Human GRM7-ROSA26 Conditional Knockin Project (CRISPR/Cas)

1. Objective

To create a human GRM7 conditional knockin at the locus of ROSA26 in C57BL/6NTac mice by CRISPR/Cas- mediated genome engineering.

2. Project summary

(1) The mouse ROSA26 gene (GenBank accession number: NR_027008.1) is located on mouse chromosome 6.
(2) The human GRM7 gene (GenBank accession number: NM_000844.4) is located on human chromosome 3.
(3) For the KI model, the “CAG-loxP-Stop-loxP-human GRM7 cDNA-polyA” cassette will be cloned into intron 1 of ROSA26 in reverse direction. The expression of human GRM7 cDNA cassette will be dependent on the expression of Cre recombination.

(4) To engineer the donor vector, homology arms will be generated by PCR using BAC clone from the C57BL/6J library as template.

(5) Cas9 and gRNA will be co-injected into fertilized eggs with donor vector for KI mice production.

(6) The pups will be genotyped by PCR followed by sequencing of PCR product.

The nucleotides sequence of human GRM7 cDNA:

```
   1 ATGGTC CAGCTG AGGAAG CTGCTC CGCGTC CTGACT TTGATG AAGTTC CCCTGC TGCGTG
  61 CTGGAG GTGCTC CTGTGC GCGCTG GCGGCG GCGGCG CGCGGC CAGGAG ATGTAC GCCCCG
 121 CACTCA ATCCGG ATCGAG GGGGAC GTCACC CTCGGG GGGCTG TTCCCC GTGCAC GCCAAG
 181 GGTCCC AGCGGA GTGCCC TGCGGC GACATC AAGAGG GAAAAC GGGATC CACAGG CTGGAA
 241 GCGATG CTCTAC GCCCTG GACCAG ATCAAC AGTGAT CCCAAC CTACTG CCCAAC GTGACG
 301 CTGGGC GCGCGG ATCCTG GACACT TGTTCC AGGGAC ACTTAC GCGCTC GAACAG TCGCTT
 361 ACTTTC GTCCAG GCGCTC ATCCAG AAGGAC ACCTCC GACGTG CGCTGC ACCAAC GGCGAA
 421 CCGCCG GTTTTC GTCAAG CCGGAG AAAGTA GTTGGA GTGATT GGGGCT TCGGGG AGTTCG
 481 GTCTCC ATCATG GTAGCC AACATC CTGAGG CTCTTC CAGATC CCCCAG ATTAGT TATGCA
 541 TCAACG GCACCC GAGCTA AGTGAT GACCGG CGCTAT GACTTC TTCTCT CGCGTG GTGCCA
 601 CCCGAT TCCTTC CAAGCC CAGGCC ATGGTA GACATT GTAAAG GCCCTA GGCTGG AATTAT
 661 GTGTCT ACCCTC GCATCG GAAGGA AGTTAT GGAGAG AAAGGT GTGGAG TCCTTC ACGCAG
 721 ATTTCC AAAGAG GCAGGT GGACTC TGCATT GCCCAG TCCGTG AGAATC CCCCAG GAACGC
 781 AAAGAC AGGACC ATTGAC TTTGAT AGAATT ATCAAA CAGCTC CTGGAC ACCCCC AACTCC
 841 AGGGCC GTCGTG ATTTTT GCCAAC GATGAG GATATA AAGCAG ATCCTT GCAGCA GCCAAA
 901 AGAGCT GACCAA GTTGGC CATTTT CTTTGG GTGGGA TCAGAC AGCTGG GGATCC AAAATA
 961 AACCCA CTGCAC CAGCAT GAAGAT ATCGCA GAAGGG GCCATC ACCATT CAGCCC AAGCGA
1021 GCCACG GTGGAA GGGTTT GATGCC TACTTT ACGTCC CGTACA CTTGAA AACAAC AGAAGA
1081 AATGTA TGGTTT GCCGAA TACTGG GAGGAA AACTTC AACTGC AAGTTG ACGATT AGTGGG
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1141 TCAAAA AAAGAA GACACA GATCGC AAATGC ACAGGA CAGGAG AGAATT GGAAAA GATTCC
1201 AACTAT GAGCAG GAGGGT AAAGTC CAGTTC GTGATT GACGCA GTCTAT GCTATG GCTCAC
1261 GCCCTT CACCAC ATGAAC AAGGAT CTCTGT GCTGAC TACCGG GGTGTC TGCCCA GAGATG
1321 GAGCAA GCTGGA GGCAAG AAGTTG CTGAAG TATATA CGCAAT GTTAAT TTCAAT GGTAGT
1381 GCTGGC ACTCCA GTGATG TTTAAC AAGAAC GGGGAT GCACCT GGGCGT TATGAC ATCTTT
1441 CAGTAC CAGACC ACAAAC ACCAGC AACCCG GGTTAC CGTCTG ATCGGG CAGTGG ACAGAC
1501 GAACTT CAGCTC AATATA GAAGAC ATGCAG TGGGGT AAAGGA GTCCGA GAGATA CCCGCC
1561 TCAGTG TGCACA CTACCA TGTAAG CCAGGA CAGAGA AAGAAG ACACAG AAAGGA ACTCCT
1621 TGCTGT TGGACC TGTGAG CCTTGC GATGGT TACCAG TACCAG TTTGAT GAGATG ACATGC
1681 CAGCAT TGCCCC TATGAC CAGAGG CCCAAT GAAAAT CGAACC GGATGC CAGGAT ATTCCC
1741 ATCATC AAACTG GAGTGG CACTCC CCCTGG GCTGTG ATTCCT GTCTTC CTGGCA ATGTTG
1801 GGGATC ATTGCC ACCATC TTTGTC ATGGCC ACTTTC ATCCGC TACAAT GACACG CCCATT
1861 GTCCGG GCATCT GGGCGG GAACTC AGCTAT GTTCTT TTGACG GGCATC TTTCTT TGCTAC
1921 ATCATC ACTTTC CTGATG ATTGCC AAACCA GATGTG GCAGTG TGTTCT TTCCGG CGAGTT
1981 TTCTTG GGCTTG GGTATG TGCATC AGTTAT GCAGCC CTCTTG ACGAAA ACAAAT CGGATT
2041 TATCGC ATATTT GAGCAG GGCAAG AAATCA GTAACA GCTCCC AGACTC ATAAGC CCAACA
2101 TCACAA CTGGCA ATCACT TCCAGT TTAATA TCAGTT CAGCTT CTAGGG GTGTTC ATTTGG
2161 TTTGGT GTTGAT CCACCC AACATC ATCATA GACTAT GATGAA CACAAG ACAATG AACCCT
2221 GAGCAA GCCAGA GGGGTT CTCAAG TGTGAC ATTACA GATCTC CAAATC ATTTGC TCCTTG
2281 GGATAT AGCATT CTTCTC ATGGTC ACATGT ACTGTG TATGCC ATCAAG ACTCGG GGTGTA
2341 CCCGAG AATTTT AACGAA GCCAAG CCCATT GGATTC ACTATG TACACG ACATGT ATAGTA
2401 TGGCTT GCCTTC ATTCCA ATTTTT TTTGGC ACCGCT CAATCA GCGGAA AAGCTC TACATA
2461 CAAACT ACCACG CTTACA ATCTCC ATGAAC CTAAGT GCATCA GTGGCG CTGGGG ATGCTA
2521 TACATG CCGAAA GTGTAC ATCATC ATTTTC CACCCT GAACTC AATGTC CAGAAA CGGAAG
2581 CGAAGC TTCAAG GCGGTA GTCACA GCAGCC ACCATG TCATCG AGGCTG TCACAC AAACCC
2641 AGTGAC AGACCC AACGGT GAGGCA AAGACC GAGCTC TGTGAA AACGTA GACCCA AACAGC
2701 CCTGCT GCAAAA AAGAAG TATGTC AGTTAT AATAAC CTGGTT ATCTAA
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The amino acid sequence of human GRM7 cDNA:

```
   1 MVQLRK LLRVLT LMKFPC CVLEVL LCALAA AARGQE MYAPHS IRIEGD VTLGGL FPVHAK
  61 GPSGVP CGDIKR ENGIHR LEAMLY ALDQIN SDPNLL PNVTLG ARILDT CSRDTY ALEQSL
 121 TFVQAL IQKDTS DVRCTN GEPPVF VKPEKV VGVIGA SGSSVS IMVANI LRLFQI PQISYA
 181 STAPEL SDDRRY DFFSRV VPPDSF QAQAMV DIVKAL GWNYVS TLASEG SYGEKG VESFTQ
