Name of Mouse model or mutation:
CXCR4-CRE-ERT2-EM2-B6N

Description:
ERT2 cassette knock in to the existing Cxcr4-CRE-EM1-B6N allele made by CRISPR/Cas9 gene editing.

Type of mutation:
Knock-in: ERT2 cassette

Delivery method:
Pronuclear injection into 1-cell stage embryo.

Genetic Background:
C57BL/6NTac

Nuclease:
Cas9 mRNA

sgRNAs:

Protospacer sequence	PAM sequence
aggggcaatggtgcgcctgc	TGG
cttaatcgccatcttccagc	AGG
tcctatactatttgaagaat	AGG

lssDNA donor sequence template (5'-3', NOTE donor delivered to embryo will be reverse complement (Codner et al., 2018: Figure 1)):

LOCUS Cxcr4_Cre_ERT2_D 1373 bp DNA linear 08-DEC-2021
FEATURES Location/Qualifiers
PCR_primer complement(3..22)
/dnas_title="p_Cxcr4_2cuts_seqR4"
/note=""
/note=""
/current=0
PCR_primer 89..108
/note="Cre_F3"

/current=0
misc_feature 1..200
/note="200 nt 5'HA"
misc_feature 184..200
/note="Cxcr4_CRE-ERT2_5_1"
misc_feature complement(200..217)
/note="Cxcr4_CRE-ERT2_5_2 (OTs in B6N)"
misc_feature 197..199
/note="SMs to kill the activity of guides 5_1 and 5_2:
cgc>AgA R=R usage from 9.4 to 12.1"
misc_feature 1..217
/note="CRE"
misc_feature 227..1171
/note="ERT2"
misc_feature 218..226
/note="Linking sequence as per Adipoq Cre-ERT2"
misc_feature 1175..1185
/note="Remains of F3"
PCR_primer 1258..1280
/dnas_title="p_Cxcr4_2cuts_seqF2"
/note=""
/note=""
/current=0
PCR_primer complement(1326..1349)
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/note="Sv40 polyA site"
misc_feature 1175..1373
/note="200 nt 3'HA"
misc_feature complement(1350..1373)
/dnas_title="Cxcr4-ERT2_RNA_R"
/note="Cxcr4-CRE-ERT2_D2_RNA_R"
source 1..1373
/dnas_title="Cxcr4_Cre_ERT2_Donor2"
ORIGIN
1 gatttacggc gctaaggatg actctggtca gagatacctg gcctggtctg gacacagtgc
61 ccgtgtcgga gccgcgcgag atatggcccg cgctggagtt tcaataccgg agatcatgca
121 agctggtggc tggaccaatg taaatattgt catgaactat atccgtaacc tggatagtga

181 aacaggggca atggtgAgAc tgctggaaga tggcgatctc gagccatctg ctggagacat
241 gagagctgcc aacctttggc caagcccgct catgatcaaa cgctctaaga agaacagcct
301 ggccttgtcc ctgacggccg accagatggt cagtgccttg ttggatgctg agccccccat
361 actctattcc gagtatgatc ctaccagacc cttcagtgaa gcttcgatga tgggcttact
421 gaccaacctg gcagacaggg agctggttca catgatcaac tgggcgaaga gggtgccagg
481 ctttgtggat ttgaccctcc atgatcaggt ccaccttcta gaatgtgcct ggctagagat
541 cctgatgatt ggtctcgtct ggcgctccat ggagcaccca gtgaagctac tgtttgctcc
601 taacttgctc ttggacagga accagggaaa atgtgtagag ggcatggtgg agatcttcga
661 catgctgctg gctacatcat ctcggttccg catgatgaat ctgcagggag aggagtttgt
721 gtgcctcaaa tctattattt tgcttaattc tggagtgtac acatttctgt ccagcaccct
781 gaagtctctg gaagagaagg accatatcca ccgagtcctg gacaagatca cagacacttt
841 gatccacctg atggccaagg caggcctgac cctgcagcag cagcaccagc ggctggccca
901 gctcctcctc atcctctccc acatcaggca catgagtaac aaaggcatgg agcatctgta
961 cagcatgaag tgcaagaacg tggtgcccct ctatgacctg ctgctggagg cggcggacgc
1021 ccaccgccta catgcgccca ctagccgtgg aggggcatcc gtggaggaga cggaccaaag
1081 ccacttggcc actgcgggct ctacttcatc gcattccttg caaaagtatt acatcacggg
1141 ggaggcagag ggtttccctg ccacagcttg atgaATAGGA ACTTCgatca taatcagcca
1201 tatcacatct gtagaggttt tacttgcttt aaaaaacctc ccacacctcc ccctgaacct
1261 gaaacataaa atgaatgcaa ttgttgttgt taacttgttt attgcagctt ataatggtta
1321 caaataaagc aatagcatca caaatttcac aaataaagca ttttttttcac tgc
//

