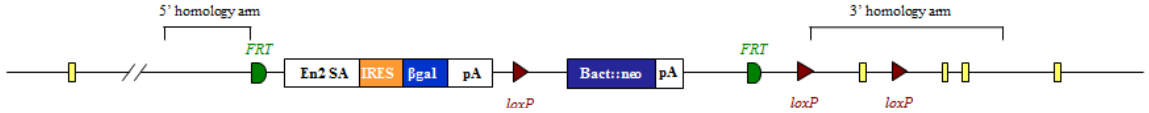
EUCOMM/KOMP-CSD 'Knockout-First' Genotyping

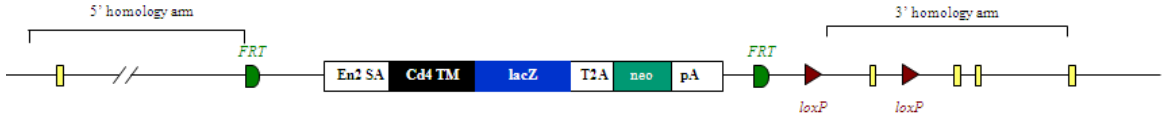
Introduction

The majority of animals produced from the EUCOMM/KOMP-CSD ES cell resource contain the Knockout-First-Reporter Tagged Insertion allele. As well as gene specific elements, the targeting cassette contains many common elements e.g. encoding beta-galactosidase (lacZ gene), neomycin-resistance (neo gene), FRT and loxP sites. Further details can be found on http://www.knockoutmouse.org/about/eucomm
The majority of cassettes will be either promoter driven where the neo gene contains its own promotor and is separated from the lacZ gene by a 3rd loxP site, or promotorless (PL) where the neo and lacZ genes are adjacent and there are only 2 loxP sites. These have further implications in our genotyping strategies detailed later.

Promotor driven line



Promotorless line



Animals containing the full Knockout-First mutant allele (Tm1a) above can be crossed to mice expressing Flp or Cre recombinase. The progeny from these mating may contain converted forms of the Tm1a allele known as Tm1b, Tm1c and Tm1d depending on the breeding strategy performed. In order to genotype animals generated from the EUCOMM/KOMP-CSD 'Knockout-First' ES cell resource, a combination of mutant assays looking for sequences occurring in the targeting cassette, and WT specific assays will need to be performed. We typically use real time qPCR and copy count the various alleles. This assay design sheet will give an outline of how to genotype these lines by gel based and qPCR based technologies, as well as including details of assays we have tried and tested.

Pcdh10-Tm1aNarl

Please note this allele was created by NARLabs so standard mutant assays may not work

Details for the tm1a allele can be found below

http://www.mousephenotype.org/data/alleles/MGI:1338042/tm1a(NARLabs)nlac/

Sequencing QC at Harwell has identified some differences compared to the standard Komp alleles

Cassette has the format LOXP-CR-FRT-NEO-FRT-LOXP

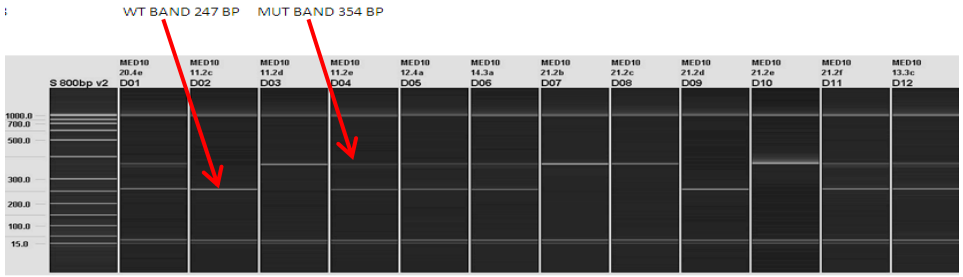
Gel based primers are located either side of the 5'loxP and give a specific sized product for the Tm1aNarl allele. If you had a Tm1bNarl allele where the critical region is removed the reverse primer location has been deleted so the gel assay will not give a mutant sized product. If the animal was a Tm1bNarl het you would only see WT sized product and the Tm1bNarl allele would be identified by a drop in the CR and the absence of NEO.

