Name of Mouse model or mutation:
KCNK13-FLOX-EM2-B6N

Description:
Floxed allele made by CRISPR/Cas9 gene editing.

Type of mutation:
Flox

Delivery method:
Pronuclear injection into 1-cell stage embryo

Genetic Background:
C57BL/6NTac

Nuclease:
Cas9 mRNA

sgRNAs:

Protospacer sequence	PAM sequence
CTGCTTTCCTAGAAAGGCTA	GGG
CCTAGAAAGGCTAGGGCTGA	GGG
GGAGGCTATCTGTAATGGTC	TGG
AGCAACCTCCATTCCTAGGA	AGG

lssDNA donor sequence (5'-3'):

LOCUS Kcnk13_flox_lssD 2456 bp DNA linear 14-MAY-2020
FEATURES Location/Qualifiers
misc_feature 130..163
/note="loxP"
misc_feature 122..129
/note="AsiSI (SfaAI)"
PCR_primer 101..121
/note="LoxPF"
misc_feature 1..100
/note="5'HA"
misc_feature 164..463
/note="Protected"
misc_feature 464..2144
/note="Exon (ENSMUSE00000861683)"

misc_feature 2295..2328
/note="loxP"
misc_feature 2329..2336
/note="MreI"
PCR_primer complement(2337..2356)
/note="LoxPR"
misc_feature 2145..2294
/note="Protected"
misc_feature 2357..2456
/note="3'HA"
source 1..2456
/dnas_title="Kcnk13_flox_lssDNA"
ORIGIN
1 GAGGGGCCTT TCCCCGCTTC ACACAATAGC ACCATATGGG CTTGCAGCAG GCCAAACTCT
61 GTATGCTGAA CCACTGCCTT TCAACAGAGA TGGGGATGCT atccgggggt accgcgtcga
121 gGCGATCGCA TAACTTCGTA TAGCATACAT TATACGAAGT TATTCTCCGG TCTCCAAATC
181 TTACACACCA GTGAGTCTCA ACCTGTGGGT CATGGTCCCT TTGGGGTTCG CATTTCAGAT
241 ATCCTGCATA TCAGGTACTT ACATTAGAAT TCATAACAGT AGCAAACTTA CAGTTATGAA
301 GTAGCAACAA GATAATCTTA CGGTGGAGGG GAGTCACCAC AGCATGAGGA ACCGCATTAA
361 AGGGTCACGG GTTTAGGAAG GTCAAGAACC TCTGCTCTAC ACCAACTCCT CTCCCAGGAA
421 GGCTTCCACT CATGCTTTTC TCCTCTGGTT TTGTCTTCTT CAGGGTTTGG GATGACAACA
481 CCAGCCACAA CGGGAGGGAA GATTTTTCTG ATCTTTTATG GTCTCATTGG ATGTGCAAGT
541 ACCATCCTCT TCTTCAACCT TTTCCTGGAG CGGCTGATCA CTGTCATCGC CTGTGTCATG
601 AGATCCTGTC ACCAGCAGCA GCTGCGCAGA CGTGGGGCGG TGACCCAGGA CAACATGAAG
661 GCTCCTGAAA AGGGGGAGGC AGACAGCCTG ACTGGCTGGA AGCCCTCTGT GTACTACGTC
721 ATGCTGATCC TATGCTTGGC ATCAGTGGCC ATCTCCTGCG GAGCCTCTGC TCTGTACACC
781 ACCATGGAGG GCTGGAGCTA CTTTGACTCG GTCTACTTCT GTTTTGTGGC TTTCAGCACC
841 ATTGGCTTCG GGGACCTGGT GAGCAGCCAG AATGCTCAGT ATGAGAGCCA AGGACTCTAC
901 CGCTTCTTCA ATTTCTTCCT CATCCTCATG GGTGTCTGCT GCATCTACTC TTTGTTTAAC
961 GTCATCTCCA TCCTGATCAA ACAGACTGTG AACTGGATCC TGAGGAAACT GGATAGCGGG
1021 TGCTTCCCAC CATGCCAAAG AGGACTCCTG CGGTCCAGGA GGAATGTGGT GATGCCGGGT
1081 AACATCCGGA ACAGGTGCAA CATCTCCATA GAGACAGACG GGGTGATGGA AAGTGACACT
1141 GATGGACGAC GTCTCTCGGG GGAGATGATC TCCATGAAGG ACACCAACAA GGTCTCCCTG
1201 GCCATCCTGC AGAAGCAGTT GTCCGAGATG GCCAATGGGG GACCCCACCA GAACAGTGCA
1261 TCCTCCCGGG ATGATGAGTT CTCAGGGGGA GTGGGAGCCT TTGCAGTAAT GAATAACAGG
1321 TTGGCAGAGA CCAGTGGAGA TAGGTAGGAA CCAGAAGTAG CTGCTATATG AAGAAAACCA
1381 GGGCATGTGG GGAATAAGAC ACTGTTACAC CACTGGTAAG CTCAGCTGTG CCTCTGGCTG
1441 TTGCAATTAA TATCTCCAGT CCAGGTTTTG AAATCTGACC TTGGCCTCAG GCAAGAGTAG
1501 CCTCTCACAG TTGAGGGCTG GAGCCTCTTT CCCTGGCTCT TACTTTACTT TTGAATTCCA
1561 AGTTTGACAT TTGGAACTGC TAAGGGCCTT GCCTTCTTCC AGAGTTGTAA TGGTCTGAGG
1621 GAGACTGGTC CAGTTTTGGT TTCTCCACCA TGATCCATTC TTAGGAAGGG AGAGCCCCCA
1681 AAAAGCCTGC TTCTGTCCTG GAACGTGAGT GGCTGAACTT CACCCAGCCC CTCAAGAAAA
1741 GGCAACAAAC ATTCCAGAGT GTCTCTGGGC CTTCTGTTTG CTGGACTTCC TTCCAAACAT
1801 ATCTATTGTT GGTTCCATTG ATGGGGACAC TATCTCATCT TCTCTTATAC CTGCTTCTCT
1861 GTTTGGGAGT CTTGGTTTCT TTTTGTTTTG TTTCCTGTTT GACTAATATA TCTTTTGGAT

