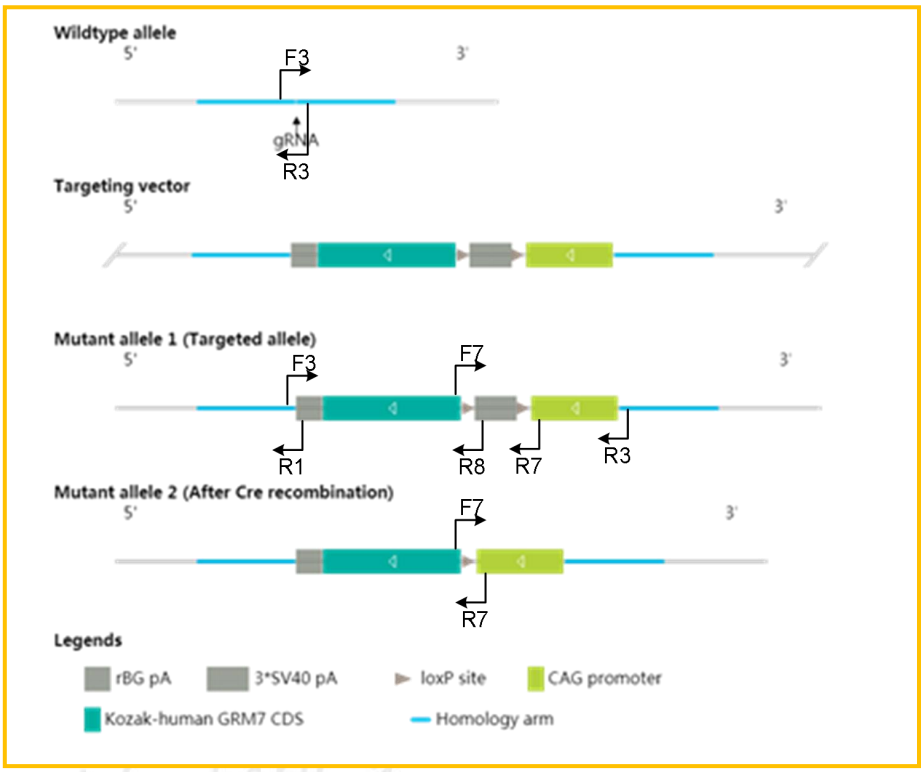
8. Breeding and Genotyping strategy

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	