3'FRT is shown to be missing bases so may not work (missing sequence in yellow)

GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC

Gel based genotyping

The gel based assays are normally run on the Qiagen QIAxcel. This is a capillary based system that provides clearer resolution and is quicker than running standard agarose gels. Different size ladders maybe loaded onto runs depending on the fragment sizes being analysed. Typically samples are run with a 50-800bp size ladder.



PCR is performed using KAPA2G Fast Hotstart Readymix (2X), although alternatives may be used.

Generic 4 primer PCR mix

KAPA2G Fast Hotstart Readymix (2X)	5µl
Primer 1F (20µM)	0.5µl
Primer 1R (20µM)	0.5µl
Primer 2F (20µM)	0.5µl
Primer 2R (20µM)	0.5µl
H_2O	2.0µl
DNA (~30ng)	1µl

60°C Cycling conditions

60TM30FA

1.	95ºC	1min
2.	95ºC	10sec
3.	**60**ºC	10sec
4.	72ºC	1sec
5.	Go to 2 for 29 cycles	
6.	72ºC	30sec
7.	16 ºC	forever

Pcdh10-Tm1aNarl gel based genotyping

Pcdh10 'Knockout'-First and WT sequences

Critical region

CACAAGACTATGAAAGCCAAGAGATGCAAAGAGAGACAGAGGGAAGAAAATGACAAGAGGAACGTCCATCCGGAGAAATGAAGAGAATGAAAGTTTTATGCTGCAGAACGGCTCCG
TGTTTTTCCAGCAAAAATTTTTCTCGCTGTCTTTAACCCCGCTCGCGTTTGCCAGCCGTTGATATCAAGAGGCATGTTCAGTGGTGCCGGCAGCATCTCCTCTACCTTCTTTTCCT
CTTCATCTTCCTCCTTCTCCTCTTCTTCCTCCTCCACATTCTCCTCCCATCAGCAGGAAGACAAACCTAGGACAGTCTTGAAATATCGAAATTTCCTCCTTGGGATTTGCCAGCGC
CGCGTTGCGCCAACACTGTCGGAATAAAGGAGGCTGACTATTGTATGATCGTTATTTTATTAATTGGTCAGTGGGAAGATTACGGCTGAGCATAGGGGACATCTGTCACCCTTTCT
GCGCTAAGATTGAAAAATGAGGCTCGATTTGGAGACGCTCTAAAATGAAGCAAAAGGAATCAAAATTTTAATACAGCCACAGGAGCCCCCCCCCCACGCAGCACACTTTTATTTAT
ATTTTTTAGATTATTATTATTCTTTTTGTGGTGGTGGTTGGGGGGCGGTGATCGGGGTGGCTGGCTGGCTACAGGGGAGCTGCTTCCTTTTCCTTTTGGAAATGATTGTGCTGTTA
TTCTTTGCCTTGCTCTGGATGGTGGACGGAGTCTTTTCCCAGCTTCATTATACTGTGCAGGAAGAGCAGGAACATGGCACTTTCGTGGGGAATATCGCTGAAGACCTGGGCTTGGA
CATTACAAAACTTTCAGCTCGCAGGTTTCAGACGGTGGCCAACTCACGGACCCCTTACTTGGACCTCAATCTGGAGACCGGGGTTCTGTACGTAAACGAGAAGATAGATCGCGAGC