1921 CTTATGTGAT AAGAAAACTA AATCCACCTT CAAACCTTTA AGCTTGGCAG TGAGTAGAGG
1981 AAGAGGGAAA CTCCATCTTG TAACTAAAGC CCACAGCTTA CTATGTCCTC TCTGAATGGT
2041 TACACGGATC GTGTCATTTT ACCAGGGCTT GGAGCCTGGT GCAGAGGCAA CTGACTATTT
2101 ATTAGAGTTG AACATTTAAT AAATTCTCAT TTTGTTAACT ACGAGGCCAT GAGTAATCAT
2161 TGCAGACAGT GTAAAGGAAG CTGGAGCTAT GAGCCTCATC ATGGAGTCTG AGCTGGAGCC
2221 TAACATTCCA GCCCACATAA TGAAGTGTGG CTTTCTGGTC CCATCTGCTT CTGAGACTGT
2281 GCCAAGGACA GAGAATAACT TCGTATAGCA TACATTATAC GAAGTTATCG CCGGCGggtc
2341 tgagctcgcc atcagtCTGA CATGCAAGAG AAAAACGTGT AAGGCATAGC AGGAGTGGTT
2401 TTTTACAAGA TTAAAATGGG TACACACAGG ACTCTCAGAA GTGATTAGGT GGACCA
//

Microinjection mixes:

Microinjection buffer (MIB; 10 mM Tris–HCl, 0.1 mM EDTA, 100 mM NaCl, pH7.5) was prepared and filtered through a 2 nm filter and autoclaved. Cas9 mRNA, sgRNAs and lssDNA donor were diluted and mixed in MIB to the working concentrations of 100 ng/µl, 50 ng/µl each and 50 ng/µl, respectively. Injected embryos were re-implanted in CD1 pseudo-pregnant females. Host females were allowed to litter and rear F_0 progeny.

Sequence details

WT

CCTTCTGCCTGGACAGTACCTCCTGGGTTTCCAAGACCCTCAGTGTAACCTTCCCAGAACCCCAAGAC
TGAGATGGATGCCCCTCTCTGCAGCTGCCAGGAAGTCCTGTACCACTTCCAAGCCCAGTACTTACCAT
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GACGTCTCTCGGGGGAGATGATCTCCATGAAGGACACCAACAAGGTCTCCCTGGCCATCCTGCAGA
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KCNK13-FLOX-EM2-B6N

