Vcan-E441A-EM1/EM2/EM3 Genotyping Strategy

MRC Harwell

Vcan-E441A CRISPR/Cas9 mutants in which SNPs are as highlighted

Vcan-WT	TAAGGACCCGGAAGCTGCAG**A**AGCTAG**G**CGTGGCCAGTACGAAAGTGTTGCACCTTCTCA
Vcan-EM1-B6N	TAAGGACCCGGAAGCTGCAG**C**AGCTAG**A**CGTGGCCAGTACGAAAGTGTTGCACCTTCTCA
Vcan-EM2-B6N	TAAGGACCCGGAAGCTGCAG**C**AGCTAG**G**CGTGGCCAGTACGAAAGTGTTGCACCTTCTCA
Vcan-EM3-B6N	TAAGGACCCGGAAGCTGCAG**C**AGCTAG**G**CGTGGCCAGTACGAAAGTGTTGCACCTTCTCA

Genotyping strategy 1

Samples are genotyped with a Wildtype (WT) assay initially which is common for all the three alleles. This is a FAM labelled assay that has an WT allele specific primer and a WT allele specific probe and their 3' end has the SNP of interest giving a primer probe overlap (Billard etal., 2012). So if the animal contains the modified allele the copy number of the WT assay drops by 1 copy.

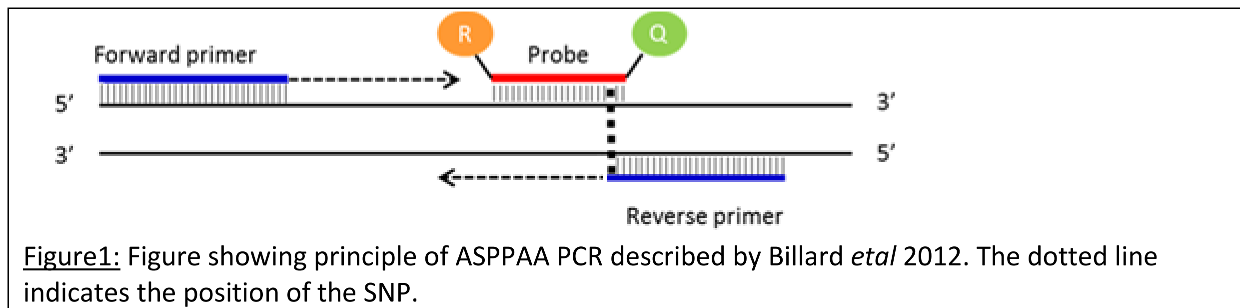


Figure1: Figure showing principle of ASPPAA PCR described by Billard *etal* 2012. The dotted line indicates the position of the SNP.

For autosomal genes that have been targeted, the following results would be expected:

Genotype of the Modified allele	WT Assay
Wildtype	2
Heterozygous	1
Homozygous mutant	0

Genotyping strategy 2

Samples are also genotyped using an Allelic Discrimination (AD) assay to detect EM2/EM3 alleles and the WT allele of Vcan-E441A CRISPR/Cas9 mutant. It is a multiplexed assay consisting of a common forward and reverse primer plus two Taqman probes, one probe (FAM labelled) is specific to wildtype allele sequence, and one probe (TET labelled) is specific to Vcan-E441A_EM2/EM3 CRISPR/Cas9 modified allele. Endpoint data is collected at the completion of the PCR process. The samples that carry the Vcan-E441A-EM1 mutant could be also be detected using this assay and they should group close to WT's as there is no specific probe targeted for them.

