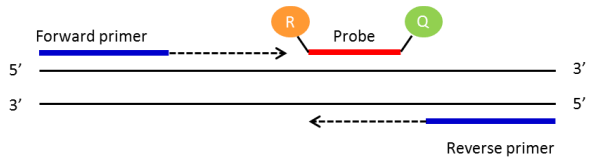
Animals have been engineered using the CRISPR/Cas9 technology. Most of the knockout alleles generated through this method will be obtained by deletion of a critical exon or by introduction of an indel (insertion/deletion) within the coding sequence of a critical exon (see picture below).



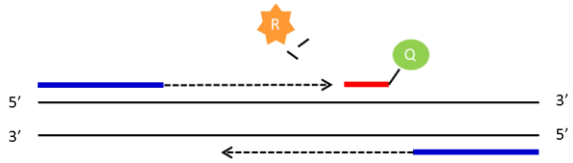


qPCR genotyping strategy

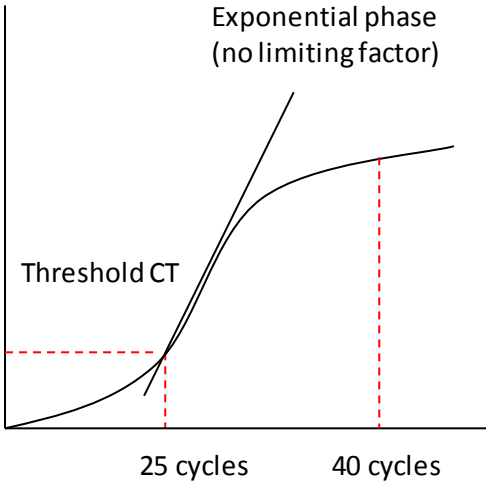
Standard PCR is the amplification of DNA between a pair of primers. Quantitative PCR employs the same principal as standard PCR, although it actually monitors the progress of the DNA synthesis as it occurs. The progress of the reaction is measured by using a Taqman probe. This is a short DNA oligo that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

Inpp5k-DEL1152 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.

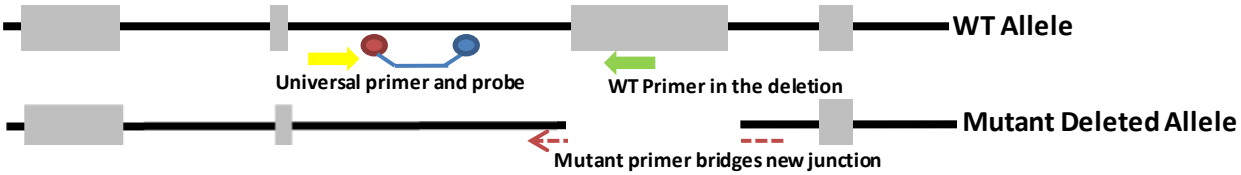


Figure1: Showing position of primers and probes

Inpp5k-DEL1152 WT1 assay (FAM labelled)

AACCCATAGGTTGAGGAGTAAGCATGCAGAGTTGCTGACTGGGCCACAGAGGATGAGTGAGGTTTCTGTAGCTGG
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Inpp5k-DEL1152-Univ-Probe (5nmol)	CAGAGTTGCTGACTGGGCCACA
Inpp5k-DEL1152-WT-R (15nmol)	GCAGGTTTACAGTCGCCCATAG
Inpp5k-DEL1152-Univ-F (15nmol)	AACCCATAGGTTGAGGAGTAAGCAT

Inpp5k-DEL1152 MUT1 assay (FAM labelled)

AACCCATAGGTTGAGGAGTAAGCATGCAGAGTTGCTGACTGGGCCACAGAGGATGAGTGAGGTTTCTGTAGCTGG
AGGGGCTAATAGGTTCCTGTCTCTCAAACGCAATCATTCTTTCCTTCTGGCCAATGGCATTTGAGCTCCCTTTGG
CAG [1152 nt deletion] GGGTTAGCTTCACACCTAGATGTTTTCTCAGTCTTATTCCTGGCC