AAATCTGTAAGCAGAGCCCCTCTTGTGTCCTGCACCTGGAGGTCTTTCTGGAGAACCCGCTGGAGCTGTTCCGAGTGGAGATCGAAGTGCTGGACATCAATGACAACCCTCCCTCC
TTCCCAGAGCCCGACCTGACAGTGGAGATCTCAGAGAGCGCCACGCCAGGCACCCGCTTTCCCTTGGAGAGCGCCTTCGACCCAGACGTGGGGACCAACTCCTTGCGAGACTACGA
GATAACCCCAAATAGCTACTTCTCGCTAGACGTACAGACCCAGGGAGATGGCAACCGATTCGCCGAACTGGTCCTGGAGAAGCCACTGGACCGAGAACAGCAAGCGGTGCACCGCT
ACGTGCTGACCGCGGTGGACGGGGGAGGAGGGGGAGGAGGAGGGGAAGGAGGGGGAGGCGGTGGGGGAGCCGGCCTGCCCCCCCAGCAGCAGCGTACTGGCACGGCCTTGCTCACC
ATCCGAGTGCTCGACTCCAACGACAATGTGCCCGCTTTTGACCAACCCGTCTACACAGTTTCCCTACCAGAGAATTCTCCCCCTGGCACGCTAGTGATCCAGCTTAATGCCACCGA
CCCTGATGAAGGCCAGAATGGCGAGGTCGTGTACTCGTTCAGTAGTCACATTTCACCCAGGGCTCGGGAGCTCTTCGGACTGTCGCCGCGCACCGGCCGGCTGGAGGTGAGCGGCG
AGCTGGACTATGAAGAGAGCCCAGTGTATCAGGTGTATGTCCAAGCCAAGGACTTGGGTCCCAATGCTGTGCCTGCGCACTGCAAGGTTCTGGTGAGAGTGCTGGATGCCAACGAC
AACGCACCAGAGATCAGCTTCAGCACAGTGAAAGAGGCGGTGAGCGAGGGTGCGGCCCCTGGCACGGTGGTGGCTCTGTTCAGCGTGACCGATCGGGACTCAGAGGAGAACGGGCA
GGTGCAGTGTGAGCTTCTGGGAGACGTGCCGTTCCGCCTCAAGTCTTCCTTCAAGAATTACTACACTATCGTGACCGAAGCCCCCTTGGACCGAGAGGCTGGGGACTCCTACACCC
TGACCGTGGTGGCCCGCGACCGGGGCGAGCCTGCACTCTCCACCAGTAAGTCGATCCAGGTTCAAGTGTCAGATGTGAATGACAATGCGCCGCGCTTCAGCCAGCCGGTCTACGAC
GTGTATGTGACAGAAAACAATGTGCCGGGTGCCTACATTTACGCGGTGAGCGCCACGGACCGCGACGAAGGGGCCAATGCAAAATTAACCTACTCTATCCTAGAGTGCCAGATCCA
AGGAATGAGTGTCTTCACCTACGTGTCCATCAACTCAGACAACGGCTACTTGTACGCCCTGAGATCCTTCGACTATGAGCAGATCAAGGACTTCAGCTTTCAAGTAGAAGCCCGGG
ACGCCGGCAGTCCCCAGGCGCTGGCCGGCAATGCCACGGTCAACATCTTGATAGTGGATCAGAACGACAACGCCCCCGCCATCGTGGCGCCCCTTCCGGGGCGCAACGGGACTCCA
GCCCGCGAGGTGCTGCCGCGCTCTGCCGAACCCGGCTACCTGCTCACTCGTGTGGCCGCAGTGGACGCGGACGACGGCGAGAACGCCAGGCTCACCTACAGCATAGTGCGGGGCAA
CGAAATGAACCTCTTTCGACTGGACTGGCGCACCGGAGAGCTCCGCACCGCGCGCCGGGTCCCAGCCAAGCGCGACCCCCA.....CTTAGAAATACAA
AATGAGCCTAATAATCTATGCAAAGGAAAAGGAATGAATAAAAGTTCTCAAGAAAGGCTAGATTTGTTACACAGACATTCCTGGTAGCCTGTCTTCATGAGAGCTATCTTATTTCA
CAGCACAGTGTTGCTATTTTGTTAATGATTCTTTCAAAGAATTTAGGATCTTAGAGACTTTTAAGGATCATTAAAATGCTAAAATGTGTTCTGCAGCACGTTTTAAATAG