Use combination of Genotyping strategies 1 and 2 to determine the correct Vcan-E441A CRISPR/Cas9 mutants

Vcan-E441A-WT3 assay (FAM labelled probe)

TGAAGACGGAGAGGAGGACTGTGTAAATGCAACGGATGTAACAACTACTCCGTCAGTGCAGTATATCAATGGGAA
GCAGCTCGTTACCACAGTGCCTAAGGACCCGGAAGCTGCAGAAGCTAGGCGTGGCCAGTACGAAAGTGTTGCACC
TTCTCAGAATTTCCCAGATAGTTCTGCAACTGACACCCATCAGTTTATACTAGCAGAAACAGAATCGTCAACTAC

Primer 1 = GGGTGTCAGTTGCAGAACTATC
Primer 2 = GACCCGGAAGCTGCAG**A**
Probe = TGGCCACG**C**CTAGCT**T**

Allele specific primer and probes with WT SNP highlighted at their 3' end

The above assay is run along with an internal Dot1l control (details as below) as reference

Dot1l internal control (VIC labelled)

CTGTTAGTAGTTGGCATCCTTATGCTTCATCTTACAGTCGACTTGAGAGCTGGCCCTGAATGGTCGTGCTGGGGC

Primer 1 = GCCCCAGCACGACCATT
Primer 2 = TAGTTGGCATCCTTATGCTTCATC
Probe = CCAGCTCTCAAGTCG

DNA extraction method:
DNA is extracted from ear clips using Applied Biosystem's Sample-to-SNP Kit and qPCR run using 1:10 dilution from the crude preparation.

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

Every sample is ran in technical duplicate. Seven WT and/or mutant controls are also ran in duplicate.

qPCR cycling conditions

95°C for 20 sec
Then 40 cycles of;
95°C for 3 sec
60°C for 30 sec

Analysis

The results are analysed using CopyCaller Softwarev2.0 from Applied Biosystem's.

Vcan-E441A-EM2 AD

Vcan-WT
GCAGTATATCAATGGGAAGCAGCTCGTTACCACAGTGCCTAAGGACCCGGAAGCTGCAGAAGCTAGGCGTGGCCA
GTACGAAAGTGTTGCACCTTCTCAGAATTTCCCAGATAGTTCTGCAACTGACACCCATCAGTTTATACTAGCAGA

Vcan-E441A-EM2 and Vcan-E441A-EM3
GCAGTATATCAATGGGAAGCAGCTCGTTACCACAGTGCCTAAGGACCCGGAAGCTGCAGCAGCTAGGCGTGGCCA
GTACGAAAGTGTTGCACCTTCTCAGAATTTCCCAGATAGTTCTGCAACTGACACCCATCAGTTTATACTAGCAGA

Primers and Probes

Primer 1	CAGCTCGTTACCACAGTGCCT
Primer 2	GGGTGTCAGTTGCAGAACTATC
Allele 1 (WT) probe (FAM-Labelled)	AAGCTGCAGAAGCTAGG
Allele 2 (Mut) probe (TET-Labelled)	TGCAGCAGCTAGGC

qPCR master mix

ABI GTX Taqman master mix	5µl
Assay (Probes 5µM each & Primers 15µM each) 20uM	2µl (of 1 in 5 dilution of stock)
ddH2O	0.5µl
DNA (1/10 dilution of ABI Sample-to-SNP prep)	2.5µl

No need to run the samples in duplicates. Two each of WT and/or mutant controls are also ran to group the samples accordingly
Allele 1 = WT on 7500 FAM-labelled. Allele 2 = MUT on 7500 TET-labelled.

7500 Settings for running Allele Discrimination Assay are as shown below

Which instrument are you using to run the experiment?
7500 (96 Wells) | ✓ 7500 Fast (96 Wells)
Set up, run, and analyze an experiment using a fast cycling 5-color, 96-well system.

What type of experiment do you want to set up?
Quantitation - Standard Curve | Quantitation - Relative Standard Curve | Quantitation - Comparative CT (ΔΔCT)
Melt Curve | ✓ Genotyping | Presence/Absence
Detect single nucleotide polymorphism variants of a target nucleic acid sequence in samples.

