(Generation of CB2-KO-eGFP mice)

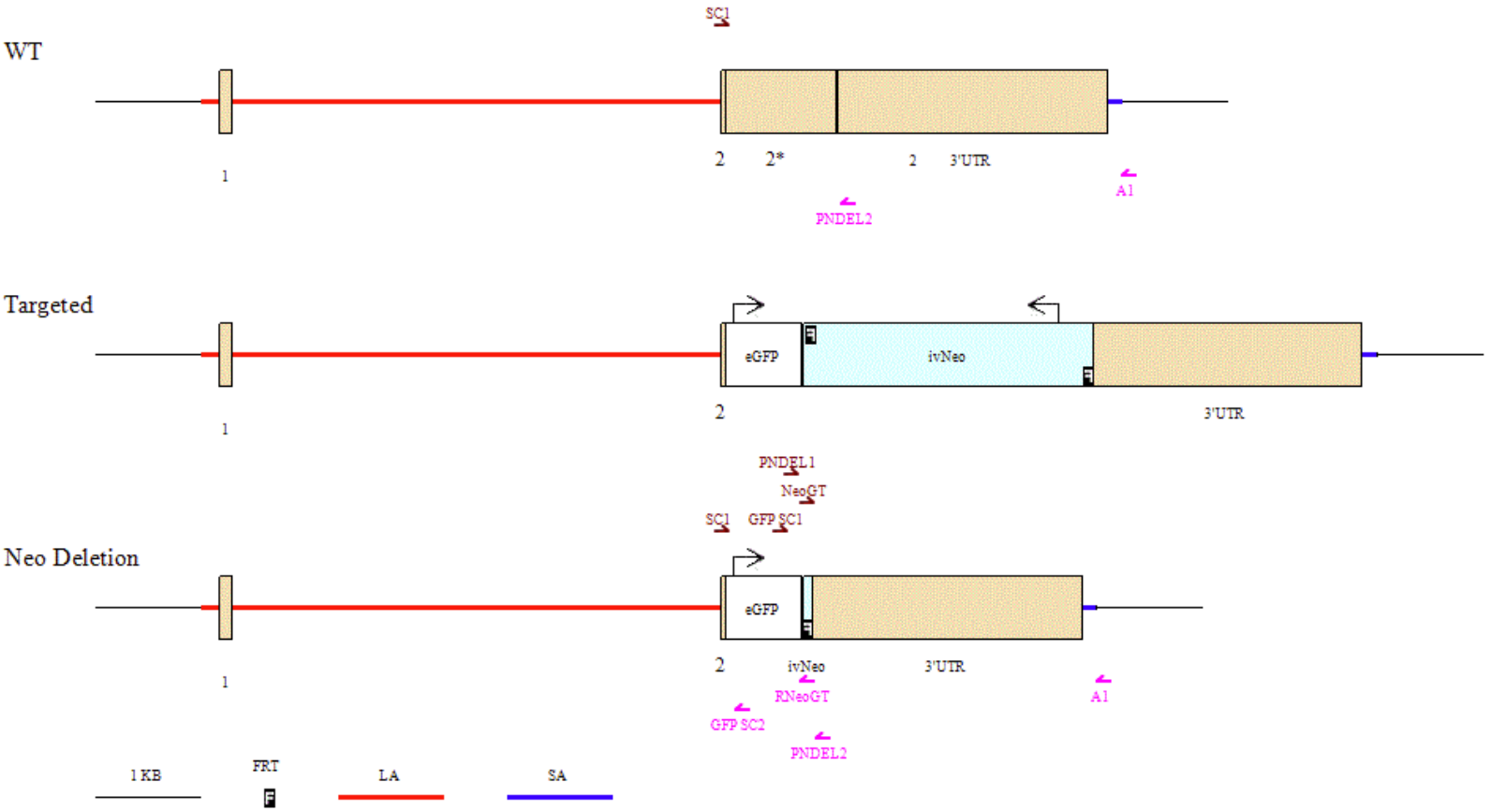
ZCNR-C: PCR Screening of Mice

Sections:

I. Identification of Germline Neo Deleted Mice.

1. Schematic and Information

Targeted iTL HF4 (129/SvEv x C57BL/6 FLP) hybrid embryonic stem cells were microinjected into CD-1 blastocysts. Resulting chimeras with a high percentage agouti coat color were mated to C57BL/6N WT mice to generate Germline Neo Deleted mice. Tail DNA was analyzed as described below from pups with agouti or black coat color.