 241 ISKEAG GLCIAQ SVRIPQ ERKDRT IDFDRI IKQLLD TPNSRA VVIFAN DEDIKQ ILAAAK
 301 RADQVG HFLWVG SDSWGS KINPLH QHEDIA EGAITI QPKRAT VEGFDA YFTSRT LENNRR
 361 NVWFAE YWEENF NCKLTI SGSKKE DTDRKC TGQERI GKDSNY EQEGKV QFVIDA VYAMAH
 421 ALHHMN KDLCAD YRGVCP EMEQAG GKKLLK YIRNVN FNGSAG TPVMFN KNGDAP GRYDIF
 481 QYQTTN TSNPGY RLIGQW TDELQL NIEDMQ WGKGVR EIPASV CTLPCK PGQRKK TQKGTP
 541 CCWTCE PCDGYQ YQFDEM TCQHCP YDQRPN ENRTGC QDIPII KLEWHS PWAVIP VFLAML
 601 GIIATI FVMATF IRYNDT PIVRAS GRELSY VLLTGI FLCYII TFLMIA KPDVAV CSFRRV
 661 FLGLGM CISYAA LLTKTN RIYRIF EQGKKS VTAPRL ISPTSQ LAITSS LISVQL LGVFIW
 721 FGVDPP NIIIDY DEHKTM NPEQAR GVLKCD ITDLQI ICSLGY SILLMV TCTVYA IKTRGV
```

781 PENFNE AKPIGF TMYTTC IVWLAF IPIFFG TAQSAE KLYIQT TTLTIS MNLSAS VALGML
841 YMPKVY IIIFHP ELNVQK RKRSFK AVVTAA TMSSRL SHKPSD RPNGEA KTELCE NVDPNS
901 PAAKKK YVSYNN LVI*

3. Targeting strategy

3.1 Gene and protein Information of mouse ROSA26

Gt(ROSA)26Sor gene trap ROSA 26, Philippe Soriano [*Mus musculus* (house mouse)]

Gene ID: 14910, updated on 13-Sep-2016

Summary

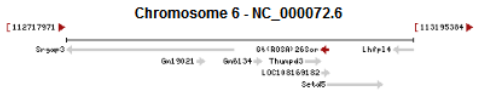
Official Symbol	Gt(ROSA)26Sor provided by MGI
Official Full Name	gene trap ROSA 26, Philippe Soriano provided by MGI
Primary source	MGI:MGI:104735
See related	Ensembl:ENSMUSG00000086429
Gene type	ncRNA
RefSeq status	VALIDATED
Organism	Mus musculus
Lineage	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Sciurognathi; Muroidea; Muridae; Murinae; Mus; Mus
Also known as	R26; ROSA26; AV258896; Gtrgeo26; Gtrosa26; Thumpd3as1
Summary	This gene produces a long non-coding RNA (lncRNA) that is under the control of a constitutive promoter. This locus is a widely used site for the integration of transgenes and reporter constructs. [provided by RefSeq, Oct 2015]
Orthologs	human all

Genomic context

Location: 6 52.73 cM — See Gt(ROSA)26Sor in Genome Data Viewer Map Viewer

Exon count: 3

Annotation release	Status	Assembly	Chr	Location
106	current	GRCm38.p4 (GCF_000001635.24)	6	NC_000072.6 (113067428..113077244, complement)
Build 37.2	previous assembly	MGSCv37 (GCF_000001635.18)	6	NC_000072.5 (113017422..113027238, complement)



3.1.1 Transcript:

This gene has 4 transcripts.

Gene: Gt(ROSA)26Sor ENSMUSG00000086429

Description	gene trap ROSA 26, Philippe Soriano [Source:MGI Symbol;Acc:MGI:104735]
Synonyms	Gtrosa26, beta geo, Gtrgeo26, ROSA26, R26, Thumpd3as1
Location	Chromosome 6: 113,067,428-113,077,333 reverse strand. GRCm38:CM000999.2
About this gene	This gene has 4 transcripts (splice variants) and is associated with 94 phenotypes.
Transcripts	Hide transcript table

Show/hide columns (1 hidden) Filter

Name	Transcript ID	bp	Protein	Biotype	CCDS	RefSeq	Flags
Gt(ROSA)26Sor-002	ENSMUST00000124246.3	1181	No protein	Antisense	-	NR_027008	TSL:1 GENCODE basic
Gt(ROSA)26Sor-003	ENSMUST00000159544.2	822	No protein	Antisense	-	-	TSL:2
Gt(ROSA)26Sor-004	ENSMUST00000167415.7	551	No protein	Antisense	-	-	TSL:1 GENCODE basic
Gt(ROSA)26Sor-001	ENSMUST00000133467.2	412	No protein	Antisense	-	-	TSL:1

3.2 Gene and protein Information of human GRM7

GRM7 glutamate metabotropic receptor 7 [*Homo sapiens* (human)]

Gene ID: 2917, updated on 7-May-2017

Summary

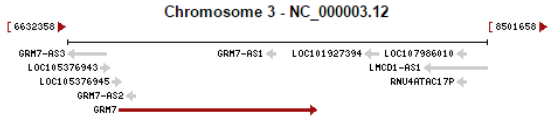
Official Symbol	GRM7 provided by HGNC
Official Full Name	glutamate metabotropic receptor 7 provided by HGNC
Primary source	HGNC:HGNC:4599
See related	Ensembl:ENSG00000196277 MIM:604101; Vega:OTTHUMG00000125549
Gene type	protein coding
RefSeq status	REVIEWED
Organism	Homo sapiens
Lineage	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo
Also known as	GLUR7; MGLU7; GPRC1G; MGLUR7; PPP1R87
Summary	L-glutamate is the major excitatory neurotransmitter in the central nervous system, and it activates both ionotropic and metabotropic glutamate receptors. Glutamatergic neurotransmission is involved in most aspects of normal brain function and can be perturbed in many neuropathologic conditions. The metabotropic glutamate receptors are a family of G protein-coupled receptors that have been divided into three groups on the basis of sequence homology, putative signal transduction mechanisms, and pharmacologic properties. Group I includes GRM1 and GRM5, and these receptors have been shown to activate phospholipase C. Group II includes GRM2 and GRM3, while Group III includes GRM4, GRM6, GRM7 and GRM8. Group II and III receptors are linked to the inhibition of the cyclic AMP cascade but differ in their agonist selectivities. Multiple transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Jun 2009]
Orthologs	mouse all

Genomic context

Location: 3p26.1 — See GRM7 in Genome Data Viewer Map Viewer

Exon count: 15

Annotation release	Status	Assembly	Chr	Location
108	current	GRCh38.p7 (GCF_000001405.33)	3	NC_000003.12 (6861097..7743032)
105	previous assembly	GRCh37.p13 (GCF_000001405.25)	3	NC_000003.11 (6902802..7783218)



3.2.1 Transcript:

This gene has 15 transcripts.