Tm1a^{NarI} gel based genotyping

External protocol Primers located either side of 5' loxP region. For tm1bNarl animals this will not work as the region has been deleted and you will need to rely on critical region assay dropping

Pcdh10-Tm1aNarl gel based primers

Pcdh10LF	GAGCTCAGAAACTGCCAGCCGAGA
Pcdh10LR	TTTCTCCGGATGGACGTTCCTCTT

See NLAC protocol

250 bp	Wildtype
350 bp	Mutant

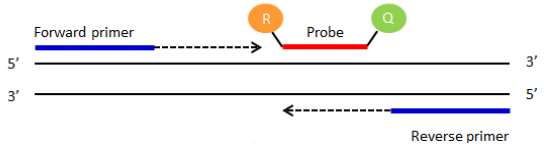
RED = Mutant sequence

TTTTCGTATTTGCTGATGCAAACAGGGGAACCTCTCTTATTCCCTGCCCATTACCTGCGGCTGGGGACTGGGAACTTTCCGCGGCAGAGTCCCCTGCAAGCGCCTCGGGACCTGCGGGCTGTTCT
TGCTCTCACACTCACACTCACTCCCGCAGCCAAAGCGAGCGTTCTGCCCCGCCCCTCCTGGCTGCCGCGTGACGTAACTACTTGACGGCCACTTTAGCCAACGAACAGTCGCTCGGTGGGTGGTG
CTCCTCGGCGATTGGTTGGCAGAATGAGGGCGCTGCGCAAAAACAGAGAAGCGGAGCGCTCGGAGCTCAGAAACTGCCAGCCGAGATCACAAGCATTGGGGCTGAAGCAGGAGGCAGGATG
GACTGAGAGGAGAAGAGGCAGGTTAGAGGGAGAGAGGCTTGCAAAGGAAACAGCAGAAAAGAAATAGCTGGTTGTGTTCACGCAAAGCCAGAGCCAGTGAAGTTGAGAATAGATAATACG
ACTCACTATAGGGCGAATTGGGCCGCATAACTTCGTATAGCATACATTATACGAAGTTATCTCGATAGCTTGAGTATTCTATAGTGTCACCTAAATCACAAGACTATGAAAGCCAAGAGATGCAA
AGAGAGACAGAGGGAAGAAAATGACAAGAGGAACGTCCATCCGGAGAAATGAAGAGAATGAAAGTTTTATGCTGCAGAACGGCTCCGTGTTTTTCCAGCAAAAATTTTTCTCGCTGTCTTTAAC
CCCGCTCGCGTTTGCCAGCCGTTGATATCAAGAGGCATGTTCAGTGGTGCCGGCAGCATCTCCTCTACCTTCTTTTCCTCTTCATCTTCCTCCTTCTCCTCTTCTTCCTCCTCCACATTCTCCTCCCATC
AGCAGGAAGACAAACCTAGGACAGTCTTGAAATATCGAAA

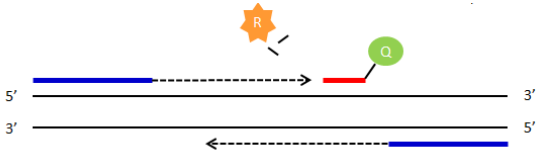
Run alongside Neo qPCR

qPCR based genotyping

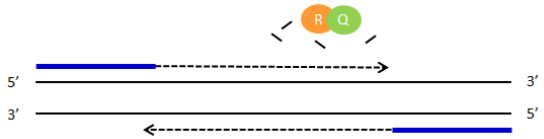
Standard PCR is the amplification of DNA between a pair of primers. Quantitative PCR employs the same principal as standard PCR, although it actually monitors the progress of the DNA synthesis as it occurs. The progress of the reaction is measured by using a Taqman probe. This is a short DNA oligo that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