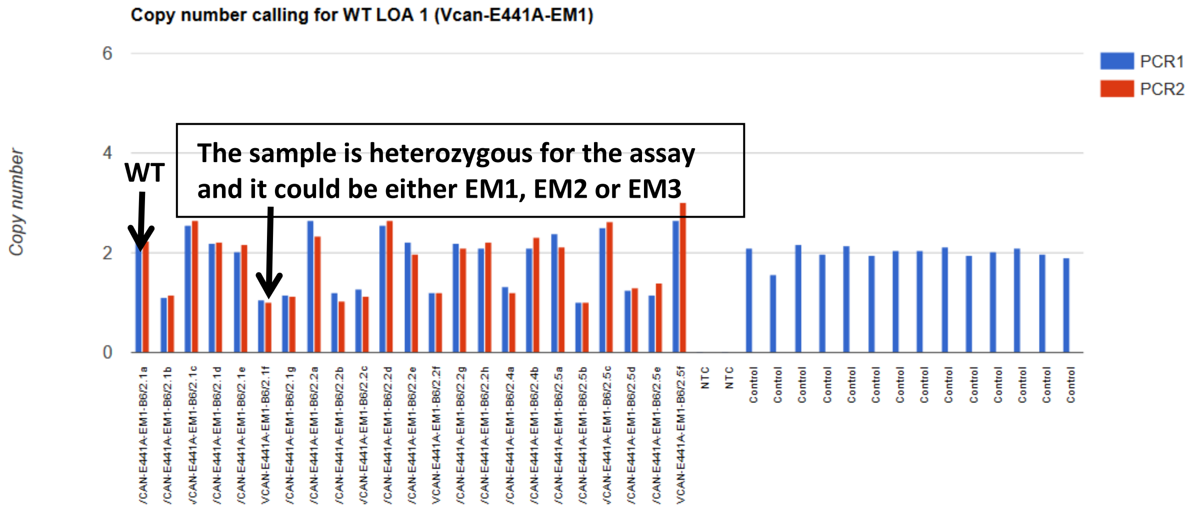
Which reagents do you want to use to detect the target sequence?
✓ TaqMan® Reagents | Other
The PCR reactions contain primers designed to amplify the target sequence and a TaqMan® probe designed to detect amplification of the target sequence.

Which ramp speed do you want to use in the instrument run?
Standard (~ 2 hours to complete a run) | ✓ Fast (~ 40 minutes to complete a run)
For optimal results with the Fast ramp speed, Applied Biosystems recommends using Fast reagents for your PCR reactions.

