

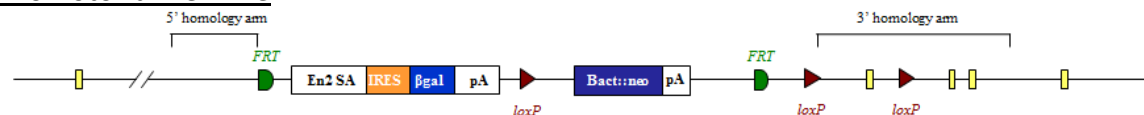


EUCOMM/KOMP-CSD 'Knockout-First' Genotyping

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 promoter and is separated from the lacZ gene by a 3rd loxP site, or promoterless (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

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/](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**

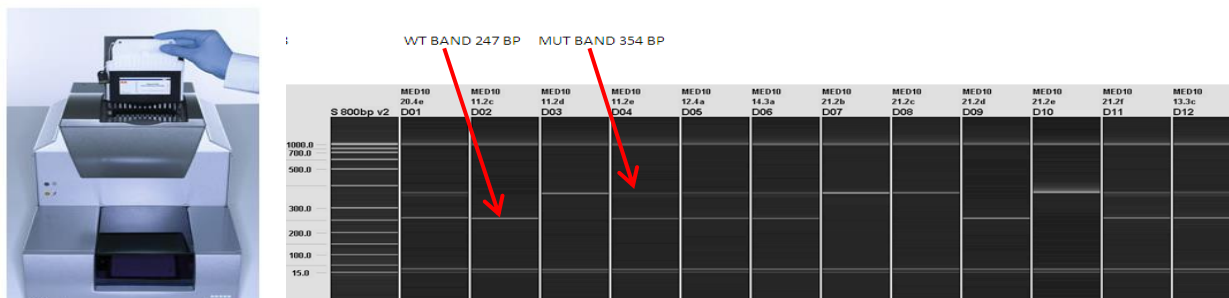
3'FRT is shown to be missing bases so may not work (missing sequence in yellow)

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 may be 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 ₂ O	2.0µl
DNA (~30ng)	1µl

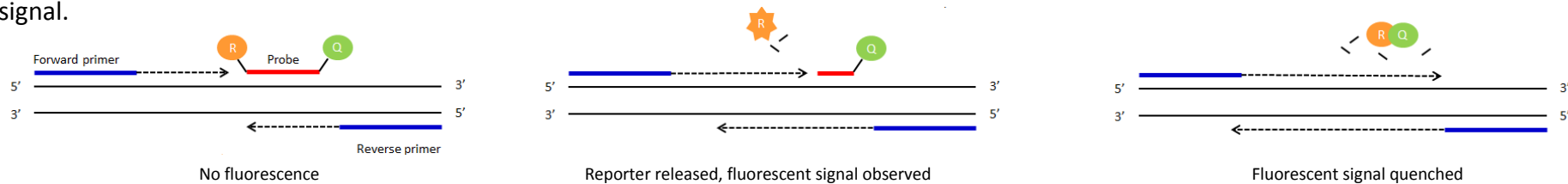
60°C Cycling conditions

60TM30FA		
1.	95°C	1min
2.	95°C	10sec
3.	60°C	10sec
4.	72°C	1sec
5.	Go to 2 for 29 cycles	
6.	72°C	30sec
7.	16 °C	forever



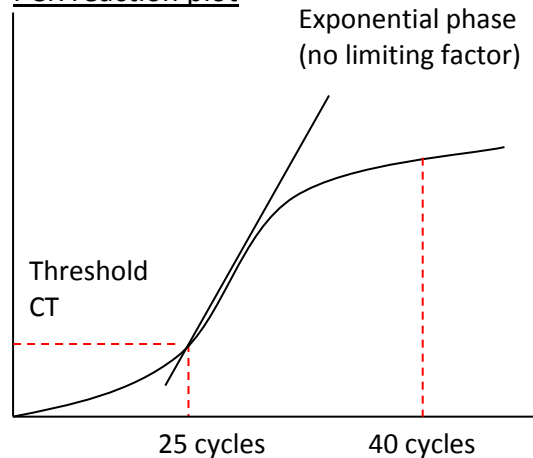
qPCR based genotyping

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.



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.