No fluorescence



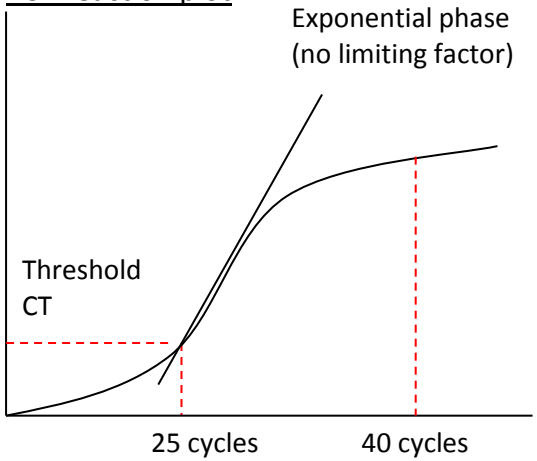
Reporter released, fluorescent signal observed



Fluorescent signal quenched

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.

qPCR assay sequences

NEO assay (FAM labelled probe)

ATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTC
AGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAG
CTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCT

Primer 1 = GGTGGAGAGGCTATTCGGC Primer 2 = GAACACGGCGGCATCAG Probe = TGGGCACAACAGACAATCGGCTG

Pcdh10-Narl-CR-LOA-wt1 assay (FAM labelled probe)

CCCTGATGAAGGCCAGAATGGCGAGGTCGTGTACTCGTTCAGTAGTCACATTTCACCCAGGGCTCGGGAGCTCTTCGGACTGTCGCCGCGCACCGGCCGGCTGGAGGTGAGCGGCG
AGCTGGACTATGAAGAGAGCCCAGTGTATCAGGTGTATGTCCAAGCCAAGGACTTGGGTCCCAATGCTGTGCCTGCGCACTGCAAGGTTCTGGTGAGAGTGCTGGATGCCAACGAC
AACGCACCAGAGATCAGCTTCAGCACAGTGAAAGAGGCGGTGAGCGAGGGTGCGGCCCCTGGCACGGTGGTGGCTCTGTTCAGCGTGACCGATCGGGACTCAGAGGAGAACGGGCA

Primer 1 = GCGCACTGCAAGGTTCTG Primer 2 = TCGCTCACCGCCTCTTTC Probe = TGAGAGTGCTGGATGCCAACGAC

All qPCR assays are run in duplex with a VIC labelled internal control, Dot1l

Dot1l internal control (VIC labelled internal control)

AACCAGTGGGCAGTGGATGTGAAGGGCAGGAAGCTCATAGGGTGACTGGCCAACCCAGGGAAGCCGGAGTGCTGCGTCTTCTGTTTCCTTGTTCTTTTCCCCTCTAGTCGTTTTCT
GTTAGTAGTTGGCATCCTTATGCTTCATCTTACAGTCGACTTGAGAGCTGGCCCTGAATGGTCGTGCTGGGGCAAGGCTTTATTTCAGGCGTAGCACACATGGTGGCCAATGGGAC
TCTGTAGGATCTGCCCACACCCATCAGGTGTGCAGGGAGACAGAGCTGAGTCAGGCTCCAGCTCTGGGGAATATGTTGAGTCACCACCTCTGTAGGGTGGTTGTGCATCATAGAAC