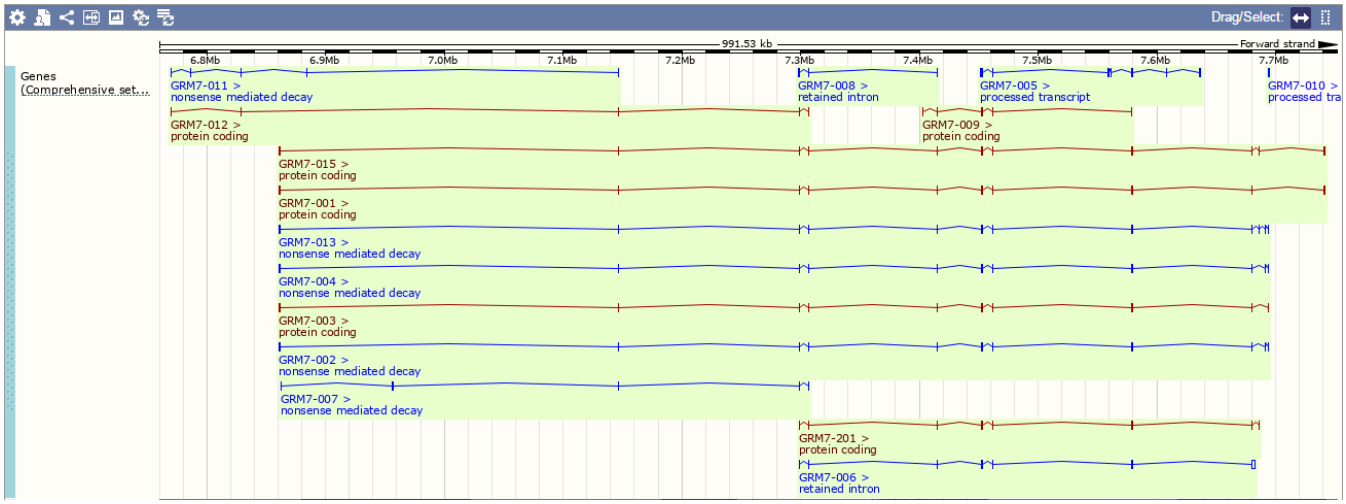
Gene: GRM7 ENSG00000196277

Description	glutamate metabotropic receptor 7 [Source:HGNC Symbol;Acc:HGNC:4599]
Synonyms	MGLUR7, GLUR7, mGlu7, PPP1R87, GPRC1G
Location	Chromosome 3: 6,770,001-7,741,533 forward strand. GRCh38:CM000665.2
About this gene	This gene has 15 transcripts (splice variants), 68 orthologues, 10 paralogues, is a member of 1 Ensembl protein family and is associated with 36 phenotypes.
Transcripts	Hide transcript table

Show/hide columns (1 hidden) — Filter

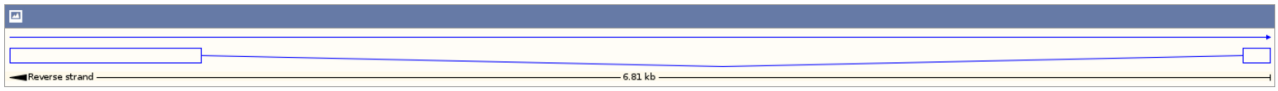
Name	Transcript ID	bp	Protein	Biotype	CCDS	UniProt	RefSeq	Flags
GRM7-001	ENST00000357716.8	4127	915aa	Protein coding	CCDS43042	Q14831	NM_000844 NP_000835	TSL:1 GENCODE basic APPRIS P2
GRM7-015	ENST00000486284.5	4241	922aa	Protein coding	-	Q14831	NM_181874 NP_870989	TSL:5 GENCODE basic APPRIS ALT2
GRM7-003	ENST00000389336.8	2901	906aa	Protein coding	-	Q14831	-	TSL:1 GENCODE basic APPRIS ALT1
GRM7-201	ENST00000402647.6	2034	677aa	Protein coding	-	A0A0A0MSC3	-	TSL:5 GENCODE basic
GRM7-009	ENST00000445087.1	683	185aa	Protein coding	-	C9JMT3	-	CDS 3' incomplete TSL:4
GRM7-012	ENST00000448328.6	595	122aa	Protein coding	-	C9JU97	-	CDS 3' incomplete TSL:4
GRM7-013	ENST00000467425.5	3289	922aa	Nonsense mediated decay	-	Q14831	-	TSL:1
GRM7-002	ENST00000440923.7	3088	913aa	Nonsense mediated decay	-	H7C3K2	-	TSL:2
GRM7-004	ENST00000389335.7	3020	911aa	Nonsense mediated decay	-	Q14831	-	TSL:1
GRM7-007	ENST00000435689.5	826	37aa	Nonsense mediated decay	-	F8WAW6	-	TSL:3
GRM7-011	ENST00000443259.1	579	68aa	Nonsense mediated decay	-	F8WCR9	-	TSL:4

GRM7-005	ENST00000458641.2	1937	No protein	Processed transcript	-	-	-	TSL:2
GRM7-010	ENST00000471242.2	160	No protein	Processed transcript	-	-	-	TSL:5
GRM7-006	ENST00000463676.6	4003	No protein	Retained intron	-	-	-	TSL:1
GRM7-008	ENST00000461677.6	546	No protein	Retained intron	-	-	-	TSL:4



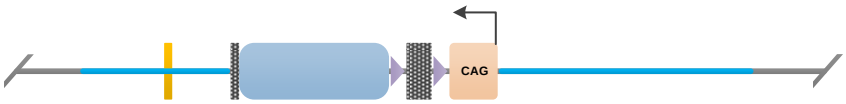
3.3 Schematic depiction of targeting strategy

Genomic region of mouse ROSA26(Transcript: Gt(ROSA)26Sor-002 ENSMUST00000124246.3) locus is diagrammed below (gene is oriented from right to left; total size is 6.81 kb).

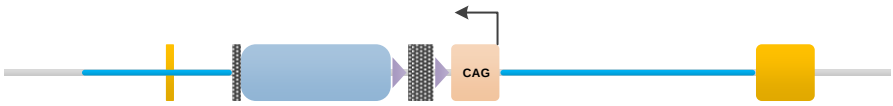


Rosa26 Wildtype allele

Donor vector



Targeted allele



Legends

3.4 gRNA target sequence

gRNA1 (matching forward strand of gene): GGCAGGCTTAAAGGCTAACCTGG
gRNA2 (matching forward strand of gene): CTCCAGTCTTTCTAGAAGATGGG

3.5 Off-target analysis

Each gRNA has a quality score, with higher quality scores indicating greater specificity. Each potential off-target site also has a score, which is calculated based on the GRM7er of mismatches with the gRNA and the distances of the mismatches from PAM sequence, with high scores indicating greater potential that the off-target site will be recognized by the gRNA. The quality score of the gRNA is calculated based on the total GRM7er of off-target sites and their scores.