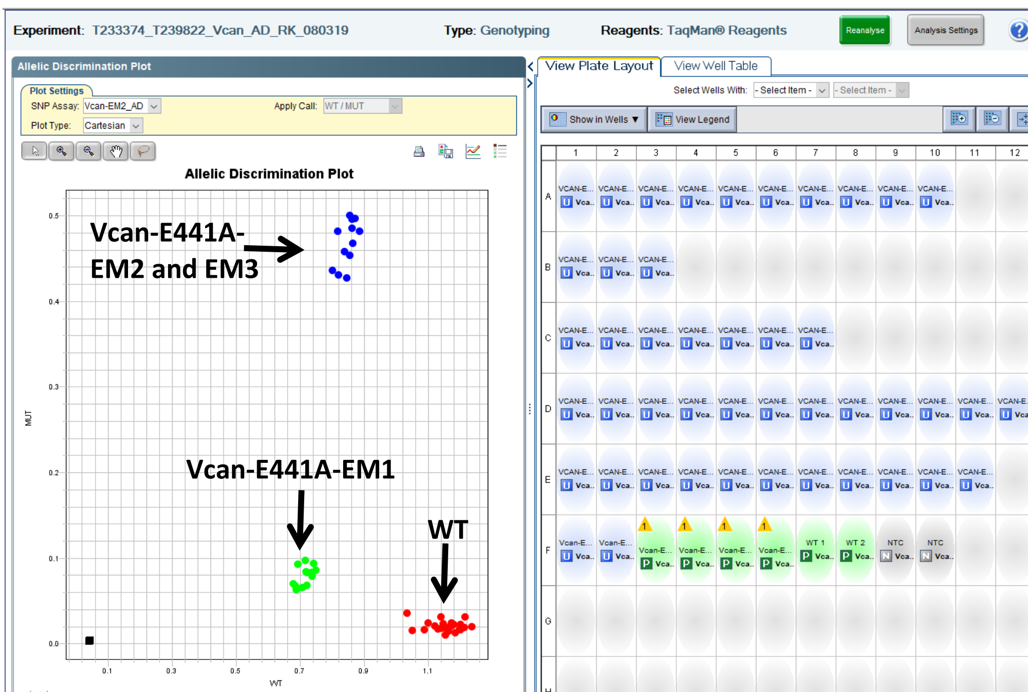
qPCR cycling conditions

95°C for 20 sec
Then 40 cycles of;
95°C for 3 sec
60°C for 30 sec

Vcan-E441A-WT3 copy called result, image showing both replicates and controls for both assays (Task 239822 Results)



Allelic Discrimination Plot and Results showing various Vcan-E441A CRISPR/Cas9 mutants



Version No.	3
Date:	20/03/2019
Created/Updated by:	Ramakrishna Kurapati
Approved by:	Daniel Ford

MRC Harwell

GEMM
GENOME EDITING MICE FOR MEDICINE

Name of Mouse model or mutation:
VCAN-E441A-EM1-B6
VCAN-E441A-EM2-B6

Description:
Point mutation model made using CRISPR/Cas9.

Type of mutation:
E441A

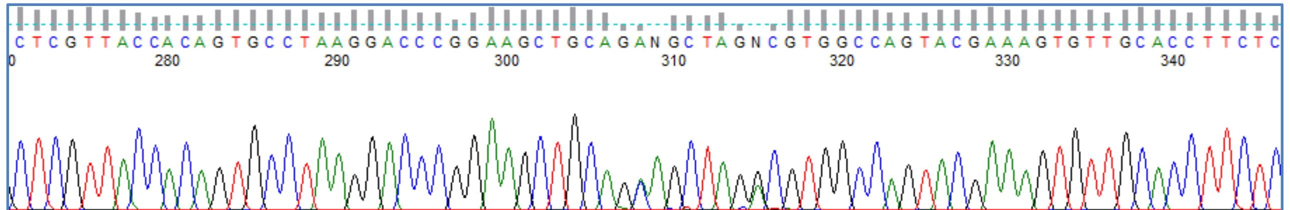
Sequence details
WT
tgtcttctgtaccctgccaaatcttccatttaagtattgtgaaaactctttgtttttttcagGTCGATTGAGTGATATGATTGT
AAGTGGTCATCCAATAGATTCAGAATCTAAAGAAGAGGAACCTTGCAGTGAAGAAACAGATCCACT
GCATGATCTGTTTGCTGAAATTTTACCCGAGTTACCAGACTCATTTGAAATAGACATATATCACAGTG
AGGAAGATGAAGACGGAGAGGAGGACTGTGTAAATGCAACGGATGTAACAACTACTCCGTCAGTG
CAGTATATCAATGGGAAGCAGCTCGTTACCACAGTGCCTAAGGACCCGGAAGCTGCAGAAGCTAGG
CGTGGCCAGTACGAAAGTGTTGCACCTTCTCAGAATTTCCCAGATAGTTCTGCAACTGACACCCATCA
GTTTATACTAGCAGAAACAGAATCGTCAACTACCATGCAATTTAAGAAATCTAAAGAAGGCACGGAA
TTGTTAGAAATCACATGGAAACCCGAGACCTACCCTGAAACACCAGACCATGTTTCAAGTGGTGAGC
CTGATGTTTTCCCTACTCTCTCATCCCATGATGGTAAAACCACCAGATGGTCAGAGTCCATCACAGAG
AGCAGTCCAAACCTTGAAAATCCAGTGCACAAACAACCTAAGCCTGTCCCTCTGTTTCCTGAAGAGTC
TTCAGGAGAGGGTGCCATTG