Primer 1 = GCCCCAGCACGACCATT Primer 2 = TAGTTGGCATCCTTATGCTTCATC Probe = CCAGCTCTCAAGTCG

qPCR genotyping set up

qPCR master mix

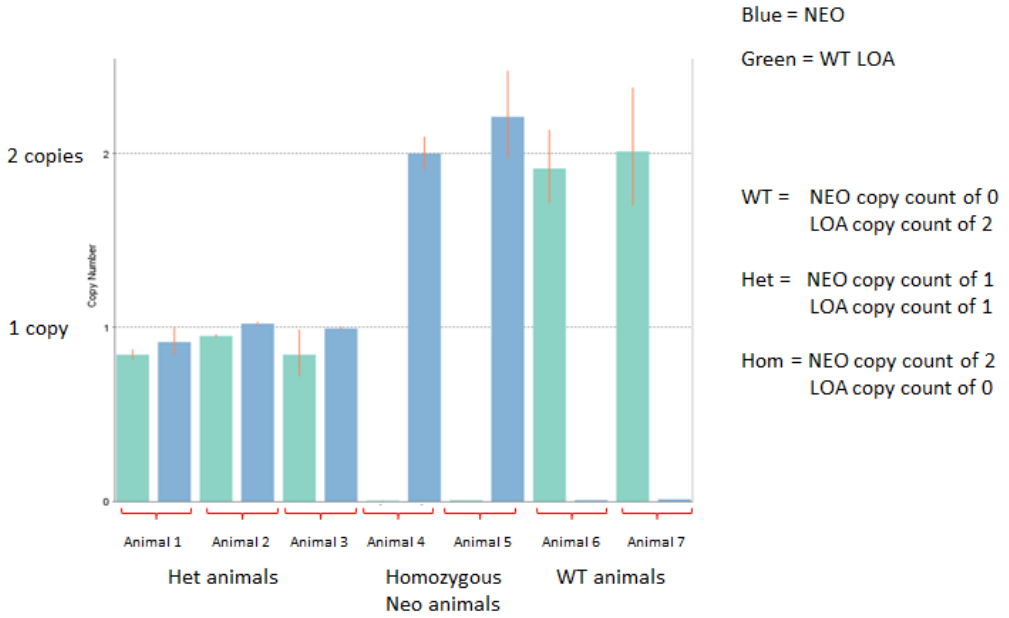
ABI GTX Taqman master mix	5µl
Primers Dot1L_2F (20µM)	0.225µl
Primers Dot1L_R (20µM)	0.225µl
Probe DotL_2M (5µM)	0.2µl
FAM Assay (probe 5µM & primers 15µM each)	0.3µl
Water	1.55µl

ALIQUOT 7.5µL

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

The results to the right have been run on an ABI 7500 and analysed using the ABI software CopyCaller. Each sample was run with a technical duplicate and for each assay, Neo and the BP-LOA, 7 controls of known copy number and a no template control, all in duplicate, were run so the software can accurately copy count.

Generic example of a NEO + BP-LOA copy called result



Allele conversions

The action of either Flp or Cre recombinase can be used to convert the KO 1st alleles described in this document. Both gel based and qPCR genotyping methods may be employed to detect animals carrying the DNA sequences coding for these proteins.

Cre genotyping

Cre gel based assays are run using primers specific to DNA sequences encoding Cre recombinase as well as a set of primers detecting a house keeping gene Slc40a1. These are run using the same cycling conditions as described in page 4.

ActinB-Cre gel based primers

Cre_F1	GCGGTCTGGCAGTAAAAACTATC
Cre_R1	GTGAAACAGCATTGCTGTCACTT
Slc40a1_sh_F (control)	CCTTTGTAACTTCCTCTGTGTC
Slc40a1_sh_R (control)	CTGAAGTCTTTCATGATAACTGCATT

Fragment sequences

Slc40a1 147bp

CCTTTGTAACTTCCTCTGTGTCTTTATTTTAGCCTAAACAGCAAAGACTTAAAAGATGGATCTTATCTGGAGAATAGTTCTATTTAGTCCAGCAAACTTTCTAGTAAATATGTTAG
ATGAAAATGCAGTTATCATGAAAGACTTCAG

Cre1 102bp

GCGGTCTGGCAGTAAAAACTATCCAGCAACATTTGGGCCAGCTAAACATGCTTCATCGTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCTGTTTCAC

Allele conversions

Cre genotyping

Cre qPCR assay (FAM labelled probe)

ATGTCCAATTTACTGACCGTACACCAAAATTTGCCTGCATTACCGGTCGATGCAACGAGTGATGAGGTTCGCAAGAACCTGATGGACATGTTCAGGGATCGCCAGGCGTTTTCTGA
GCATACCTGGAAAATGCTTCTGTCCGTTTGCCGGTCGTGGGCGGCATGGTGCAAGTTGAATAACCGGAAATGGTTTCCCGCAGAACCTGAAGATGTTCGCGATTATCTTCTATATC
TTCAGGCGCGCGGTCTGGCAGTAAAAACTATCCAGCAACATTTGGGCCAGCTAAACATGCTTCATCGTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCTGTTTCACTGGTT