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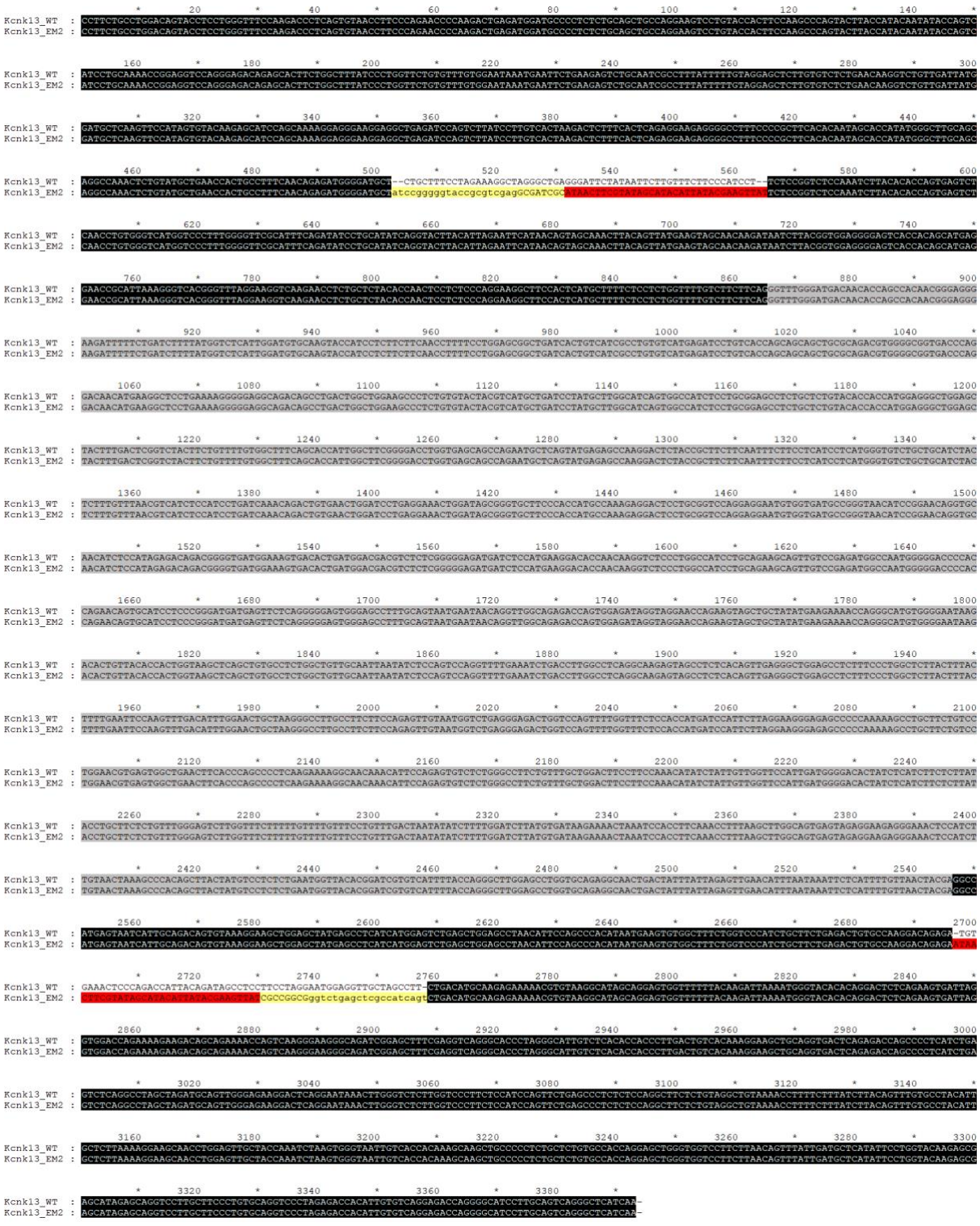
TGTCACCACAAAGCAAGCTGCCCCCTCTGCTCTGTGCCACCAGGAGCTGGGTGGTCCTTCTTAACAG

TTTATTGATGCTCATATTCCTGGTACAAGAGCGAGCATAGAGCAGGTCCTTGCTTCCCTGTGCAGGTC
CCTAGAGACCACATTGTGTCAGGAGACCAGGGGCATCCTTGCAGTCAGGGCTCATCAA

LoxP sites are underlined and genotyping handles (restriction enzyme site plus primer unique to each LoxP site) in italics. Floxed exon highlighted in bold.

Nucleotide Alignment:

Alignment below shows how WT and floxed allele align. LoxP sites highlighted in red, genotyping handles (restriction enzyme site plus primer unique to each LoxP site) in yellow and floxed exon in grey.