Pcdh10-Tm1aNarl

qPCR assay sequences

NEO assay (FAM labelled probe)

ATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTG **GGTGGAGAGGCTATTCGGC** TATGAC **TGGGCACAACAGACAATCGGCT** CT **CTGATGCCGCCGTGTT** CCGCTGTC
AGCGCAGGGGCGCCCGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAAGTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAG
CTGTGCTCGACGTTGTCACTGAAGCGGGAAGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCT
Primer 1 = GGTGGAGAGGCTATTCGGC Primer 2 = GAACACGGCGGCATCAG Probe = TGGGCACAACAGACAATCGGCTG

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

CCCTGATGAAGGCCAGAATGGCGAGGTCGTGTACTCGTTTCAGTAGTCACATTTACCCAGGGCTCGGGAGCTCTTCGGACTGTCGCCGCGCACCCGGCCGGCTGGAGGTGAGCGGCG
AGCTGGACTATGAAGAGAGCCCAGTGTATCAGGTGTATGTCCAAGCCAAGGACTTGGGTCCCAATGCTGTGCCT **GCGCACTGCAAGGTTCTG** **TGAGAGTGCTGGATGCCAACGAC**
AACGCACCAGAGATCAGCTTCAGCACAGT **GAAAGAGGCGGTGAGCGA** GGGTGCGGCCCTGGCACGGTGGTGGCTCTGTTTCAGCGTGACCGATCGGGACTCAGAGGAGAACGGGCA
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)

AACCAGTGGGCAGTGGATGTGAAGGGCAGGAAGCTCATAGGGTGACTGGCCAACCCAGGGAAGCCGGAGTGCTGCGTCTTCTGTTTCCTTGTTCCTTTTCCCCTCTAGTCGTTTTCT
GTTAG **TAGTTGGCATCCTTATGCTTCATC** TTACAGT **CGACTTGAGAGCTG** CCTG **AATGGTCGTGCTGGGGC** AAGGCTTTATTTTCAGGCGTAGCACACATGGTGGCCAATGGGAC
TCTGTAGGATCTGCCACACCCATCAGGTGTGCAGGGAGACAGAGCTGAGTCAGGCTCCAGCTCTGGGGAATATGTTGAGTCACCACCTCTGTAGGGTGGTGTGCATCATAGAAC
Primer 1 = GCCCCAGCACGACCATT Primer 2 = TAGTTGGCATCCTTATGCTTCATC Probe = CCAGCTCTCAAGTCTG



Pcdh10-Tm1aNarl

qPCR genotyping set up

qPCR master mix

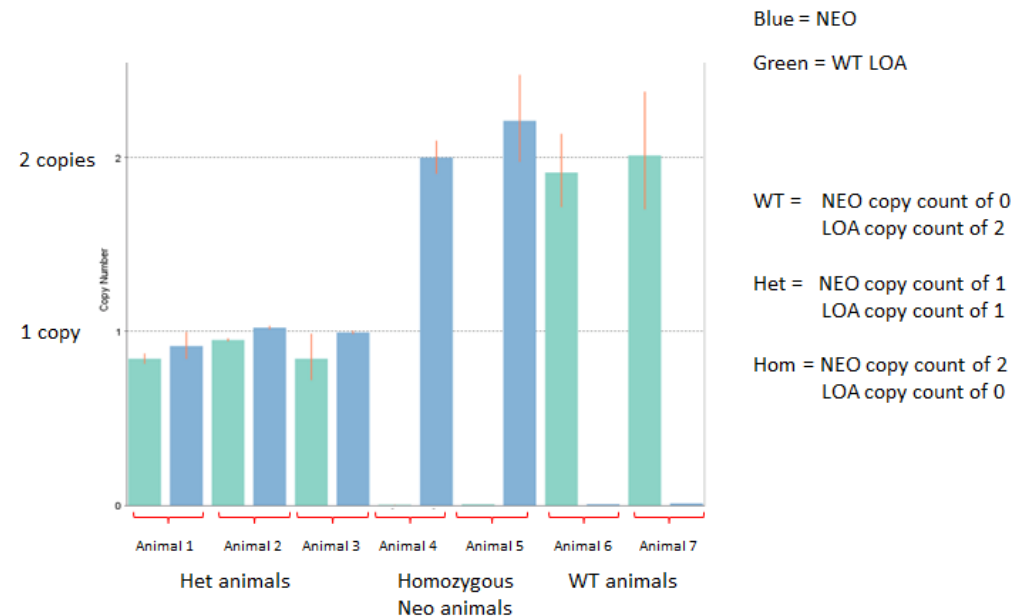
ABI GTX Taqman master mix	5µl
Primers Dot1L_2F (20µM)	0.225µl
Primers Dot1L_R (20µM)	0.225µl
Probe DotL_2M (5µM)	0.2µl
FAM Assay (probe 5µM & primers 15µM each)	0.3µl
Water	1.55µl