Off-target analysis for gRNA1:

guide #1 **quality score: 81**

guide sequence: GGCAGGCTTAAAGGCTAACC **TGG**
on-target locus: chr6:+113026024
number of offtarget sites: 103 (7 are in genes)

top 20 genome-wide off-target sites ☐ show all exonic

sequence	score	mismatches	UCSC gene	locus
GGCAGGCCTAAAAGCTAACCAAG	2.5	2MMs [8:13]		chr14:+10436339
TGGAGGATTAAAGGCTAACCCAG	1.7	3MMs [1:3:7]		chr11:-121391073
GGAAAGCTCAAAGGCTAACCAGG	1.5	3MMs [3:5:9]		chr6:-60539842
GGTAGGCTTTAAGGCTAACTTGG	1.3	3MMs [3:10:20]		chr2:-104207052
TGCTGGATTCAAGGCTAACCAAG	0.9	4MMs [1:4:7:10]		chr1:-165370296
TCCAGGCTTAAAGGTTAACCTGG	0.8	3MMs [1:2:15]		chrX:-103384222
AAAAGGCTTAAAGGCTTACCTGG	0.6	4MMs [1:2:3:17]		chr10:-25118669
GGCAGGAGCTAAGGCTAACCTGG	0.5	4MMs [7:8:9:10]		chr10:-105117147
TGAAGGTTTAAGGGCTAACCTGG	0.5	4MMs [1:3:7:12]		chr1:-72722738
GGCAGCCCTGCAGGCTAACCAGG	0.4	4MMs [6:8:10:11]		chr7:+106544641

Off-target analysis for gRNA2:

guide #8 **quality score: 58**

guide sequence: CTCCAGTCTTTCTAGAAGAT **GGG**
on-target locus: chr6:+113025988
number of offtarget sites: 301 (22 are in genes)

top 20 genome-wide off-target sites — show all exonic

sequence	score	mismatches	UCSC gene	locus
CTGCTGTCTTTCTAGAAGATGGG	5.4	2MMs [3:5]		chr14:+16134369
CTTCAGTATCTCTAGAAGATAAG	2.4	3MMs [3:8:10]		chr2:-114730277
GTCCTGGCTTTCTAGAAGATGGG	1.7	3MMs [1:5:7]		chr5:+69947282
CTCAAGGCTGTCTAGAAGATTGG	1.6	3MMs [4:7:10]		chr7:-88830861
CCCCAGTTTTTCTAGAAGTTGAG	1.1	3MMs [2:8:19]		chr5:+101598417
CTCAAACCTTTCTAGAAGATGAG	1.0	3MMs [4:6:7]		chr2:+143437609
CTCCAGCAGTTCTAGAAGATCAG	1.0	3MMs [7:8:9]		chr12:-82957101
TTCTAGGGTTTCTAGAAGATCAG	0.9	4MMs [1:4:7:8]		chr6:+140459402
TTCCAGTGTTTCTACAAGATCGG	0.8	3MMs [1:8:15]		chrX:+85361248
AGCCCATCTTTCTAGAAGATGAG	0.8	4MMs [1:2:5:6]		chr10:-69287964

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Vector Report

Quote: ROSAM-190212-ACD-01-tac
Project: Human GRM7-ROSA26 Conditional Knockin

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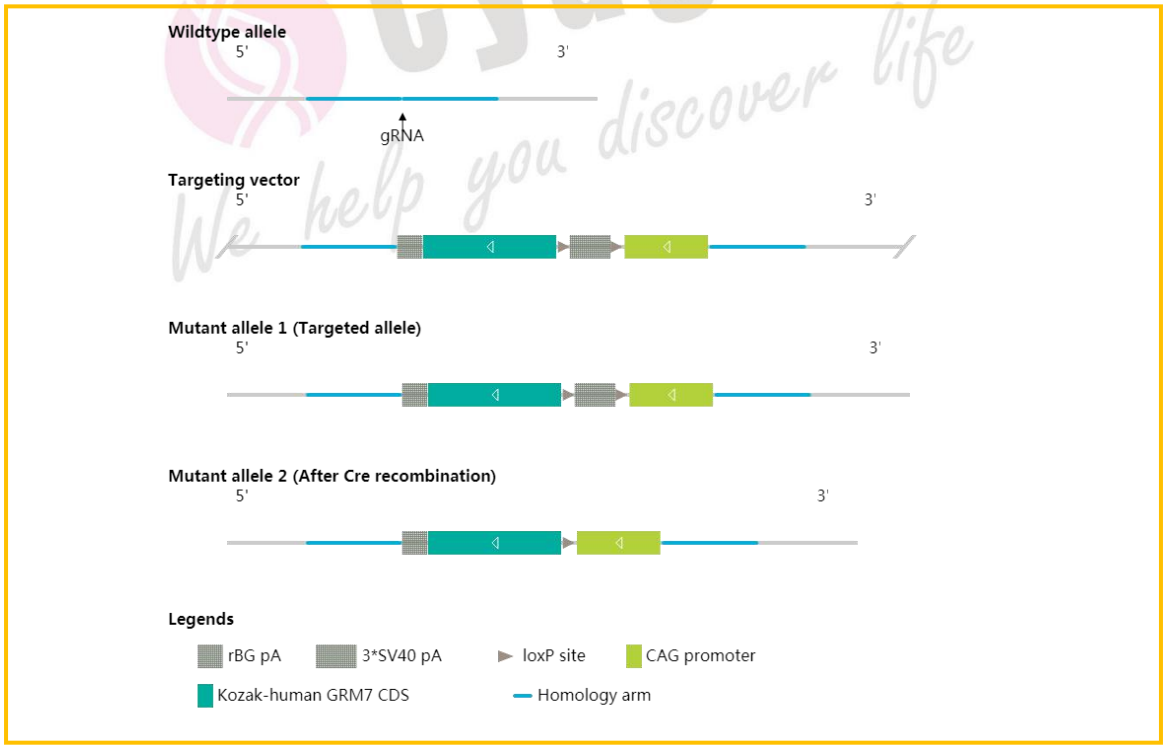
Tel: 800-921-8930; Fax: 408-969-0336
2255 Martin Ave., Suite E. Santa Clara, CA 95050

1. Objective

To create a human GRM7 conditional knockin at the locus of ROSA26 in C57BL/6NTac mice by CRISPR/Cas- mediated genome engineering.

2. Summary

- The mouse ROSA26 gene (GenBank accession number: NR_027008.1) is located on mouse chromosome 6.
- The human GRM7 gene (GenBank accession number: NM_000844.4) is located on human chromosome 3.
- For the KI model, the "CAG-loxP-Stop-loxP-human GRM7 cDNA-polyA" cassette was cloned into intron 1 of ROSA26 in reverse direction. The expression of human GRM7 cDNA cassette will be dependent on the expression of Cre recombination.
- Cas9 and gRNA will be co-injected into fertilized eggs with donor vector for KI mice production.
- The pups will be genotyped by PCR followed by sequencing of PCR product.