VCAN-E441A-EM1-B6
tgtcttctgtaccctgccaaatcttccatttaagtattgtgaaaactctttgtttttttcagGTCGATTGAGTGATATGATTGT
AAGTGGTCATCCAATAGATTCAGAATCTAAAGAAGAGGAACCTTGCAGTGAAGAAACAGATCCACT
GCATGATCTGTTTGCTGAAATTTTACCCGAGTTACCAGACTCATTTGAAATAGACATATATCACAGTG
AGGAAGATGAAGACGGAGAGGAGGACTGTGTAAATGCAACGGATGTAACAACTACTCCGTCAGTG
CAGTATATCAATGGGAAGCAGCTCGTTACCACAGTGCCTAAGGACCCGGAAGCTGCAG**C**AGCTAG**A**
CGTGGCCAGTACGAAAGTGTTGCACCTTCTCAGAATTTCCCAGATAGTTCTGCAACTGACACCCATCA
GTTTATACTAGCAGAAACAGAATCGTCAACTACCATGCAATTTAAGAAATCTAAAGAAGGCACGGAA
TTGTTAGAAATCACATGGAAACCCGAGACCTACCCTGAAACACCAGACCATGTTTCAAGTGGTGAGC
CTGATGTTTTCCCTACTCTCTCATCCCATGATGGTAAAACCACCAGATGGTCAGAGTCCATCACAGAG
AGCAGTCCAAACCTTGAAAATCCAGTGCACAAACAACCTAAGCCTGTCCCTCTGTTTCCTGAAGAGTC
TTCAGGAGAGGGTGCCATTG

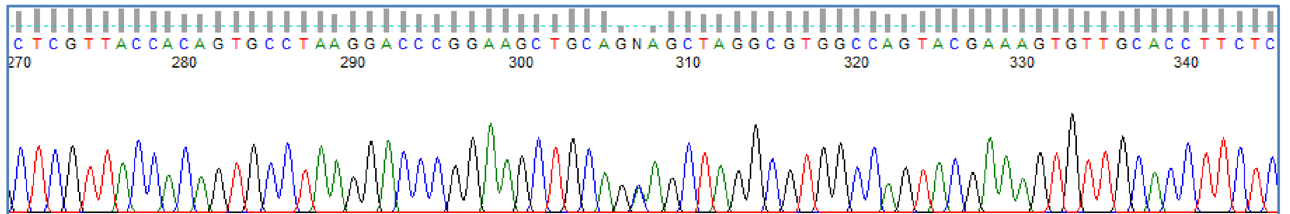
VCAN-E441A-EM2-B6

tgtcttctgtaccctgccaaatcttccatttaagtattgtgaaaactctttgtttttttcagGTCGATTGAGTGATATGATTGT
AAGTGGTCATCCAATAGATTCAGAATCTAAAGAAGAGGAACCTTGCAGTGAAGAAACAGATCCACT
GCATGATCTGTTTGCTGAAATTTTACCCGAGTTACCAGACTCATTTGAAATAGACATATATCACAGTG
AGGAAGATGAAGACGGAGAGGAGGACTGTGTAAATGCAACGGATGTAACAACTACTCCGTCAGTG
CAGTATATCAATGGGAAGCAGCTCGTTACCACAGTGCCTAAGGACCCGGAAGCTGCAG**C**AGCTAGG
CGTGGCCAGTACGAAAGTGTTGCACCTTCTCAGAATTTCCCAGATAGTTCTGCAACTGACACCCATCA
GTTTATACTAGCAGAAACAGAATCGTCAACTACCATGCAATTTAAGAAATCTAAAGAAGGCACGGAA
TTGTTAGAAATCACATGGAAACCCGAGACCTACCCTGAAACACCAGACCATGTTTCAAGTGGTGAGC
CTGATGTTTTCCCTACTCTCTCATCCCATGATGGTAAAACCACCAGATGGTCAGAGTCCATCACAGAG
AGCAGTCCAAACCTTGAAAATCCAGTGCACAAACAACCTAAGCCTGTCCCTCTGTTTCCTGAAGAGTC
TTCAGGAGAGGGTGCCATTG