Inpp5k-DEL1152-Univ-Probe (5nmol)	CAGAGTTGCTGACTGGGCCACA
Inpp5k-DEL1152-MUT-R (15nmol)	GGTGTGAAGCTAACCCCTGC
Inpp5k-DEL1152-Univ-F (15nmol)	AACCCATAGGTTGAGGAGTAAGCAT

Dot1l internal control (VIC labelled)

TCATAGGGTGACTGGCCAACCCAGGGAAGCCGGAGTGCTGCGTCTTCTGTTTCCTTGTTCTTTTCCCCTCTAGTC
GTTTTCTGTTAGTAGTTGGCATCCTTATGCTTCATCTTACAGTCGACTTGAGAGCTGGCCCTGAATGGTCGTGCT
GGGGCAAGGCTTTATTTCAGGCGTAGCACACATGGTGGCCAATGGGACTCTGTAGGATCTGCCCACACCCATCAG

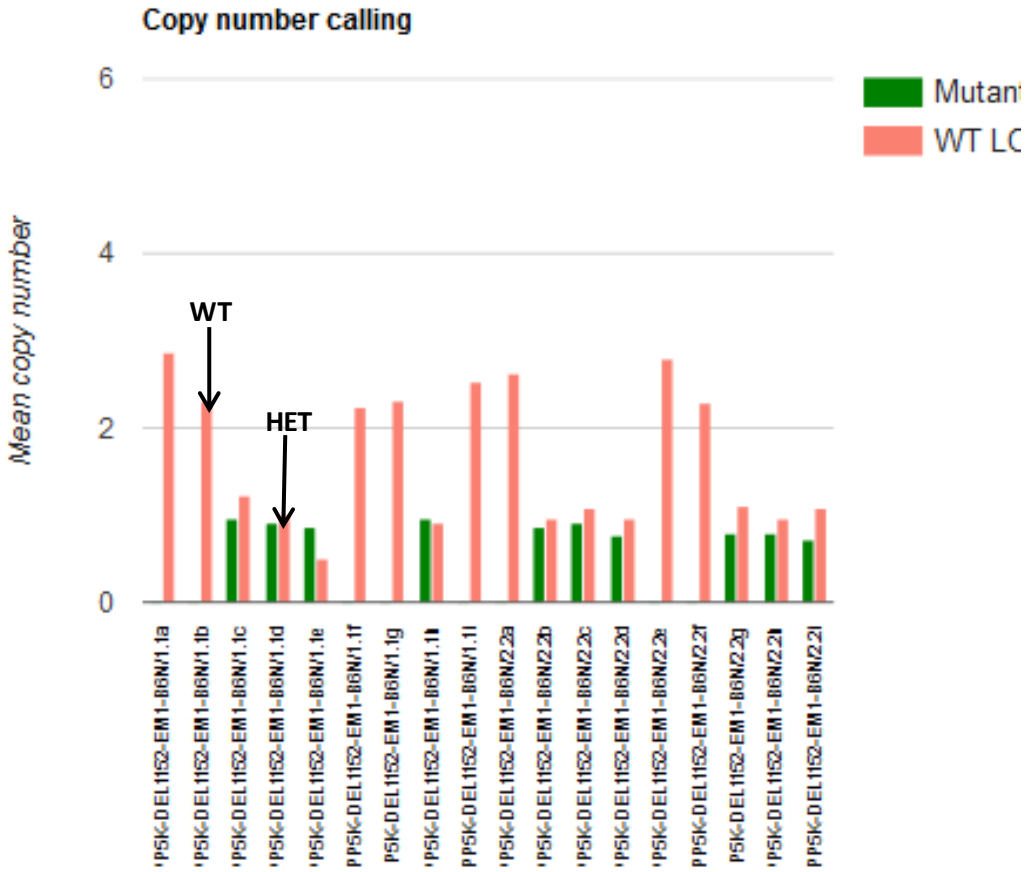
Primer 1 = GCCCCAGCACGACCATT
Primer 2 = TAGTTGGCATCCTTATGCTTCATC
Probe = CCAGCTCTCAAGTCG

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

Inpp5k-DEL1152 Assay copy called result, image showing both WT and Mutant assays

Task 207650 Results



Version No.	1
Date:	23.05.2018
Created/Updated by:	Ramakrishna Kurapati
Approved by:	Deen Quwailid

Name of Mouse model or mutation:
INPP5K-FLOX-EM3-B6N

Description:
Floxed model made using CRISPR/Cas9.