Pronuclear 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 ssODNs 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

Cxcr4-CRE-EM1-B6N sequence (allele targeted)

ccacgaccaagtgacagcaatgctgtttcactggttatgcggcggatccgaaaagaaaacgttgatgccggtgaacgtgcaaaaca
ggctctagcgttcgaacgcactgatttcgaccaggttcgttcactcatggaaaatagcgatcgctgccaggatatacgtaatctggca
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aactcatcaatgtatcttatcatgtctggatccCACTTATGCAAAGACATATATAATATATATATATATATATGA
TAAAGAACTTTTTTATGTTACACATTTTCCAGATATAAGAGACTGACCAGTCTTGTACAGTTTTTTTTT
TTTATTGACTGTTGGGAGTTTATGTTCCTCTAGTTTTTGTGAGGTTTGACTTAATTTATATAAATACTT
TTTTTTGTTTGTTTGTTTCATGTGAATGAGTGTCTAGGCAGGACCTGTGGCCAAGTTCTTAGTAGCTG
TTTATCTGTGTGTAGGACTGTAGAACTGTAGAGGAAGAAACTGAACATTCCAGAATGTGTGGTAAAT
TGAATAAAGCTAGCCGTGATCC

Sequence highlighted in red is to be replaced by the ERT2 cassette. Nucleotides highlighted in yellow background and red are bases that are changed for silent mutation in the mutant allele.