Primers for PCR Screening:

Forward Oligos

SC1: 5′- GAT TCT TTT CTC CTT GCC CAC AGC -3′
GFP SC1: 5′- AGG TGA ACT TCA AGA TCC GCC ACA -3′
PNDEL1: 5′- CAA CCA CTA CCT GAG CAC CC -3′
NEOGT: 5′- GTC CGT GTC GCG AAG TTC CTA TAC TTT C -3′

Reverse Oligos

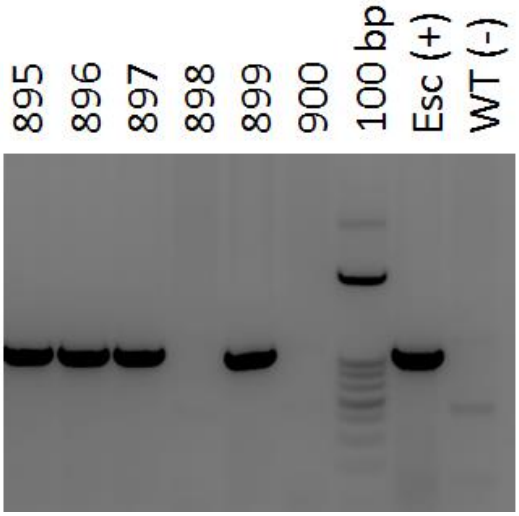
GFP SC2: 5′- AGA TGA ACT TCA GGG TCA GCT TGC -3′
RNEOGT: 5′- GAA AGT ATA GGA ACT TCG CGA CAC GGA C -3′
PNDEL2: 5′- CGA GGG AGT GAA CTG AAC GG -3′
A1: 5′- CGG GTT CTC TGT GCT ATA CCT CCA G -3′

newFLP1: 5′- ACA GAG ACA AAG ACA AGC GTT AGT AGG -3′
newFLP2: 5′- ATT TCC CAC AAC ATT AGT CAA CTC CGT TAG G-3'

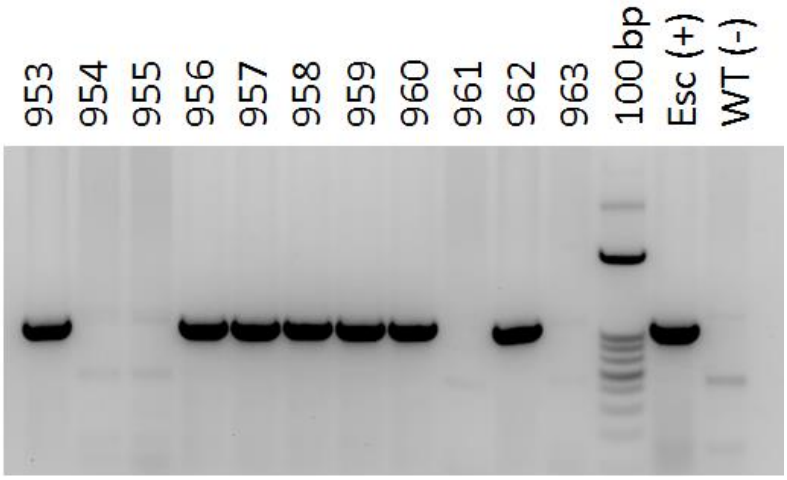
*The FLP primers cannot be seen in the schematic above.

2. Screening for Knockin Reporter

A PCR was performed to confirm the knockin cassette using the SC1 and RNEOGT primers. SC1 is on the long homology arm upstream of the knockin cassette. RNEOGT is inside the remaining Neo cassette downstream of the knockin cassette. This reaction amplifies a 837 bp product when the knockin cassette is present.