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:

LoxPF	AAAGGAGGGAAGGAGGCTGA
LoxPR	AGAGAAGCCTGGAGAGAGGG
Taq Polymerase used	Roche Expand Long Range dNTP pack
Annealing Temperature (°C)	61
Elongation time (min)	3
WT product size (bp)	N/A
Mutant product size (bp)	2256
Notes	PCR used to screen for floxed alleles. 3% DMSO used in reactions.

Geno_Kcnk13_Flox_F3	CCTTCTGCCTGGACAGTACC
Geno_Kcnk13_Flox_R3	TTGATGAGCCCTGACTGCAA
Taq Polymerase used	ThermoFisher SuperFi II
Annealing Temperature (°C)	64
Elongation time (min)	1.75
WT product size (bp)	3387
Mutant product size (bp)	3393
Notes	Sequenced with Geno_Kcnk13_Flox_F3, Geno_Kcnk13_Flox_R3, LoxPF and LoxPR as well as Geno_Kcnk13_Flox_SeqF3 (CCGGGATGATGAGTTCTCAG) Geno_Kcnk13_Flox_SeqF4 (TGGGCCTTCTGTTTGCTGGACTTC) Geno_Kcnk13_Flox_SeqR1 (GCCAACCTGTTATTCATTACTGC) Geno_Kcnk13_Flox_SeqR2 (AGCATGACGTAGTACACAGAGGG)

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 sex chromosome.

For further coverage of the critical region, the following PCR was conducted, and amplicons sequenced:

Geno_Kcnk13_Flox_F6	CCCAGGACAACATGAAGGCT
Geno_Kcnk13_Flox_R6	TCACGTTCCAGGACAGAAGC
Taq Polymerase used	ThermoFisher SuperFi II
Annealing Temperature (°C)	60
Elongation time (min)	0.75
WT product size (bp)	1065
Mutant product size (bp)	1065
Notes	

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

Off-target site	Sequence	Type	Primers used (5'-3')
15:25961892-25961914	CTGCTTTCCTGGAAAGGCTT GGG	Intronic	Kcnk13_OT1_F1: ACATCTCCCAGACTTTGTCCAG Kcnk13_OT1_R1: TAGGGACAGAGTCAAGACCTAAGA
8:32406613-32406635	CCTAGAAAGGCTGGGGCTCA AGG	Intergenic	Kcnk13_OT2_F1: AACCACATCAAGACCATCTCCATT Kcnk13_OT2_R1: TGATTTTTGCCCTAGTCCCAGT
4:123644946-123644968	CTTAGAAAGGCTAGGGCTGG AGG	Intronic	Kcnk13_OT3_F4: CTGCCAGCCTGAAGTACACA Kcnk13_OT3_R4: TGGCCTCTAACTTGGGACCT Sequenced with: Kcnk13_OT3_F1: TTCTCTGCAAGAGCAGCTAG Kcnk13_OT3_R1: TCTTAGAAGAGTACCTGTCTGG Kcnk13_OT3_R5: CAGAAGTCCAGAGGTCAGGC
2:129607223-129607245	CCTAGAAAGGATAGGGCTGG TGG	Intronic	Kcnk13_OT4_F1: CTGTCCAACCAGTCTCGCAT Kcnk13_OT4_R1: GTTGCTGCACTGTCAAAGGG
8:9072092-9072114	AACAACCTCCATTCCTAGAA CGG	Intergenic	Kcnk13_OT5_F1: GATGTTAATGATTTCTTCGGAGCAT

			Kcnk13_OT5_R1: TGGTCAGACCTGAACAACCC

All amplicons were sent for Sanger sequencing. No evidence of off-target activity was detected.

Additional integrations of the donor sequence

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	KCNK13-FLOX-DON-MUT5’
Forward Primer (5’-3’)	GCAGCAGGCCAAACTCTGT
Reverse Primer (5’-3’)	GACCATGACCCACAGGTTGAG
Probe (5’-3’)	TCGAGGCGATCGCATAACTTCG
Label	FAM

This ddPCR assay is specific to the KCNK13 Flox donor and only MUT alleles are expected to be 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.

Assay name	KCNK13-FLOX-3'-MUT1
Forward Primer (5’-3’)	GTGCCAAGGACAGAGAATAACTTC
Reverse Primer (5’-3’)	CCACTCCTGCTATGCCTTACAC
Probe (5’-3’)	AAGTTATCGCCGGCGGGTCTGA
Label	FAM

This ddPCR assay is specific to the KCNK13 Flox donor and only MUT alleles are expected to be 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.

