

Name of Mouse model or mutation:**Ccdc103-H154P-EM1-B6****Description:**

Point mutation model made using CRISPR/Cas9.

Type of mutation:

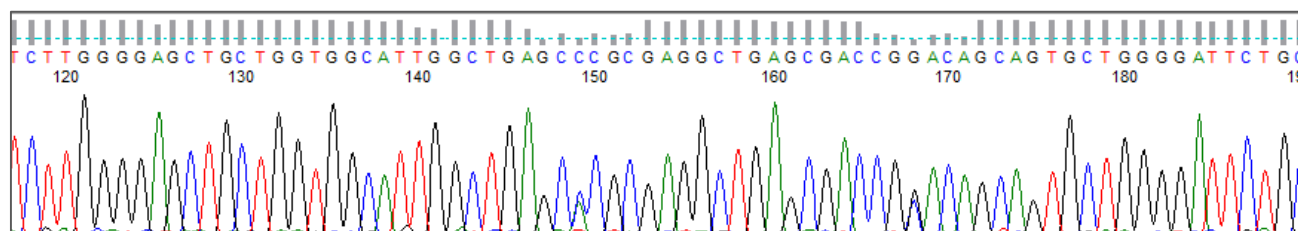
SNP: H154P

Sequence details**WT**

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AAATCACCCCTTCCAGCCCACCACCTCTGCAGAATTTACCGTGATTGGCGGCGACACTTAAGAAGTG
GGCCAGAGCGCTACCAGGCCCTACTTCAGCTTGGGGGGCCCAAGCTGGGCCACCTCTTCCAGATGG
ATGTGGGCTTTGGACTTCTTGGGGAGCTGCTGGTGGCATTGGCTGAGC<ACGCGAGGCTGAGCGAC
CGGA>CAGCAGTGTGGGGATTCTGCACAGCCTGGCTAACACTGGGCGGTTCAACTTGAACCTGAG
CCTGCTGAGCCATGCAGAGCGAGAGAGCTGCCAGCGCCTGTTTCAGAAGTTGCAAGCCATGAGCAC
CACCAGACCCATGCAGGAGGGCCTCACTGTGGAGGAGCCATCTGCTGGGCTACAGGGAGAAGAAG
GGCTCCTACAGGAGCTGCTAGAACTGTATGGAGTCCATTGATAGGACTGTTTGCCTCAGCATTCC
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CCDC103-H154P-EM1-B6

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AAATCACCCCTTCCAGCCCACCACCTCTGCAGAATTTACCGTGATTGGCGGCGACACTTAAGAAGTG
GGCCAGAGCGCTACCAGGCCCTACTTCAGCTTGGGGGGCCCAAGCTGGGCCACCTCTTCCAGATGG
ATGTGGGCTTTGGACTTCTTGGGGAGCTGCTGGTGGCATTGGCTGAGCCCGCGAGGCTGAGCGACC
GCACAGCAGTGTGGGGATTCTGCACAGCCTGGCTAACACTGGGCGGTTCAACTTGAACCTGAGCC
TGCTGAGCCATGCAGAGCGAGAGAGCTGCCAGCGCCTGTTTCAGAAGTTGCAAGCCATGAGCACCA
CCAGACCCATGCAGGAGGGCCTCACTGTGGAGGAGCCATCTGCTGGGCTACAGGGAGAAGAAGGG
CTCCTACAGGAGCTGCTAGAACTGTATGGAGTCCATTGATAGGACTGTTTGCCTCAGCATTCC
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CCDC103-H154P-EM1-B6 Heterozygous F1 animal sequence trace:

Nucleotide Alignment:

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280      *      300      *      320      *      340      *      360      *      380      *      400      *
Ccdc103_WT : AAATCACCCCTTCCAGCCCACCACCTCTGCAGAATTTTACCGTGATTGGCGGGCAGACTTAAGAAGTGGGCCAGAGCGCTACCAGGCCCTACTTCAGCTTGGGGGGCCCAAGCTGGGGCCACCTCTTCCAGATGGATGT
Ccdc103_H154P : AAATCACCCCTTCCAGCCCACCACCTCTGCAGAATTTTACCGTGATTGGCGGGCAGACTTAAGAAGTGGGCCAGAGCGCTACCAGGCCCTACTTCAGCTTGGGGGGCCCAAGCTGGGGCCACCTCTTCCAGATGGATGT
AAATCACCCCTTCCAGCCCACCACCTCTGCAGAATTTTACCGTGATTGGCGGGCAGACTTAAGAAGTGGGCCAGAGCGCTACCAGGCCCTACTTCAGCTTGGGGGGCCCAAGCTGGGGCCACCTCTTCCAGATGGATGT

420      *      440      *      460      *      480      *      500      *      520      *      540      *
Ccdc103_WT : GGGCTTTGGACTTCTTGGGGAGCTGCTGGTGGCATTGGCTGAGCCACGCGAGGCTGAGCGACCGACAGCAGTGCTGGGGATTCTGCACAGCCTGGCTAACACTGGGCGGTTCAACTTGAACCTGAGCCTGCTGAGCC
Ccdc103_H154P : GGGCTTTGGACTTCTTGGGGAGCTGCTGGTGGCATTGGCTGAGCCACGCGAGGCTGAGCGACCGACAGCAGTGCTGGGGATTCTGCACAGCCTGGCTAACACTGGGCGGTTCAACTTGAACCTGAGCCTGCTGAGCC
GGGCTTTGGACTTCTTGGGGAGCTGCTGGTGGCATTGGCTGAGCCACGCGAGGCTGAGCGACCGACAGCAGTGCTGGGGATTCTGCACAGCCTGGCTAACACTGGGCGGTTCAACTTGAACCTGAGCCTGCTGAGCC

560      *      580      *      600      *      620      *      640      *      660      *      680      *
Ccdc103_WT : ATGCAGAGCGAGAGAGCTGCCAGCGCCTGTTTCAGAAGTTGCAAGCCATGAGCACCACCAGACCCATGCAGGAGGGCCTCACTGTGGAGGAGCCATCTGCTGGGCTACAGGGAGAAGAAGGGCTCCTACAGGAGCTG
Ccdc103_H154P : ATGCAGAGCGAGAGAGCTGCCAGCGCCTGTTTCAGAAGTTGCAAGCCATGAGCACCACCAGACCCATGCAGGAGGGCCTCACTGTGGAGGAGCCATCTGCTGGGCTACAGGGAGAAGAAGGGCTCCTACAGGAGCTG
ATGCAGAGCGAGAGAGCTGCCAGCGCCTGTTTCAGAAGTTGCAAGCCATGAGCACCACCAGACCCATGCAGGAGGGCCTCACTGTGGAGGAGCCATCTGCTGGGCTACAGGGAGAAGAAGGGCTCCTACAGGAGCTG

700      *      720      *
Ccdc103_WT : CTAGAACTGTATGGAGTCCATTGATAGGACTGTTGCCCCTCAGCATTC : 460
Ccdc103_H154P : CTAGAACTGTATGGAGTCCATTGATAGGACTGTTGCCCCTCAGCATTC : 460
CTAGAACTGTATGGAGTCCATTGATAGGACTGTTGCCCCTCAGCATTC

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Predicted Protein Alignment:

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280      *      300      *      320      *      340      *      360      *      380      *      400      *
Ccdc103_WT : QPTTSAEFYRDWRRHLRSGPERYQALLQLGGPKLGHLPQMDVGFLLGELLVALAEARLSDRTAVLGILHSLANTGRFNLNLSLLSHAERESCQRLFQKIQAMSTTRPMQEGLTVEEPSAGLQGEGLLELLELY
Ccdc103_H154P : QPTTSAEFYRDWRRHLRSGPERYQALLQLGGPKLGHLPQMDVGFLLGELLVALAEARLSDRTAVLGILHSLANTGRFNLNLSLLSHAERESCQRLFQKIQAMSTTRPMQEGLTVEEPSAGLQGEGLLELLELY
QPTTSAEFYRDWRRHLRSGPERYQALLQLGGPKLGHLPQMDVGFLLGELLVALAEARLSDRTAVLGILHSLANTGRFNLNLSLLSHAERESCQRLFQKIQAMSTTRPMQEGLTVEEPSAGLQGEGLLELLELY

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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_Ccdc103_F1	AAATCACCTTCCAGCCCAC
Geno_Ccdc103_R1	GGAATGCTGAGGGCAAACAG
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	62
Elongation time (min)	1
WT product size (bp)	460
Mutant product size (bp)	460

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.