VCAN-E441A-EM1-B6 Heterozygous F1 animal sequence trace:



VCAN-E441A-EM2-B6 Heterozygous F1 animal sequence trace:



Nucleotide Alignment:

```
                         *        20         *        40         *        60         *        80         *       100
Vcan_WT         : tgtcttctgtaccctgccaaatcttccatttaagtattgtgaaaactctttgtttttttcagGTCGATTGAGTGATATGATTGTAAGTGGTCATCCAATA
Vcan_E441A_EM1  : tgtcttctgtaccctgccaaatcttccatttaagtattgtgaaaactctttgtttttttcagGTCGATTGAGTGATATGATTGTAAGTGGTCATCCAATA
Vcan_E441A_EM2  : tgtcttctgtaccctgccaaatcttccatttaagtattgtgaaaactctttgtttttttcagGTCGATTGAGTGATATGATTGTAAGTGGTCATCCAATA
                  TGTCTTCTGTACCCTGCCAAATCTTCCATTTAAGTATTGTGAAAACTCTTTGTTTTTTTCAGGTCGATTGAGTGATATGATTGTAAGTGGTCATCCAATA

                         *       120         *       140         *       160         *       180         *       200
Vcan_WT         : GATTCAGAATCTAAAGAAGAGGAACCTTGCAGTGAAGAAACAGATCCACTGCATGATCTGTTTGCTGAAATTTTACCCGAGTTACCAGACTCATTTGAAA
Vcan_E441A_EM1  : GATTCAGAATCTAAAGAAGAGGAACCTTGCAGTGAAGAAACAGATCCACTGCATGATCTGTTTGCTGAAATTTTACCCGAGTTACCAGACTCATTTGAAA
Vcan_E441A_EM2  : GATTCAGAATCTAAAGAAGAGGAACCTTGCAGTGAAGAAACAGATCCACTGCATGATCTGTTTGCTGAAATTTTACCCGAGTTACCAGACTCATTTGAAA
                  GATTCAGAATCTAAAGAAGAGGAACCTTGCAGTGAAGAAACAGATCCACTGCATGATCTGTTTGCTGAAATTTTACCCGAGTTACCAGACTCATTTGAAA

                         *       220         *       240         *       260         *       280         *       300
Vcan_WT         : TAGACATATATCACAGTGAGGAAGATGAAGACGGAGAGGAGGACTGTGTAAATGCAACGGATGTAACAACTACTCCGTCAGTGCAGTATATCAATGGGAA
Vcan_E441A_EM1  : TAGACATATATCACAGTGAGGAAGATGAAGACGGAGAGGAGGACTGTGTAAATGCAACGGATGTAACAACTACTCCGTCAGTGCAGTATATCAATGGGAA
Vcan_E441A_EM2  : TAGACATATATCACAGTGAGGAAGATGAAGACGGAGAGGAGGACTGTGTAAATGCAACGGATGTAACAACTACTCCGTCAGTGCAGTATATCAATGGGAA
                  TAGACATATATCACAGTGAGGAAGATGAAGACGGAGAGGAGGACTGTGTAAATGCAACGGATGTAACAACTACTCCGTCAGTGCAGTATATCAATGGGAA

                         *       320         *       340         *       360         *       380         *       400
Vcan_WT         : GCAGCTCGTTACCACAGTGCCTAAGGACCCGGAAGCTGCAGAAGCTAGGCGTGGCCAGTACGAAAGTGTTGCACCTTCTCAGAATTTCCCAGATAGTTCT
Vcan_E441A_EM1  : GCAGCTCGTTACCACAGTGCCTAAGGACCCGGAAGCTGCAGCAGCTAGACGTGGCCAGTACGAAAGTGTTGCACCTTCTCAGAATTTCCCAGATAGTTCT
Vcan_E441A_EM2  : GCAGCTCGTTACCACAGTGCCTAAGGACCCGGAAGCTGCAGCAGCTAGGCGTGGCCAGTACGAAAGTGTTGCACCTTCTCAGAATTTCCCAGATAGTTCT
                  GCAGCTCGTTACCACAGTGCCTAAGGACCCGGAAGCTGCAGcAGCTAGgCGTGGCCAGTACGAAAGTGTTGCACCTTCTCAGAATTTCCCAGATAGTTCT

                         *       420         *       440         *       460         *       480         *       500
Vcan_WT         : GCAACTGACACCCATCAGTTTATACTAGCAGAAACAGAATCGTCAACTACCATGCAATTTAAGAAATCTAAAGAAGGCACGGAATTGTTAGAAATCACAT
Vcan_E441A_EM1  : GCAACTGACACCCATCAGTTTATACTAGCAGAAACAGAATCGTCAACTACCATGCAATTTAAGAAATCTAAAGAAGGCACGGAATTGTTAGAAATCACAT
Vcan_E441A_EM2  : GCAACTGACACCCATCAGTTTATACTAGCAGAAACAGAATCGTCAACTACCATGCAATTTAAGAAATCTAAAGAAGGCACGGAATTGTTAGAAATCACAT
                  GCAACTGACACCCATCAGTTTATACTAGCAGAAACAGAATCGTCAACTACCATGCAATTTAAGAAATCTAAAGAAGGCACGGAATTGTTAGAAATCACAT

                         *       520         *       540         *       560         *       580         *       600
Vcan_WT         : GGAAACCCGAGACCTACCCTGAAACACCAGACCATGTTTCAAGTGGTGAGCCTGATGTTTTCCCTACTCTCTCATCCCATGATGGTAAAACCACCAGATG
Vcan_E441A_EM1  : GGAAACCCGAGACCTACCCTGAAACACCAGACCATGTTTCAAGTGGTGAGCCTGATGTTTTCCCTACTCTCTCATCCCATGATGGTAAAACCACCAGATG
Vcan_E441A_EM2  : GGAAACCCGAGACCTACCCTGAAACACCAGACCATGTTTCAAGTGGTGAGCCTGATGTTTTCCCTACTCTCTCATCCCATGATGGTAAAACCACCAGATG
                  GGAAACCCGAGACCTACCCTGAAACACCAGACCATGTTTCAAGTGGTGAGCCTGATGTTTTCCCTACTCTCTCATCCCATGATGGTAAAACCACCAGATG

                         *       620         *       640         *       660         *       680
Vcan_WT         : GTCAGAGTCCATCACAGAGAGCAGTCCAAACCTTGAAAATCCAGTGCACAAACAACCTAAGCCTGTCCCTCTGTTTCCTGAAGAGTCT
Vcan_E441A_EM1  : GTCAGAGTCCATCACAGAGAGCAGTCCAAACCTTGAAAATCCAGTGCACAAACAACCTAAGCCTGTCCCTCTGTTTCCTGAAGAGTCT
Vcan_E441A_EM2  : GTCAGAGTCCATCACAGAGAGCAGTCCAAACCTTGAAAATCCAGTGCACAAACAACCTAAGCCTGTCCCTCTGTTTCCTGAAGAGTCT
                  GTCAGAGTCCATCACAGAGAGCAGTCCAAACCTTGAAAATCCAGTGCACAAACAACCTAAGCCTGTCCCTCTGTTTCCTGAAGAGTCT
```