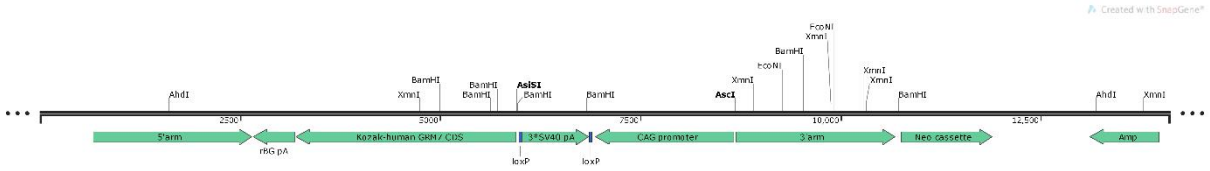


3. Method

Mouse genomic fragments containing homology arms (HAs) were amplified from BAC clone by using high fidelity Taq DNA polymerase, and were sequentially assembled into a targeting vector together with recombination sites and selection markers shown below.

Diagram

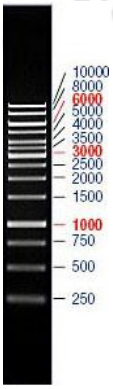
Linearized targeting vector



4. Result

Your targeting vector was digested by restriction enzymes for confirmation purposes. Units below are all in kilo-base pair (kb).

Enzyme digestion of final vector



Marker

1. AhdI/EcoNI: 7.7/3.3/2.5/0.7
2. AscI/XmnI: 5.1/3.9/3.5/1.0/0.4/0.2
3. AsisI/BamHI: 8.4/2.7/1.2/0.9/0.6/0.2/0.1

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2255 Martin Ave., Suite E. Santa Clara, CA 95050

5. Sequence of the Final Targeting Vector

Homology arm KI region loxP site Exon Sequence confirmed region

```
   1  CTAAAT TGTAAG CGTTAA TATTTT GTTAAA ATTCGC GTTAAA TTTTTG TTAAAT CAGCTC
  61  ATTTTT TAACCA ATAGGC CGAAAT CGGCAA AATCCC TTATAA ATCAAA AGAATA GACCGA
 121  GATAGG GTTGAG TGTTGT TCCAGT TTGGAA CAAGAG TCCACT ATTAAA GAACGT GGACTC
 181  CAACGT CAAAGG GCGAAA AACCGT CTATCA GGGCGA TGGCCC ACTACG TGAACC ATCACC
 241  CTAATC AAGTTT TTTGGG GTCGAG GTGCCG TAAAGC ACTAAA TCGGAA CCCTAA AGGGAG
 301  CCCCCG ATTTAG AGCTTG ACGGGG AAAGCC GGCGAA CGTGGC GAGAAA GGAAGG GAAGAA
 361  AGCGAA AGGAGC GGGCGC TAGGGC GCTGGC AAGTGT AGCGGT CACGCT GCGCGT AACCAC
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13621  CTCTTA CTGTCA TGCCAT CCGTAA GATGCT TTTCTG TGACTG GTGAGT ACTCAA CCAAGT
13681  CATTCT GAGAAT AGTGTA TGCGGC GACCGA GTTGCT CTTGCC CGGCGT CAATAC GGGATA
13741  ATACCG CGCCAC ATAGCA GAACTT TAAAAG TGCTCA TCATTG GAAAAC GTTCTT CGGGGC
13801  GAAAAC TCTCAA GGATCT TACCGC TGTTGA GATCCA GTTCGA TGTAAC CCACTC GTGCAC
13861  CCAACT GATCTT CAGCAT CTTTTA CTTTCA CCAGCG TTTCTG GGTGAG CAAAAA CAGGAA
13921  GGCAAA ATGCCG CAAAAA AGGGAA TAAGGG CGACAC GGAAAT GTTGAA TACTCA TACTCT
13981  TCCTTT TTCAAT ATTATT GAAGCA TTTATC AGGGTT ATTGTC TCATGA GCGGAT ACATAT
14041  TTGAAT GTATTT AGAAAA ATAAAC AAATAG GGGTTC CGCGCA CATTTC CCCGAA AAGTGC
14101  CAC
```

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Animal Report

Quote: ROSAM-190212-ACD-01-tac
Project: Human GRM7-ROSA26 Conditional Knockin

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www.cyagen.com

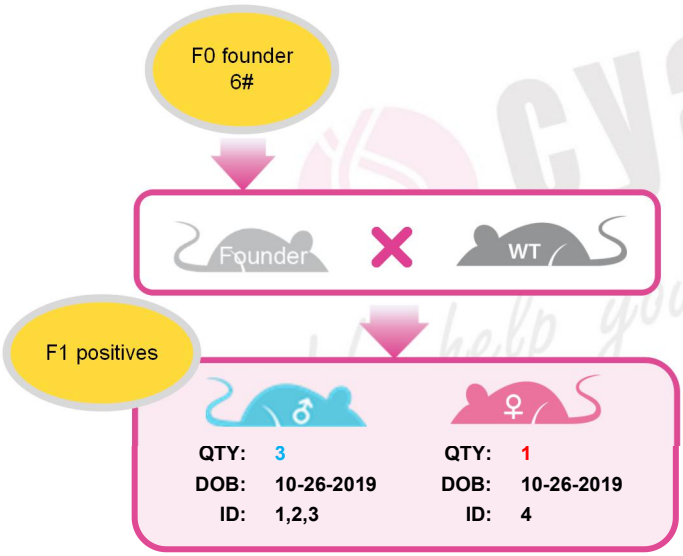
1. Method

The gRNA to mouse ROSA26 gene, the donor vector containing “CAG-loxP-Stop-loxP-human GRM7 cDNA-polyA” cassette, and Cas9 mRNA were co-injected into fertilized mouse eggs to generate targeted conditional knockin offspring. F0 founder animals were identified by PCR followed by sequence analysis, which were bred to wildtype mice to test germline transmission and F1 animal generation.