Primer 1 = CGCAAGAACCTGATGGACATG Primer 2 = ACCGGCAAACGGACAGAA Probe = TTCAGGGATCGCCAGGCGTTT

qPCR set up for the Cre assay is exactly the same as previously, where the assay is run in duplex with a VIC labelled Dot1l internal control.

Allele conversions

Flpe genotyping

Flpe gel based assays are run using primers specific to DNA sequences encoding Flpe recombinase as well as a set of primers detecting a house keeping gene Slc40a1. These are run using the same cycling conditions as described in page 4.

Flpe gel based primers

ICS_Flpe_F	TCTTTAGCGCAAGGGGTAGGATCG
ICS_Flpe_R	GTCCTGGCCACGGCAGAAGC
Slc40a1_sh_F (control)	CCTTTGTAACTTCCTCTGTGTC
Slc40a1_sh_R (control)	CTGAAGTCTTTCATGATAACTGCATT

Fragment sequences

Slc40a1 147bp

CCTTTGTAACTTCCTCTGTGTCTTTATTTTAGCCTAAACAGCAAAGACTTAAAAGATGGATCTTATCTGGAGAAtagttctatttagtcCAGCAAACTTTCTAGTAAATATGTTAG
ATGAAAATGCAGTTATCATGAAAGACTTCAG

ICS-Flpe1 332bp

TCTTTCTTTAGCGCAAGGGGTAGGATCGATCCACTTGTATATTTGGATGAATTTTTGAGGAATTCTGAACCAGTCCTAAAACGAGTAAATAGGACCGGCAATTCTTCAAGCAACAA
ACAGGAATACCAATTATTAAAAGATAACTTAGTCAGATCGTACAACAAGGCTTTGAAGAAAAATGCGCCTTATCCAATCTTTGCTATAAAGAATGGCCCAAAATCTCACATTGGAA
GACATTTGATGACCTCATTTCTGTCAATGAAGGGCCTAACGGAGTTGACTAATGTTGTGGGAAATTGGAGCGATAAGCGTGCTTCTGCCGTGGCCAGGAC

Allele conversions

Flpe genotyping

Flpe qPCR assay (FAM labelled probe)

GGTAGGATCGATCCACTTGTATATTTGGATGAATTTTTGAGGAATTCTGAACCAGTCCTAAAACGAGTAAATAGGACCGGCAATTCTTCAAGCAACAAACAGGAATACCAATTATT
AAAAGATAACTTAGTCAGATCGTACAACAAGGCTTTGAAGAAAAATGCGCCTTATCCAATCTTTGCTATAAAGAATGGCCCAAAATCTCACATTGGAAGACATTTGATGACCTCAT
TTCTGTCAATGAAGGGCCTAACGGAGTTGACTAATGTTGTGGGAAATTGGAGCGATAAGCGTGCTTCTGCCGTGGCCAGGACAACGTATACTCATCAGATAACAGCAATACCTGAT
CACTACTTCGCACTAGTTTCTCGGTACTATGCATATGATCCAATATCAAAGGAAATGATAGCATTGAAGGATGAGACTAATCCAATTGAGGAGTGGCAGCATATAGAACAGCTAAA
GGGTAGTGCTGAAGGAAGCATACGATACCCCGCATGGAATGGGATAATATCACAGGAGGTACTAGACTACCTTTCATCCTACATAAATAGACGCATATAAT

Primer 1 = GTGGGAAATTGGAGCGATAAGC Primer 2 = ACCGAGAAACTAGTGCGAAGTAG Probe = CTTCTGCCGTGGCCAGGACAAC

qPCR set up for the Flpe assay is exactly the same as previously, where the assay is run in duplex with a VIC labelled Dot1l internal control.