ALIQUOT 7.5µL

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

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	GCGGTCTGGCAGTAAAACTATC
Cre_R1	GTGAAACAGCATTGCTGTCACCTT
Slc40a1_sh_F (control)	CCTTTGTAACCTCCTCTGTGTC
Slc40a1_sh_R (control)	CTGAAGTCTTTCATGATAACTGCATT

Fragment sequences

Slc40a1 147bp

CCTTTGTAACCTCCTCTGTGTC TTTATTTTAGCCTAAACAGCAAAGACTTAAAAGATGGATCTTATCTGGAGAATAGTTCTATTTAGTCCAGCAAACCTTCTAGTAAATATGTTAG
ATGAA AATGCAGTTATCATGAAAGACTTCAG

Cre1 102bp

GCGGTCTGGCAGTAAAACTATC CAGCAACATTTGGGCCAGCTAAACATGCTTCATCGTCGGTCCGGGCTGCCACGACCA AGTGACAGCAATGCTGTTTCAC



Allele conversions

Cre genotyping

Cre qPCR assay (FAM labelled probe)

ATGTCCAATTTACTGACCGTACACCAAATTTGCCTGCATTACCGGTTCGATGCAACGAGTGATGAGGTT **CGCAAGAACCTGATGGACATG** **TTCAGGGATCGCCAGGCGTTT** TCTGA
GCATACCTGGAAAATGC **TTCGTCCGTTTGCCGGT** CGTGGCGGCATGGTGAAGTTGAATAACCGGAAATGGTTTCCCGCAGAACCTGAAGATGTTTCGCGATTATCTTCTATATC
TTCAGGCGCGCGGTCTGGCAGTAAAACTATCCAGCAACATTTGGGCCAGCTAAACATGCTTCATCGTTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCTGTTTCACTGGTT

Primer 1 = **CGCAAGAACCTGATGGACATG**

Primer 2 = **ACCGCAAACGGACAGAA**

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)	CCTTTGTAATTCCTCTGTGTC
Slc40a1_sh_R (control)	CTGAAGTCTTTCATGATAACTGCATT

Fragment sequences

Slc40a1 147bp

CCTTTGTAATTCCTCTGTGTCTTTATTTTGTAGCCTAAACAGCAAAGACTTAAAAGATGGATCTTATCTGGAGAAtagttctatttagtcCAGCAAACCTTCTAGTAAATATGTTAG
ATGAAAATGCAGTTATCATGAAAGACTTCAG

ICS-Flpe1 332bp

TCTTTCTTTAGCGCAAGGGGTAGGATCGATCCACTTGTATATTTGGATGAATTTTTGAGGAATTCTGAACCAGTCCTAAAACGAGTAAATAGGACCGGCAATTCTTCAAGCAACAA
ACAGGAATACCAATTATTTAAAAGATAACTTAGTCAGATCGTACAACAAGGCTTTGAAGAAAAATGCGCCTTATCCAATCTTTGCTATAAAGAATGGCCAAAATCTCACATTGGAA
GACATTTGATGACCTCATTCTGTCAATGAAGGGCTAACGGAGTTGACTAATGTTGTGGAAATTGGAGCGATAAGCGT**GCTTCTGCCGTGGCCAGGAC**



Allele conversions

Flpe genotyping

Flpe qPCR assay (FAM labelled probe)

GGTAGGATCGATCCACTTGTATATTTGGATGAATTTTTGAGGAATTCTGAACCAGTCCAAAACGAGTAAATAGGACCGGCAATTCCTCAAGCAACAAACAGGAATACCAATTATT
AAAAGATAACTTAGTCAGATCGTACAACAAGGCTTTGAAGAAAAATGCGCCTTATCCAATCTTGTATAAAGAATGGCCAAAATCTCACATTGGAAGACATTTGATGACCTCAT
TTCTGTCAATGAAGGGCCTAACGGAGTTGACTAATGTTGTGGGAAATTGGAGCGATAAGCGTGCTTCTGCCGTGGCCAGGACAAGTATACTCATCAGATAACAGCAATACCTGAT
CACTACTTCGCACTAGTTTCTCGGTACTATGCATATGATCCAATATCAAAGGAAATGATAGCATTGAAGGATGAGACTAATCCAATTGAGGAGTGGCAGCATATAGAACAGCTAAA
GGGTAGTGCTGAAGGAAGCATACGATACCCCGCATGGAATGGGATAATATCACAGGAGGTACTAGACTACCTTTCATCCTACATAAATAGACGCATATAAT

Primer 1 = GTGGGAAATTGGAGCGATAAGC

Primer 2 = ACCGAGAACTAGTGCGAAGTAG

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.