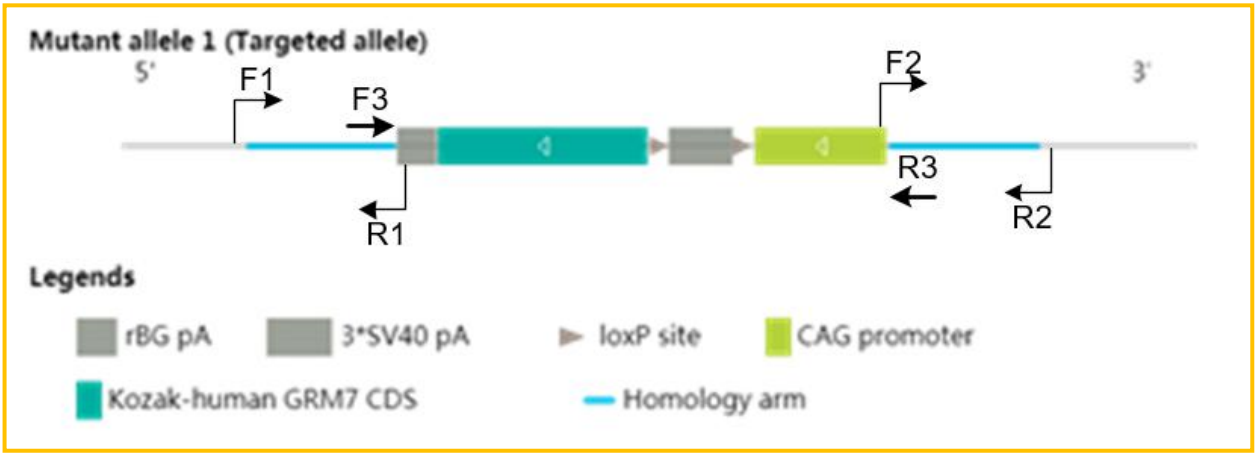
2. gRNA target sequence

gRNA(matching reverse strand of gene): GGATTTAGCCACATCCATAGTGG

3. Diagram: Breeding Scheme



4. Genotyping Strategy



5. PCR Screening

PCR Primers 1 (Annealing Temperature 60.0 ºC):

5'arm forward primer (F1): 5'-CAATACCTTTCTGGGAGTTCTCTG-3'
5'KI reverse primer (R1): 5'-GCATCTGACTTCTGGCTAATAAAG-3'

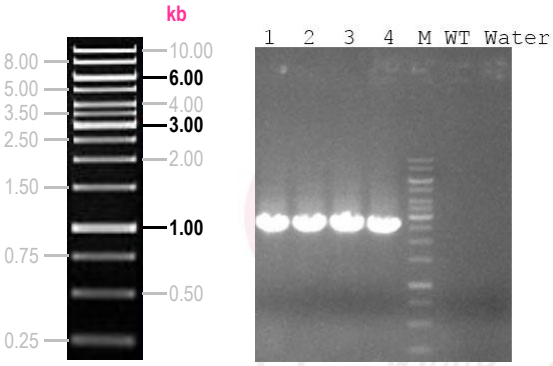
PCR Primers 2 (Annealing Temperature 60.0 ºC):

3'KI forward primer (F2): 5'-GATGGGGAGAGTGAAGCAGAACG-3'
3'arm reverse primer (R2): 5'-GAACAAGGTAGTATAAAGCTGGTAG-3'

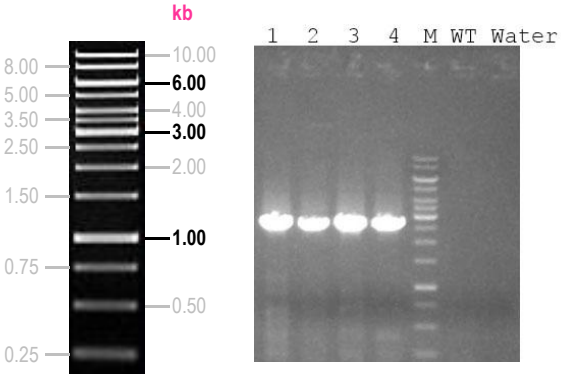
PCR Results:

F1 animals 1, 2, 3 and 4 were identified positive by PCR screening.

Marker **Positive F1 for PCR Primers 1 (MT: 2.6 kb)**



Marker **Positive F1 for PCR Primers 2 (MT: 2.7 kb)**



6. Sequencing Confirmation

Sequencing Primer for PCR product 1:

5'Sequence primer (F3): 5'-CAGACTTGTGGGATACAGAAGAC-3'

Sequencing Primer for PCR product 2:

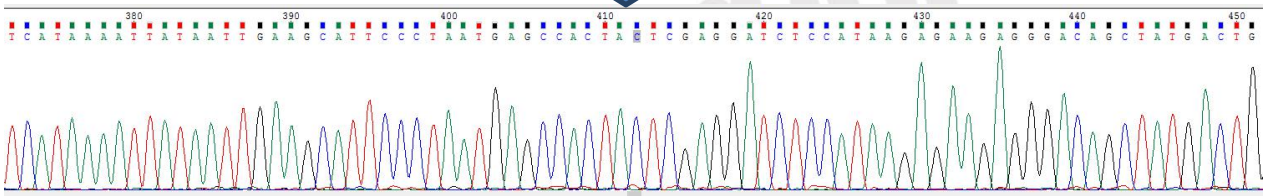
3'Sequence primer (R3): 5'-TGGAAATCAGGCTGCAAATCTCAG-3'

Sequencing Results:

Mouse ID: 1 (One example of positive mice)

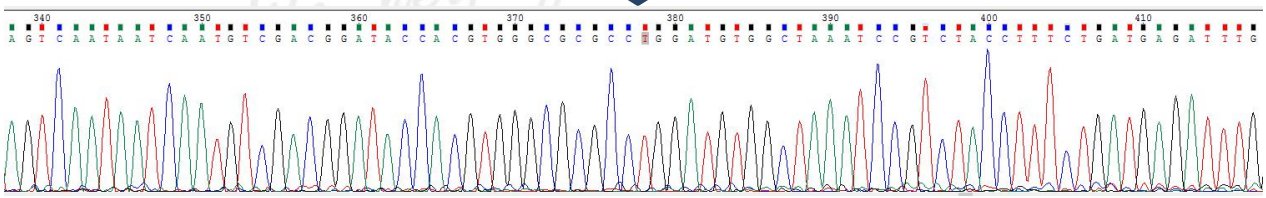
5'arm KI

TCATAAAATTATAATTGAAGCATTCCCTAATGAGCCACTACTCGAGGATCTCCATAAGAGAAGAGGGACAGCTATGACTG



KI 3'arm

AGTCAATAATCAATGTCGACGGATACCACGTGGGCGCGCCTGGATGTGGCTAAATCCGTCTACCTTTCTGATGAGATTTG



Note:

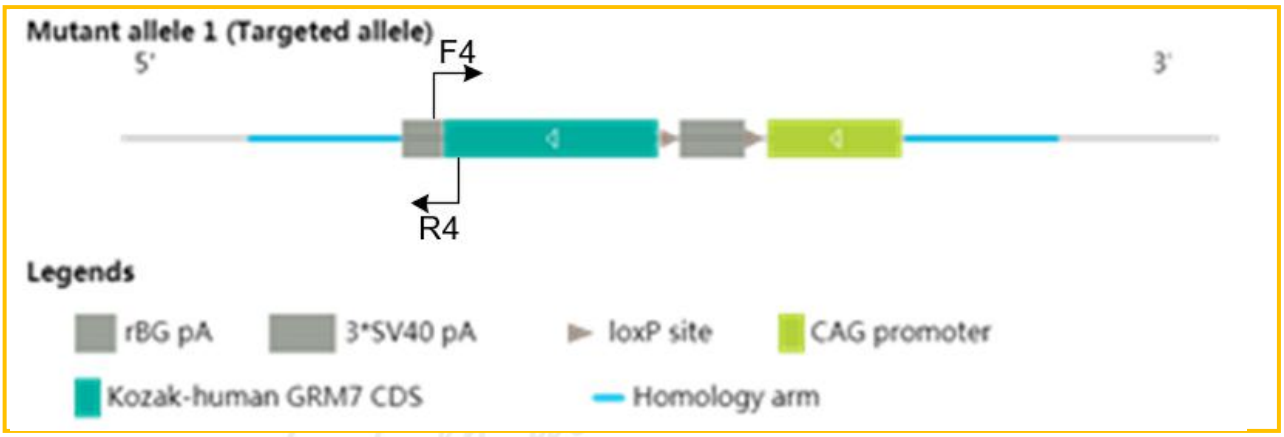
1) PCR was carried out in 50 μL volume for 33 cycles under standard conditions, with all two primers listed above added to each reaction.

2) Taq DNA polymerase used was LongAmp Taq DNA polymerase (NEB M0323V).

3) Two controls used in PCR genotyping are:

- Water control: No DNA template added.
- Wildtype control: 400 ng of mouse genomic DNA.