CXCR4-CRE-ERT2-EM2-B6N

ccacgaccaagtgacagcaatgctgtttcactggttatgcggcggatccgaaaagaaaacgttgatgccggtgaacgtgcaaaaca
ggctctagcgttcgaacgcactgatttcgaccaggttcgttcactcatggaaaatagcgatcgctgccaggatatacgtaatctggca
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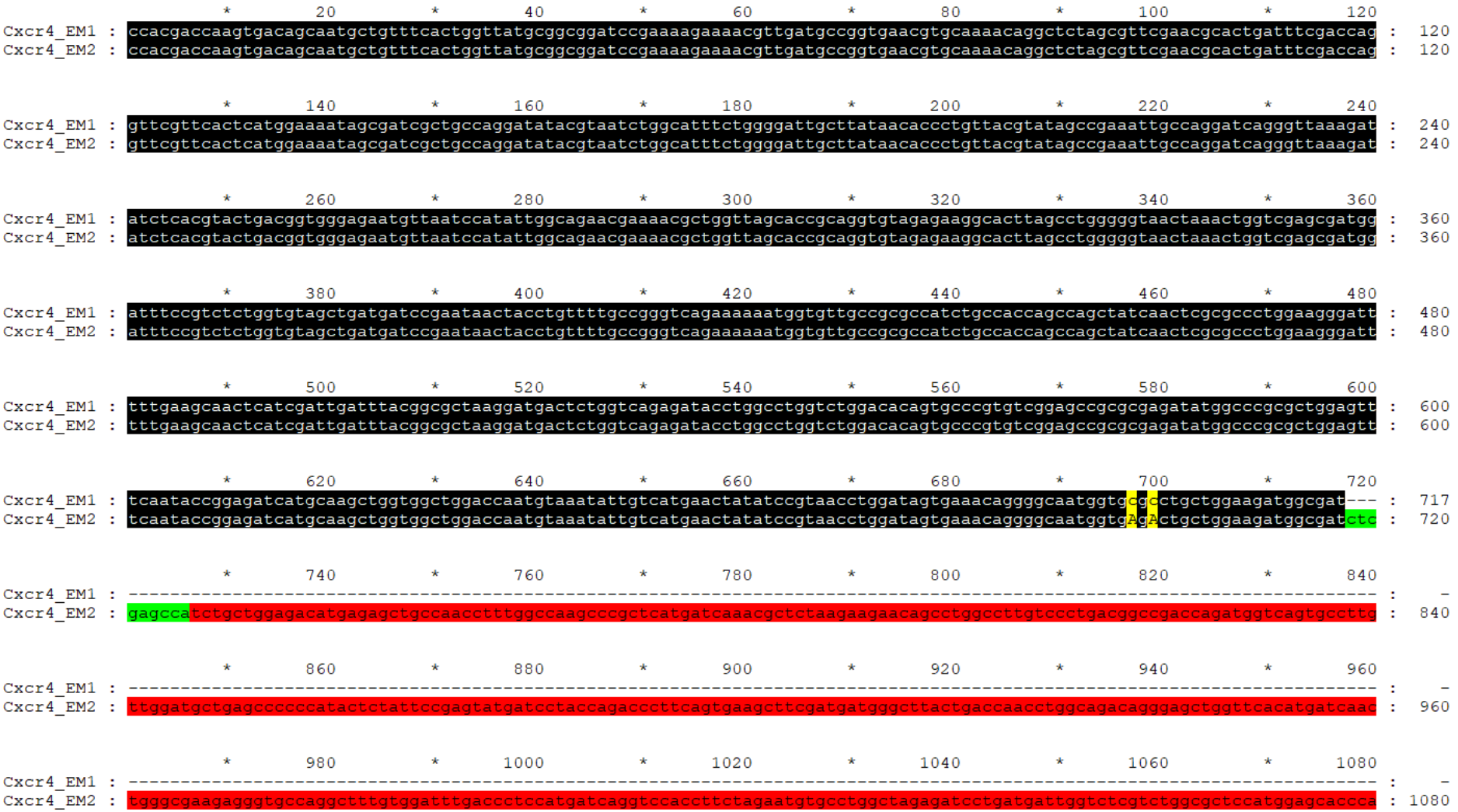