Predicted Protein Alignment:

```
                             *        20         *        40         *        60         *        80         *       100
Vcan_WT          : RLSDMIVSGHPIDSESKEEEPCSEETDPLHDLFAEILPELPDSFEIDIYHSEEDEDGEEDCVNATDVTTTPSVQYINGKQLVTTVPKDPEAAEARRGQYE
Vcan_E441A_EM1 : RLSDMIVSGHPIDSESKEEEPCSEETDPLHDLFAEILPELPDSFEIDIYHSEEDEDGEEDCVNATDVTTTPSVQYINGKQLVTTVPKDPEAAAARRGQYE
Vcan_E441A_EM2 : RLSDMIVSGHPIDSESKEEEPCSEETDPLHDLFAEILPELPDSFEIDIYHSEEDEDGEEDCVNATDVTTTPSVQYINGKQLVTTVPKDPEAAAARRGQYE
                   RLSDMIVSGHPIDSESKEEEPCSEETDPLHDLFAEILPELPDSFEIDIYHSEEDEDGEEDCVNATDVTTTPSVQYINGKQLVTTVPKDPEAAaARRGQYE

                             *       120         *       140         *       160         *       180         *       200
Vcan_WT          : SVAPSQNFPDSSATDTHQFILAETESSTTMQFKKSKEGTELLEITWKPETYPETPDHVSSGEPDVFPTLSSHDGKTTRWSESITESSPNLENPVHKQPKP
Vcan_E441A_EM1 : SVAPSQNFPDSSATDTHQFILAETESSTTMQFKKSKEGTELLEITWKPETYPETPDHVSSGEPDVFPTLSSHDGKTTRWSESITESSPNLENPVHKQPKP
Vcan_E441A_EM2 : SVAPSQNFPDSSATDTHQFILAETESSTTMQFKKSKEGTELLEITWKPETYPETPDHVSSGEPDVFPTLSSHDGKTTRWSESITESSPNLENPVHKQPKP
                   SVAPSQNFPDSSATDTHQFILAETESSTTMQFKKSKEGTELLEITWKPETYPETPDHVSSGEPDVFPTLSSHDGKTTRWSESITESSPNLENPVHKQPKP

Vcan_WT          : VPLFPEES
Vcan_E441A_EM1 : VPLFPEES
Vcan_E441A_EM2 : VPLFPEES
                   VPLFPEES
```