4) If DNA sample is not very pure or without enough PCR extension time, the long fragment PCR product may not be amplified. You can use the primers below:



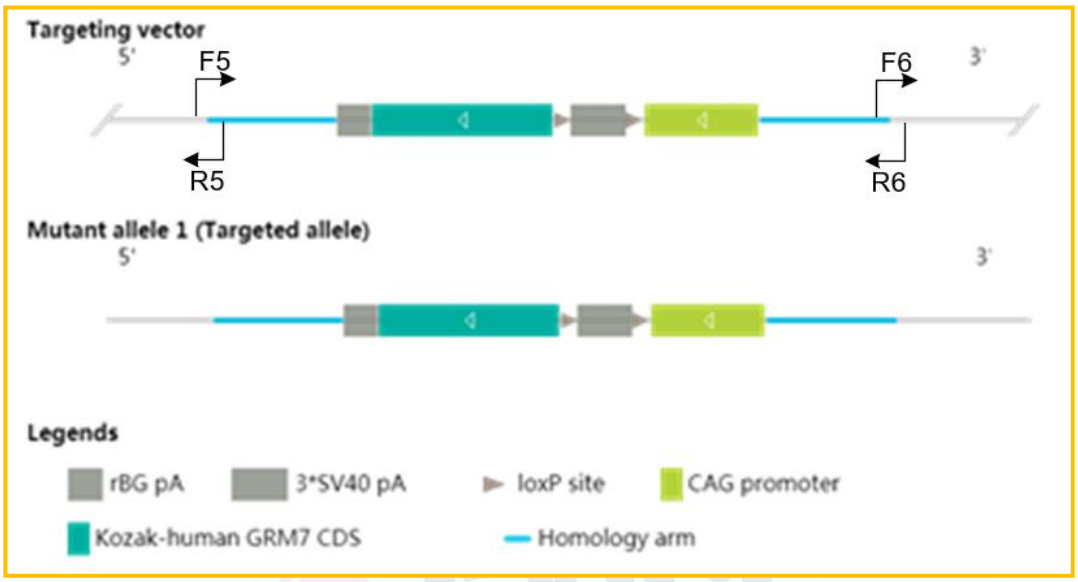
Primers (Annealing Temperature 60.0 ºC):

Forward primer (F4): 5'-CTTTATTAGCCAGAAGTCAGATGC-3'
Reverse primer (R4): 5'-ACCACGCTTACAATCTCCATGAAC-3'

Expected PCR Product:

Targeted allele: 442 bp

7. Analysis of Potential Vector Random Integration:



➢ **Pair1 Primers Used:**

Forward primer (F5): 5'-ATTCAGGCTGCGCAACTGTTG-3'
Reverse primer (R5): 5'-CATAGTCTAACTCGCGACACTG-3'
Internal control PCR primer F: 5'-CATGCCAATGGTTCACTCTAAGGT-3'
Internal control PCR primer R: 5'-TCTCTATGTCCCAAAGTGCAGACAC-3'
Annealing Temp: 60 ℃

Expected PCR Product:

Alleles with random integration: 451 bp Internal control PCR product size: 335 bp

➢ **Pair2 Primers Used:**

Forward primer (F6): 5'-GAGTGGCCTTTAGGCTTGAATTG-3'
Reverse primer (R6): 5'-GCATCAGAGCAGCCGATTGTC-3'
Internal control PCR primer F: 5'-CATGCCAATGGTTCACTCTAAGGT-3'
Internal control PCR primer R: 5'-TCTCTATGTCCCAAAGTGCAGACAC-3'
Annealing Temp: 60 ℃

Expected PCR Product:

Alleles with random integration: 585 bp Internal control PCR product size: 335 bp

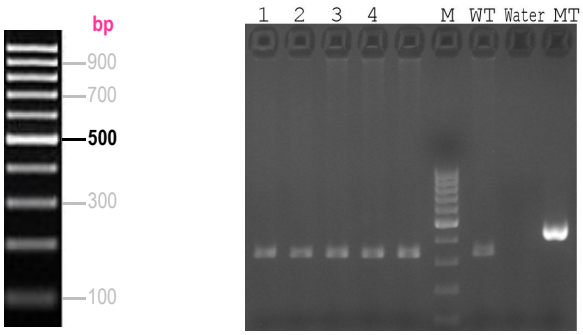
Note:

The internal control PCR targets the endogenous mouse Rgs7 (G protein signaling 7) locus.

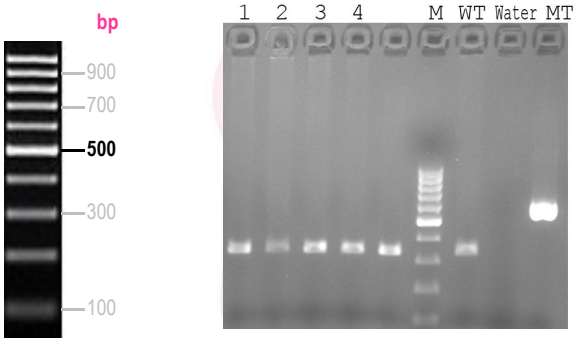
PCR Results:

F1 animals 1, 2, 3 and 4 were identified positive and without random insertion by vector backbone PCR screening.

Marker **Positive F1 PCR analysis (MT: 451 bp; WT: 335 bp) (Pair1 primers)**



Marker **Positive F1 PCR analysis (MT: 585 bp; WT: 335 bp) (Pair2 primers)**



Note:

1) PCR was carried out in 25 μL volume for 35 cycles under standard conditions, with all primers listed above added to each reaction.

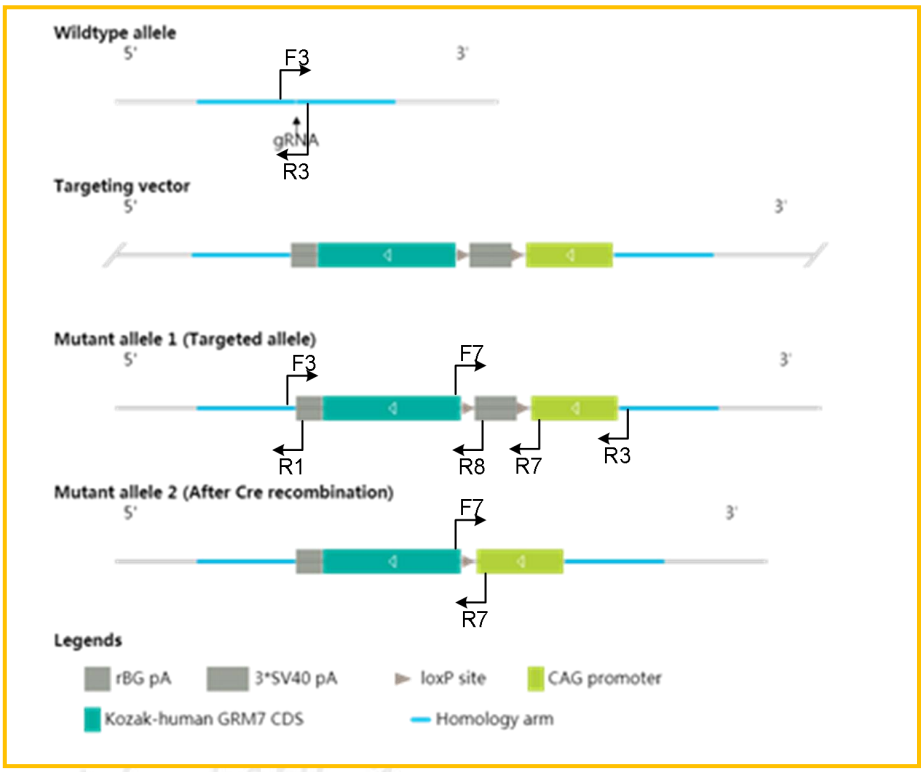
2) Taq DNA polymerase used was P112-01.