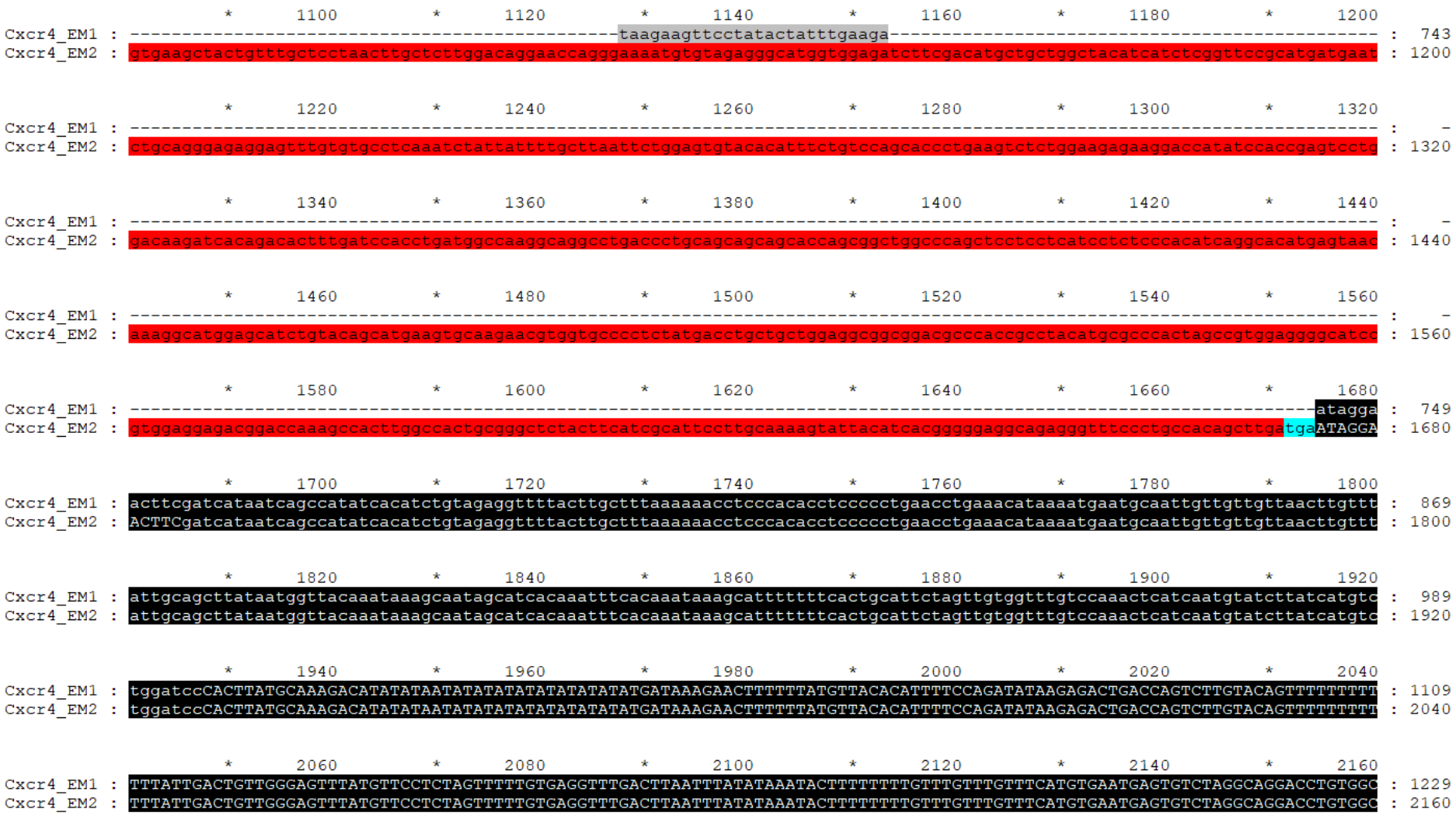
gtctggatccCACTTATGCAAAGACATATATAATATATATATATATATATGATAAAGAACTTTTTTATG
TTACACATTTTCCAGATATAAGAGACTGACCAGTCTTGTACAGTTTTTTTTTTTTATTGACTGTTGGGA
GTTTATGTTCCTCTAGTTTTTGTGAGGTTTGACTTAATTTATATAAATACTTTTTTTTGTTTGTTTGTTTC