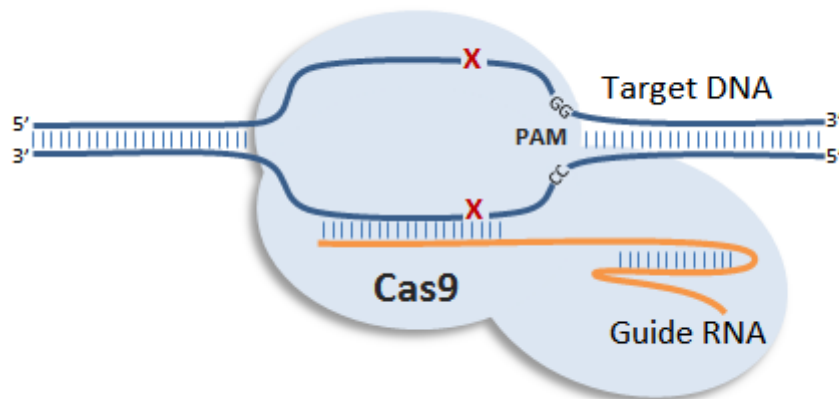
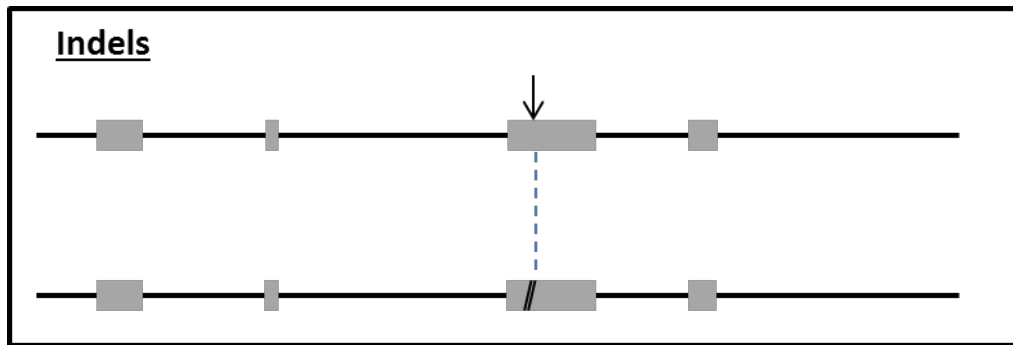
Copy counting of the donor sequence was carried out by ddPCR at the F1 stage to confirm donor oligos were inserted once on target into the genome. The following Taqman assay was used to detect the donor sequence:

Assay name	CCDC103H154PDONORMUT2
Forward Primer	GTGGCATTGGCTGAGCC
Reverse Primer	CAAGTTGAACCGCCCAGTGTT
Probe	CTGAGCGACCGCACAGCAGT
Label	FAM-BHQ1

The ddPCR assay is specific to the H154P mutation of Ccdc103 and the WT gene is not recognised by this assay. Therefore, WT controls are expected to call at 0 copies and a single integration for a correct mutation is expected to call at 1 copy for F1 (HET) animals.

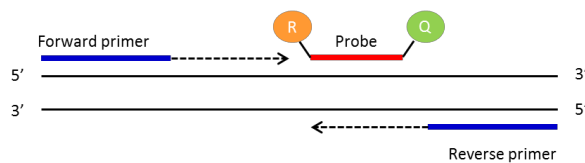
Ccdc103-H154P Genotyping Strategy

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).

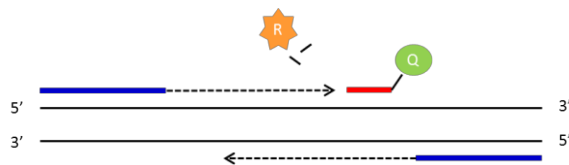


qPCR genotyping strategy

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 *et al.*, 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.