Assay name	KCNK13-CR-LOA-WT1
Forward Primer (5’-3’)	TGGCAGTGAGTAGAGGAAGA
Reverse Primer (5’-3’)	CACGATCCGTGTAACCATTCAG
Probe (5’-3’)	CTCCATCTTGTAACTAAAGCCCACAGC

Label	FAM

This ddPCR assay is universal to KCNK13 - both WT and MUT alleles are recognised by this assay. Therefore, WT controls are expected to call at 2 copies and a single integration for a correct mutation is expected to call at 2 copies for F1 (HET) animals.

Reference Assay Name	Dot1l
Forward primer (5'-3')	GCCCCAGCACGACCATT
Reverse primer (5'-3')	TAGTTGGCATCCTTATGCTTCATC
Probe (5'-3')	CCCAACAGGCCTGGATTCTCAATGC
Label	VIC

VIC-labelled reference assay for Dot1l gene.

No random integrations were detected in the animals selected for breeding.

Kcnk13-Flox

Allele Description

This is a CRISPR/Cas9 induced mutation creating a conditional knock-out by floxing critical exon, ENSMUSE00000861683 of *Kcnk13*. The stock was generated at MRC Harwell via pronuclear injection of CRISPR/Cas9 reagents into 1-cell stage embryos.

qPCR Copy Counting Genotyping Strategy

The genotyping strategy presented here has been optimized for reagents and conditions used by the Genotyping Core at MRC Harwell. To genotype animals, we recommend researchers validate the assay independently. PCR cycling temperature and times may require additional optimization based on the specific genotyping reagents used.

Samples are genotyped using qPCR copy counting with both a wildtype loss of allele (WT-LOA) and a mutant assay against a known reference assay (*Dot1l* on chromosome 10; 2 copies present). Samples for this line are genotyped using the following primers and probe:

- Universal probe and Universal primer designed 5' of the deleted region.
- Wildtype specific primer situated within the deleted region.
- Mutant specific primer that binds to the inserted LoxP sequence

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

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

Kcnk13-Flox

Kcnk13-Flox-WT1 assay (FAM labelled)

GGGGCCTTTCCCCGCTTCACACAATAGCACCATATGGGCTTGCAGCAGGCCAAACTCTGTATGCTGAA**CCACT**
GCCTTTCAACAGAGATGGGGATGCTCTGCTTTCCT**AGAAAGGCTAGGGCTGAGGGATTCTA**TAATTCTTGTTT
CTTCCCATC**CTTCTCCGGTCTCCAAATCTTAC**ACACCAGTGAGTCTCAACCTGTGGGTCATGGTCCCTTTGGGG

Probe sequence is in bold and shaded grey
Primer sequences are in bold and underlined

Oligo Kcnk13-Flox	5' label	Sequence 5' → 3'	3' label	Oligo Type
Kcnk13-Flox-WT_F	n/a	**CCACTGCCTTTCAACAGAGA**	n/a	WT Forward
Kcnk13-Flox-WT_PROBE	FAM	**AGAAAGGCTAGGGCTGAGGGATTCTA**	Zen-IBFQ	WT Probe
Kcnk13-Flox-WT_R	n/a	**GTAAGATTTGGAGACCGGAGAAG**	n/a	WT Reverse

Kcnk13-Flox -MUT1 assay (FAM labelled)

ATTGCAGACAGTGTAAAGGAAGCTGGAGCTATGAGCCTCATCATGGAGTCTGAGCTGGAG**CCTAAC**
ATTCCAGCCCACATAA**TGAAGTGTGGCTTTCTGGTCCCAT**CTGCTTCTGAGACTGTGCCAAGGACAG
AGAataacttcgtatagcataca**ttatacgaagttatCGCCGGC**GGGTCTGAGCTCGCCATCAGTCTGACATGCA

Lower case letters denote the inserted LoxP sequence
Probe sequence is in bold and shaded grey
Primer sequences are in bold and underlined

Oligo Kcnk13-Flox	5' label	Sequence 5' → 3'	3' label	Oligo Type
Kcnk13-Flox-MUT_F	n/a	**CCTAACATTCCAGCCCACATAA**	n/a	Mutant Forward
Kcnk13-Flox-MUT_PROBE	FAM	**TGAAGTGTGGCTTTCTGGTCCCAT**	Zen-IBFQ	Mutant Probe
Kcnk13-Flox-MUT_R	n/a	**GCCGGCGATAACTTCGTATAA**	n/a	Mutant Reverse