Type of mutation:
Floxed exons: ENSMUSE00001230278 and ENSMUSE00001223975

Delivery method:
Pronuclear injection into 1-cell stage embryo.

Genetic Background:
C57BL/6NTac

Nuclease:
Cas9 mRNA

sgRNAs:

Protospacer sequence	PAM sequence
TGCCAGGTACTGCCAGCTAT	GGG
GGTTTACAGTCGCCCATAGC	TGG
CTAGGTGTGAAGCTAACCCC	TGG
AGTCTGAGCGATGAGTCCAG	GGG

lssDNA donor sequence (5'-3'):

```
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                     /note="AsiSI (SfaAI)"
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                     /note="LoxPF"
                     /current=0
     misc_feature    1..100
                     /note="5' homology arm"
     misc_feature    164..1232
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     exon            544..651
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     exon            938..1046
                     /note="ENSMUSE00001223975"
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    misc_feature    1267..1274
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                    /note="LoxPR"
                    /current=0
    misc_feature    1295..1394
                    /note="3' homology arm"
    conflict        272..272
                    /note="Erroneous base (C>T change)"
    source          1..1394
                    /dnas_title="Inpp5k donor EM3"
ORIGIN
      1 taagcatgca gagttgctga ctgggccaca gaggatgagt gaggtttctg tagctggagg
     61 ggctaatagg ttcctgtctc tcaaacgcaa tcattctttc atccgggggt accgcgtcga
    121 gGCGATCGCA TAACTTCGTA TAGCATACAT TATACGAAGT TATctgtaaa cctgcctaga
    181 agagccagtt ggagggaggg aagagattcc tgctttccag gcatgaaagg gaaggtgaaa
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    301 catagtgaga ttaacaacgt taatctcaga atttaggagg cagaagaagg ctgatctccg
    361 taaggagttg aggccagcca gggctgcata gtgagacaca gcctcaaaag caccaaacaa
    421 acaaacatgc agacacattt cccatgatca ctgcagcgga agccttgcac atacctaggt
    481 ctttgcctgc tatctggaaa aatcttagcc tccatcccct gtgacttaac atcctttcca
    541 cagCGTACAT GTTGTGACGT GGAATGTGGC CTCCGCAGCC CCCACTGTAG ACCTCAGTGA
    601 CCTACTTCAA CTGAACAACC AAGACCTGAA TCTGGACATA TATATCATTG Ggtaagtgtc
    661 tgaagggccc ctgcaccccc tatccctgta gagggctgat gtaacctgtc ctcgcttgtg
    721 ttagagtctg aaggggagga gctttcatcc tccctgggtc cagtcttgtg tttcttgtcc
    781 tgggtcagca atggttatgg gttggaggtc aacagccaca cagctaggaa agggaggcct
    841 cttggtcttc agaatgcagg cctgagagtc tgatgatggc tgtgggagag tcagtatcct
    901 ccaggccagg ggtgatcctc tgccttggaa tggtcagTTT GCAGGAAATG AATTTTGGAA
    961 TCATAAGCCT TCTTTCTGAT GCTGCTTTTG AAGACCCATG GAGCAGTCTC TTCATGGATA
   1021 TGCTTTCCCC ACTGAACTTT GTCAAGgtaa gttaaatttc tgggcagtgg aggaatgtgt
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   1261 AGTTATCGCC GGCGggtctg agctcgccat cagtttagct tcacacctag atgttttctc
   1321 agtcttattc ctggcctacc ctctggtctc tccttactgc ccagcatgaa atctcttgag
   1381 tgtagcactt gcta
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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 donor template 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.