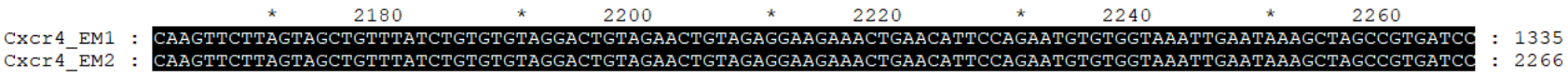
ATGTGAATGAGTGTCTAGGCAGGACCTGTGGCCAAGTTCTTAGTAGCTGTTTATCTGTGTGTAGGAC
TGTAGAACTGTAGAGGAAGAAACTGAACATTCCAGAATGTGTGGTAAATTGAATAAAGCTAGCCGT
GATCC

Sequence highlighted in red and underlined is the ERT2 cassette. Sequence highlighted in red is the stop codon. Sequence highlighted in bold is **a linking sequence**. Nucleotides highlighted with a yellow background, in bold and red as the **silent mutations** introduced to prevent the reprocessing of the engineered allele.

Nucleotide Alignment:




* 1100 * 1120 * 1140 * 1160 * 1180 * 1200
Cxcr4_EM1 : ----------taagaagttcctatactatttgaaga---------- : 743
Cxcr4_EM2 : gtgaagctactgtttgctcctaacttgctcttggacaggaaccagggaaaatgtgtagagggcatggtggagatcttcgacatgctgctggctacatcatctcggttccgcatgatgaat : 1200
* 1220 * 1240 * 1260 * 1280 * 1300 * 1320
Cxcr4_EM1 : ---------- : -
Cxcr4_EM2 : ctgcagggagaggagtttgtgtgcctcaaatctattatttttgcttaattctggagtgtacacatttctgtccagcaccctgaagtctctggaagagaaggaccatatccaccgagtcctg : 1320
* 1340 * 1360 * 1380 * 1400 * 1420 * 1440
Cxcr4_EM1 : ---------- : -
Cxcr4_EM2 : gacaagatcacagacactttgatccacctgatggccaaggcaggcctgaccctgcagcagcagcaccagcggctggcccagctcctcctcatcctctcccacatcaggcacatgagtaac : 1440
* 1460 * 1480 * 1500 * 1520 * 1540 * 1560
Cxcr4_EM1 : ---------- : -
Cxcr4_EM2 : aaaggcatggagcatctgtacagcatgaagtgcaagaacgtggtgcccctctatgacctgctgctggaggcggcggacgcccaccgcctacatgcgcccactagccgtggaggggcatcc : 1560
* 1580 * 1600 * 1620 * 1640 * 1660 * 1680
Cxcr4_EM1 : ----------atagga : 749
Cxcr4_EM2 : gtggaggagacggaccaaagccacttggccactgcgggctctacttcatcgcattccttgcaaaagtattacatcacgggggaggcagagggtttccctgccacagcttgatgaATAGGA : 1680
* 1700 * 1720 * 1740 * 1760 * 1780 * 1800
Cxcr4_EM1 : acttcgatcataatcagccatatcacatctgtagaggttttacttgctttaaaaaacctcccacacctccccctgaacctgaaacataaaatgaatgcaattgttgttgttaacttgttt : 869
Cxcr4_EM2 : ACTTCgatcataatcagccatatcacatctgtagaggttttacttgctttaaaaaacctcccacacctccccctgaacctgaaacataaaatgaatgcaattgttgttgttaacttgttt : 1800
* 1820 * 1840 * 1860 * 1880 * 1900 * 1920
Cxcr4_EM1 : attgcagcttataatggttacaaataaagcaatagcatcacaaatttcacaaataaagcatttttttcactgcattctagttgtggtttgtccaaactcatcaatgtatcttatcatgtc : 989
Cxcr4_EM2 : attgcagcttataatggttacaaataaagcaatagcatcacaaatttcacaaataaagcatttttttcactgcattctagttgtggtttgtccaaactcatcaatgtatcttatcatgtc : 1920
* 1940 * 1960 * 1980 * 2000 * 2020 * 2040
Cxcr4_EM1 : tggatccCACTTATGCAAAGACATATATAATATATATATATATATATGATAAAGAACTTTTTTATGTTACACATTTTCCAGATATAAGAGACTGACCAGTCTTGTACAGTTTTTTTTT : 1109
Cxcr4_EM2 : tggatccCACTTATGCAAAGACATATATAATATATATATATATATATGATAAAGAACTTTTTTATGTTACACATTTTCCAGATATAAGAGACTGACCAGTCTTGTACAGTTTTTTTTT : 2040
* 2060 * 2080 * 2100 * 2120 * 2140 * 2160
Cxcr4_EM1 : TTTATTGACTGTTGGGAGTTTATGTTCCTCTAGTTTTTGTGAGGTTTGACTTAATTTATATAAATACTTTTTTTTTGTTTGTTTGTTTCATGTGAATGAGTGTCTAGGCAGGACCTGTGGC : 1229
Cxcr4_EM2 : TTTATTGACTGTTGGGAGTTTATGTTCCTCTAGTTTTTGTGAGGTTTGACTTAATTTATATAAATACTTTTTTTTTGTTTGTTTGTTTCATGTGAATGAGTGTCTAGGCAGGACCTGTGGC : 2160