Dot1l internal control (VIC labelled)

CTGATGGGTGTGGGCAGATCCTACAGAGTCCCATTGGCCACCATGTGTGCTACGCCTGAAATAAAGCCTT**GCC CCAGCACGACCATT**CAGGG**CCAGCTCTCAAGTCG**ACTGTAA**GATGAAGCATAAGGATGCCAACTA**CTAACA GAAAACGACTAGAGGGGAAAAGAACAAGGAAACAGAAGACGCAGCACTCCGGCTTCCCTGGGTTGGCCAGT CACCCTATGA

Oligo Kcnk13-Flox	5' label	Sequence 5' → 3'	3' label	Oligo Type
Dot1l_Forward	n/a	**GCCCCAGCACGACCATT**	n/a	WT Forward
Dot1l_Probe	VIC	**CCAGCTCTCAAGTCG**	BHQ	WT Probe
Dot1l_Reverse	n/a	**TAGTTGGCATCCTTATGCTTCATC**	n/a	WT Reverse

Probe sequence is in bold and shaded grey
Primer sequences are in bold and underlined

DNA extraction method

DNA is extracted from ear clips using Applied Biosystems Taqman Sample-to-SNP Kit and qPCR run using 1:10 dilution from the crude preparation.

qPCR master mix 1X

Applied Biosystems GTX Taqman master mix	5 µl
Dot1l_Forward (20 µM)	0.225 µl
Dot1l_Reverse (20 µM)	0.225 µl
Dot1l_Probe (5 µM)	0.2 µl
FAM Assay (probe 5 µM & primers 15 µM each)	0.3 µl
ddH20	1.55 µl
DNA (1:10 dilution of ABI Sample-to-SNP prep)	2.5 µl

Each sample is ran in technical duplicate. Seven WT and/or mutant controls are also included in duplicate along with non-template controls.

qPCR cycling conditions

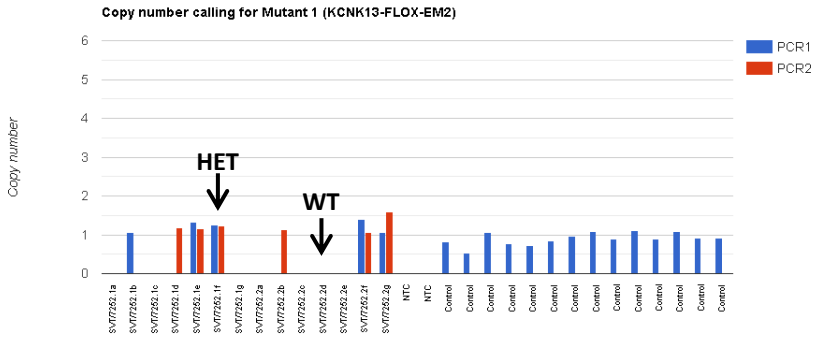
qPCR instrument: Applied Biosystems 7500/7900 or ThermoFisher QuantStudio 7

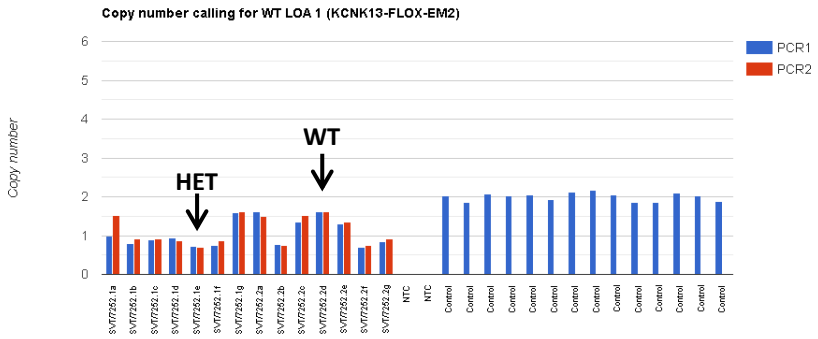
95°C for 20 sec
Then 40 cycles of;
95°C for 3 sec
60°C for 30 sec

Analysis

The results are analysed using CopyCaller software v2.0 from Applied Biosystems or in-house software that is based on CopyCaller v2.0.

Kcnk13-Flox'-WT1 and Kcnk13-Flox-MUT1 assays copy called results, image showing copy number chart for WT and Mutant assays (Task 315569 results)





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