3) Three controls used in PCR genotyping are:

- Positive control: 0.3 ng donor vector DNA.
- Wildtype control: 400 ng of mouse genomic DNA.
- Water control: No DNA template added.

8. Breeding and Genotyping strategy

8.1 Targeting Strategy



8.2 Cyagen Delivered

Heterozygous recombinant mice

www.cyagen.com
Tel: 800-921-8930; Fax: 408-969-0336
2255 Martin Ave., Suite E. Santa Clara, CA 95050

8.3 Method

(Suggested Breeding and Genotyping Assay for Tissue-specific Knockin Mice Generation)

Step 1: Inter-cross heterozygous targeted mice to generate homozygous targeted mice

Primers:

F3: 5'-CAGACTTGTGGGATACAGAAGAC-3'
R3: 5'-TGGAAATCAGGCTGCAAATCTCAG-3'
R1: 5'-GCATCTGACTTCTGGCTAATAAAG-3'

Homozygous: one band with 841 bp
Heterozygous: two bands with 841 bp and 648 bp
WT: one band with 648 bp

Step 2: Breed a homozygous targeted mouse with a tissue-specific Cre delete mouse to generate mice that are heterozygous for a targeted allele and a hemizygous/heterozygous for the Cre transgene

Primers:

F3: 5'-CAGACTTGTGGGATACAGAAGAC-3'
R3: 5'-TGGAAATCAGGCTGCAAATCTCAG-3'
R1: 5'-GCATCTGACTTCTGGCTAATAAAG-3'

Homozygous: one band with 841 bp
Heterozygous: two bands with 841 bp and 648 bp
WT: one band with 648 bp

PCR for Cre transgene:

Forward: 5'-GAACGCACTGATTTCGACCA-3'
Reverse: 5'-GCTAACCAGCGTTTTCGTTC-3'

Cre amplicon: 204 bp

Step 3: Breed heterozygous, Cre+ mice with homozygous mice. Approximately 25% of the progeny from this mating will be homozygous for the targeted allele and hemizygous/heterozygous for the Cre transgene. The pups can be screened by the same assay as described above. The tissue-specific gene deletion can be confirmed by adding one additional primer to the PCR assay:

PCR for constitutive KI allele:

F7: 5'-ACGCAGCAGGGGAACTTCATCAAAG-3'
R7: 5'-GGCAACGTGCTGGTTATTGTG-3'
R8: 5'-AGATCTGCAAGCTAATTCCTGC-3'

With Cre activity: one band with 224 bp
No Cre activity: two bands with 169 bp and 1101 bp

Note: If DNA sample is not very pure or without enough PCR extension time, the 1101 bp PCR product may not be amplified.

9. PCR Conditions Attachment

9.1 DNA Extraction

➢ Method One:

We recommend that using TaKaRa MiniBEST Universal Genomic DNA Extraction kit (Ver.5.0_Code No. 9765) to gain high purity of genomic DNA.

a. Add 180 μL of Buffer GL, 20 μL of Proteinase K and 10 μL of RNase A per tail piece (2-5 mm) in a microcentrifuge tube. Be careful not to cut too much tail.

b. Incubate the tube at 56℃ overnight.

c. Spin in microcentrifuge at 12,000 rpm for 2 minutes to remove impurities.

d. Add 200 μL Buffer GB and 200 μL absolute ethyl alcohol with sufficient mixing.

e. Place the spin Column in a collection tube. Apply the sample to the spin and centrifuge at 12,000 rpm for 2 min. Discard flow-through.

f. Add 500 μL Buffer WA to the spin column and centrifuge at 12,000 rpm for 1 min. Discard flow-through.

g. Add 700 μL Buffer WB to the spin column and centrifuge at 12,000 rpm for 1 min. Discard flow-through. (Note: Make sure the Buffer WB has been premixed with 100% ethanol. When adding Buffer WB, add to the tube wall to wash off the residual salt.)

h. Repeat step g.

i. Place the spin Column in a collection tube and centrifuge at 12,000 rpm for 2 min.

j. Place the spin Column in a new 1.5ml tube. Add 50~200 μL sterilized water or elution buffer to the center of the column membrane and let the column stand 5min. (Note: Heating sterilized water or elution buffer up to 65℃ can increase the yield of elution.)

k. To elute DNA, centrifuge the column at 12,000 rpm for 2 min. To increase the yield of DNA, add the flow-through and/or 50~200 μL sterilized water or elution buffer to the center of the spin column membrane and let the column stand 5 min. Centrifuge at 12,000 rpm for 2 min.

l. Quantify to genomic DNA. Eluted genomic DNA can be quantified by electrophoresis or electrophoresis.

➢ Method Two:

A low-cost and sample method to gain rough genomic DNA.

a. Add 100 μL of tail digestion buffer per tail piece (2-5 mm) in a microcentrifuge tube. Be careful not to cut too much tail.

b. Incubate the tube at 56℃ overnight.

c. Incubate the tube at 98℃ for 13 minutes to denature the Proteinase K.

d. Spin in microcentrifuge at top speed for 15 minutes. Use an aliquot of supernatant straight from the tube (2 μL in a 50 μL reaction) for PCR.

Final concentration of tail digestion buffer:

➢ 50 mM KCl
➢ 10 mM Tris-HCl (pH 9.0)

- 0.1 % Triton X-100
- 0.4 mg/mL Proteinase K

9.2 Long fragment PCR reaction

PCR Mixture:

Component	x1	
Mouse tail genomic DNA	2	μl
Forward primer (10 μM)	2	μl
Reverse primer (10 μM)	2	μl
dNTPs (2.5 mM)	6	μl
5X LongAmp Taq Reaction	10	μl
LongAmp Taq DNA Polymerase	2	μl
ddH_2O	26	μl
Total	50	μl

Cycling Condition:

Step	Temp.	Time	Cycles
Initial denaturation	94 °C	3 min	
Denaturation	94 °C	30 s	33 x
Annealing	60 °C	30 s	
Extension	65 °C	50 s/kb	
Additional extension	65 °C	10 min	

9.3 Short fragment PCR reaction

PCR Mixture 1:

Component	x1	
Mouse tail genomic DNA	1	μl
Forward primer (10 μM)	1	μl
Reverse primer (10 μM)	1	μl
Premix Taq Polymerase	12.5	μl
ddH_2O	9.5	μl
Total	25	μl

PCR Mixture 2:

Component	x1	
Mouse tail genomic DNA	1	μl
Forward primer (10 μM)	1	μl
Reverse primer (10 μM)	1	μl
Internal control PCR primer F	0.5	μl
Internal control PCR primer R	0.5	μl
Premix Taq Polymerase	12.5	μl
ddH_2O	8.5	μl
Total	25	μl

Cycling Condition:

Step	Temp.	Time	Cycles
Initial denaturation	94 °C	3 min	
Denaturation	94 °C	30 s	35 x
Annealing	60 °C	35 s	
Extension	72 °C	35 s	
Additional extension	72 °C	5 min	