* 2180 * 2200 * 2220 * 2240 * 2260
Cxcr4_EM1 : CAAGTTCTTAGTAGCTGTTTATCTGTGTGTAGGACTGTAGAACTGTAGAGGAAGAAACTGAACATTCCAGAATGTGTGGTAAATTGAATAAAGCTAGCCGTGATCC : 1335
Cxcr4_EM2 : CAAGTTCTTAGTAGCTGTTTATCTGTGTGTAGGACTGTAGAACTGTAGAGGAAGAAACTGAACATTCCAGAATGTGTGGTAAATTGAATAAAGCTAGCCGTGATCC : 2266

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_Cxcr4_CRE-ERT2_F6 (5'-3')	CCACGACCAAGTGACAGCAAT
Geno_Cxcr4_CRE-ERT2_R6 (5'-3')	GGATCACGGCTAGCTTTATTCA
Taq Polymerase used	Roche KAPA Hotstart PCR Kit
Annealing Temperature (°C)	56
Elongation time (min)	2
WT product size (bp)	1335
Mutant product size (bp)	2266
Notes	This PCR sits externally to the donor template applied.

Geno_Cxcr4_CRE-ERT2_F3 (5'-3')	TCAAACGCTCTAAGAAGAACAGC
Geno_Cxcr4_CRE-ERT2_R7 (5'-3')	GATGTGGGAGAGGATGAGGA
Taq Polymerase used	ThermoFisher SuperFi II PCR Kit
Annealing Temperature (°C)	60
Elongation time (min)	0.5
WT product size (bp)	N/A
Mutant product size (bp)	650
Notes	This PCR is used to screen for the presence of the ERT2 cassette.

Cre_F4 (5'-3')	tccgtctctggtgtagctga
Geno_Cxcr4_CRE-ERT2_R7 (5'-3')	GATGTGGGAGAGGATGAGGA
Taq Polymerase used	ThermoFisher SuperFi II PCR Kit
Annealing Temperature (°C)	60
Elongation time (min)	0.75
WT product size (bp)	N/A

Mutant product size (bp)	1063
Notes	This PCR is used to screen whether the 5’ end of the donor has gone in on-target.

Geno_Cxcr4_CRE-ERT2_F8 (5’-3’)	ggctacatcatctcggttcc
Geno_Cxcr4_CRE-ERT2_R8 (5’-3’)	ATAAGTGggatccagacatg
Taq Polymerase used	ThermoFisher SuperFi II PCR Kit
Annealing Temperature (°C)	60
Elongation time (min)	1
WT product size (bp)	N/A
Mutant product size (bp)	765
Notes	This PCR is used to screen whether the 3’ end of the donor has gone in on-target.

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.

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	ERT2-MUT1
Forward Primer (5’-3’)	GCTTCGATGATGGGCTTACT
Reverse Primer (5’-3’)	CCTGATCATGGAGGGTCAAATC
Probe (5’-3’)	TGGTTCACATGATCAACTGGGCGA
Label	FAM

This ddPCR assay is specific to the donor used to create the engineered mutation and only mutant 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.

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 additional donor integrations were detected in the animals taken forward to establish the colony.