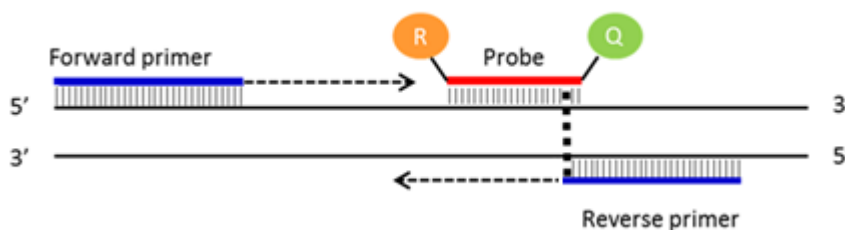
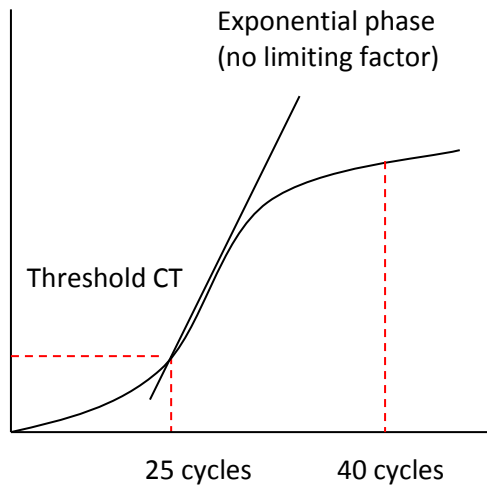


Figure1: Figure showing principle of ASPPAA PCR. The dotted line indicates the position of the SNP.

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



Ccdc103-H154P 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

Ccdc103-H154P CRISPR/Cas9 mutant in which H154P is highlighted in underlined characters

WT ACGCGAGGCTGAGCGACCGG
Mutant CCGCGAGGCTGAGCGACCGC

Ccdc103-H154P-WT1 assay (FAM labelled probe)

CCACCTCTTCCAGATGGATGTGGGCTTTGGACTTCTTGGGGAGCTGCTGGTGGCATTGGCTGAGCACGCGAGGCTGAGCGACCGGACAGCAGTGTGGGGATTCTGCACAGCCTGGCTAACACTGGGCGGTTCAACTTGAACCTGAGCCT

Primer 1 = GTGGCATTGGCTGAGCAC

Primer 2 = AATCCCCAGCACTGCTGTC

Probe = AGGCTGAGCGACCGGAC

} Allele specific primer and probes

CCDC103H154PDONORMUT2 assay (FAM labelled probe)

CCACCTCTTCCAGATGGATGTGGGCTTTGGACTTCTTGGGGAGCTGCTGGTGGCATTGGCTGAGCCCGCGAGGCTGAGCGACCGCACAGCAGTGCTGGGGATTCTGCACAGCCTGGCTAACACTGGGCGGTTCAACTTGAACCTGAGCCT

Primer 1 = GTGGCATTGGCTGAGCC

Primer 2 = CAAGTTGAACCGCCAGTGTT

Probe = CTGAGCGACCGCACAGCAGT

Dot1l internal control (VIC labelled)

CCCCCTAGTCGTTTTCTGTTAGTAGTTGGCATCCTTATGCTTCATCTTACAGTCGACTTGAGAGCTGCCCCTGATGGTTCGTGCTGGGGCAGGCTTTATTTACAGCGTAGCACACATGGTGGCCAATGGGACTCTGTAGGATCTGCC

