Vcan-E441A-EM1/EM2/EM3 Genotyping Strategy

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

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Vcan-WT        TAAGGACCCGGAAGCTGCAGAAGCTAGGCGTGGCCAGTACGAAAGTGTTGCACCTTCTCA
Vcan-EM1-B6N   TAAGGACCCGGAAGCTGCAGCAGCTAGACGTGGCCAGTACGAAAGTGTTGCACCTTCTCA
Vcan-EM2-B6N   TAAGGACCCGGAAGCTGCAGCAGCTAGGCGTGGCCAGTACGAAAGTGTTGCACCTTCTCA
Vcan-EM3-B6N   TAAGGACCCGGAAGCTGCAGCAGCTAGGCGTGGCCAGTACGAAAGTGTTGCACCTTCTCA
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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.

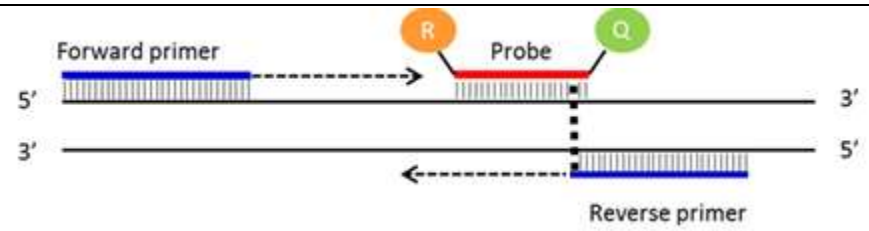


Figure1: Figure showing principle of ASPPAA PCR described by Billard *etal* 2012. The dotted line indicates the position of the SNP.

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 = GACCCGGAAGCTGCAGA
Probe = TGGCCACGCCTAGCTT

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.

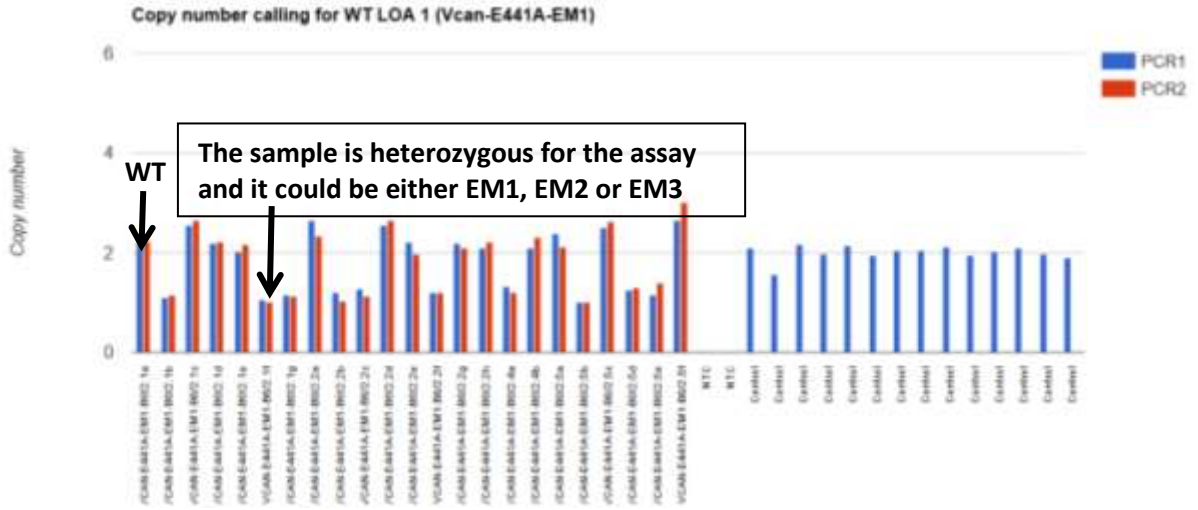
qPCR cycling conditions

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



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

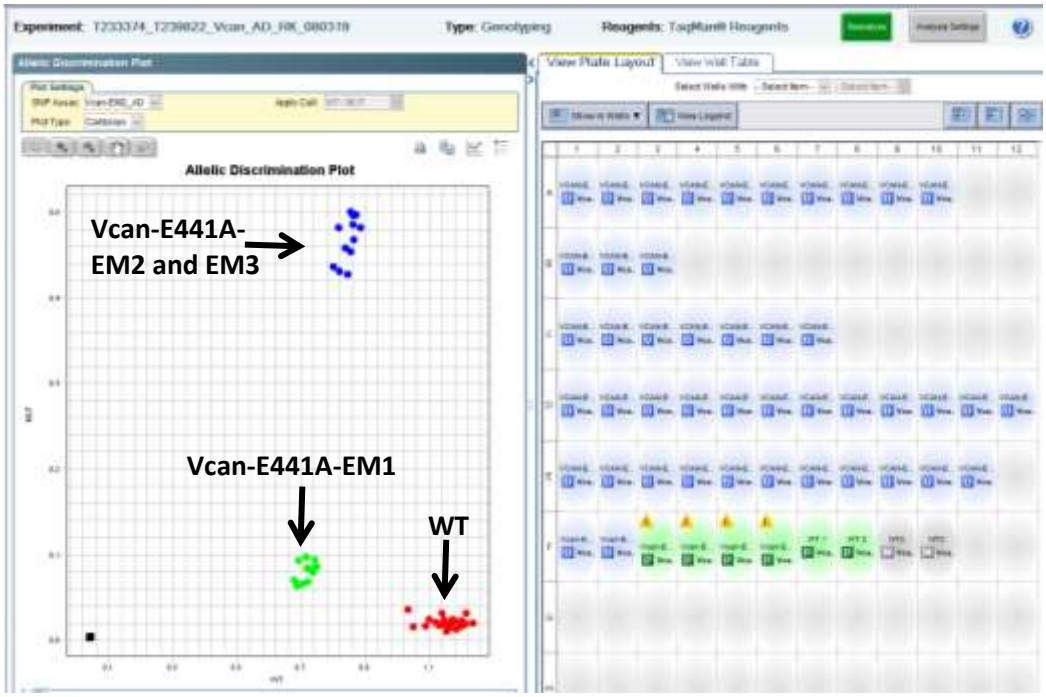
ABI GTX Taqman master mix	5µl
Assay (Probes 5µM each & Primers 15µM each) 20uM	2µl (of 1 in 5 dilution of stock)
ddH2O	0.5µl
DNA (1/10 dilution of ABI Sample-to-SNP prep)	2.5µl

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.

qPCR cycling conditions

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

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



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