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_Vcan _F1 (5'-3')	TGTCTTCTGTACCCTGCCAAA
Geno_ Vcan_R1 (5'-3')	CAATGGCACCCTCTCCTGAA
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	61
Elongation time (min)	1
WT product size (bp)	707
Mutant product size (bp)	707
Notes	

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 located on a sex chromosome.

Off-target site with ≤2 mismatches for the guide used were checked with the following primers:

Off-target site	Sequence	Type	Primers used (5'-3)
2:122701403-122701425	**C**CCCGG**C**AGCTGCAGAAGCT AGG	Intronic	Geno_Vcan_OT1_F1 CAGTGCCTTTTACTGTAGGGT Geno_Vcan_OT1_R1 AGTAACTCTTCCTCAGCGTTCG
2:166453554-166453576	A**T**CCGGAAGCTGCAGAAGC**A** AGG	Intergenic	Geno_Vcan_OT2_F1 CGCTGTGGGAACCCAGACT Geno_Vcan_OT2_R1 CTGAATCCTCACCCTGGTAAAACT
9:58210201-58210223	ACCC**A**GAAGC**A**GCAGAAGCT AGG	Intergenic	Geno_Vcan_OT3_F1 CTGACCATCAGGATCCACGG Geno_Vcan_OT3_R1 TACATCCTGGGCTTGTCACC

All amplicons were sent for Sanger sequencing. No evidence of off-target cutting was observed at these sites in the correct mutants.

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	VCAN-E441A-DONOR-UNIV1
Forward Primer	ACAGTGCCTAAGGACCCGGAAG
Reverse Primer	GGGTGTCAGTTGCAGAACTATC
Probe	CGTGGCCAGTACGAAAGTGTTGCA
Label	FAM-BHQ1

Reference Assay Name	Dot1l
Forward primer	GCCCCAGCACGACCATT
Reverse primer	TAGTTGGCATCCTTATGCTTCATC
Probe	CCCAACAGGCCTGGATTCTCAATGC
Label	VIC