Primer 1 = GCCCCAGCACGACCATT

Primer 2 = TAGTTGGCATCCTTATGCTTCATC

Probe = CCAGCTCTCAAGTCG

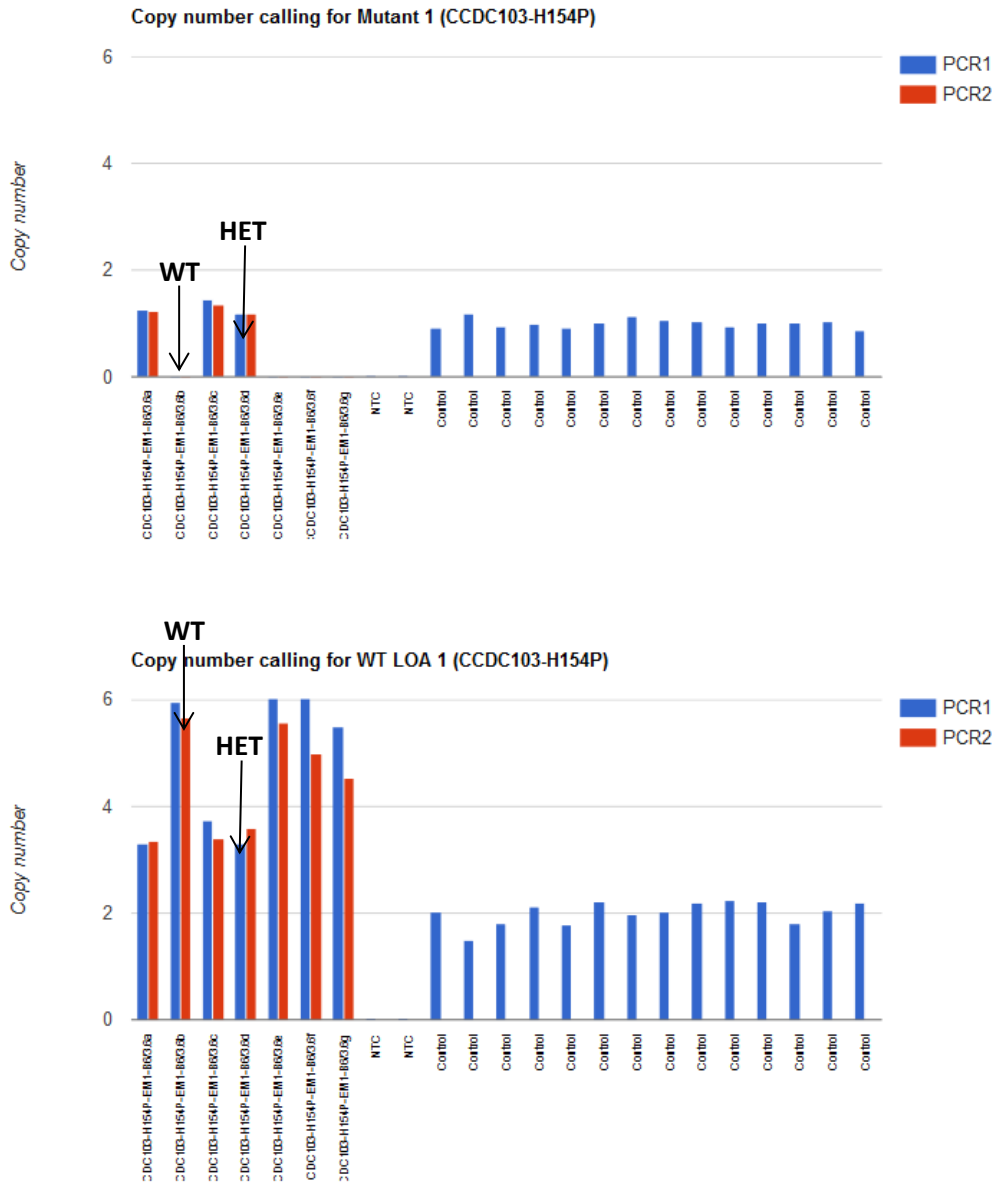
qPCR master mix

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



Ccdc103-H154P copy called result, image showing both replicates and controls for both WT and Mutant assays

Task 199854 Results



Please be aware to use B6N/B6J as your WT controls as there is a natural polymorphism between B6N and B6J and the copy numbers might be affected if you use C3H controls

Version No. 2
Date: 16.04.2018
Created/Updated by: Ramakrishna Kurapati
Approved by: Debbie Williams