SC1/ RNEOGT (837 bp)

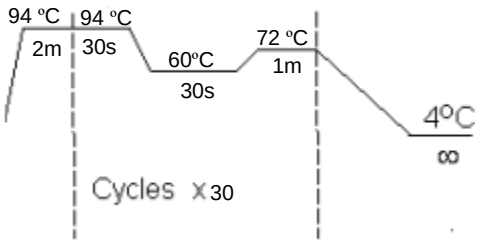


SC1/ RNEOGT (837 bp)

PCR Parameters for SC1/ RNEOGT:

EconoTaq Plus Green 2x Master Mix (Lucigen catalog# 30033-1)

11.00 µL ddH_20
12.50 µL EconoTaq Plus Green 2x Master Mix
0.25µL 100 µM Primer
1.00 µL DNA



After a 2 minute hot start at 94°C the samples were run using the above conditions. The PCR product was run on a 2% gel with a 100 bp ladder as reference. The expanded ES clone, which was used as a positive control, is denoted by a (+) in the gel photograph above.

Below is sequencing of representative mouse #897 using primer GFP SC2.

eGFP sequence is in violet text, and genomic sequence is in plain text with 3' UTR shaded.

```
Query  18     CCTCGCCGGACACGCTGAACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGATGG  77
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16319  CCTCGCCGGACACGCTGAACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGATGG  16260

Query  78     GCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATGGATGGGCTTTGGCTTCTTC  137
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16259  GCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATGGATGGGCTTTGGCTTCTTC  16200

Query  138    TACTGGAGCTGTCCCAGAAGACTGGGCTGTGGGCAAGGAGAAAAGAATC  186
              ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16199  TACTGGAGCTGTCCCAGAAGACTGGGCTGTGGGCAAGGAGAAAAGAATC  16151

Query: Sequencing data from PCR products
Sbjct: Respective targeted allele sequence
```

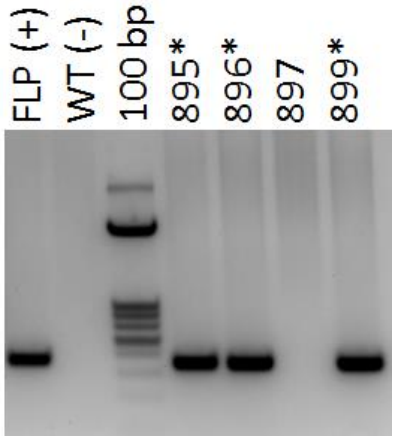
Below is sequencing of representative mouse #899 using primer GFP SC1.

eGFP sequence is in violet text and the remaining Neo cassette is in red text with the FRT site underlined.

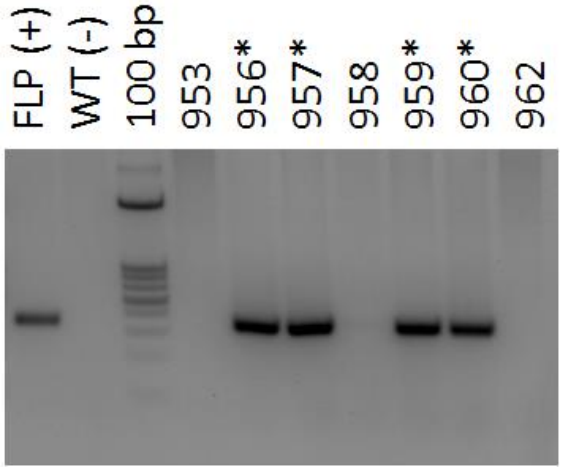
```
Query  11     AGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG  70
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16745  AGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG  16804

Query  71     CTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAG  130
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16805  CTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAG  16864

Query  131    CGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGAC  190
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16865  CGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGAC  16924

Query  191    GAGCTGTACAAGTAACGTACGTTCGTGGGATTGTGTCCGTGTCGCGAAGTTCCTATACTT  250
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16925  GAGCTGTACAAGTAACGTACGTTCGTGGGATTGTGTCCGTGTCGCGAAGTTCCTATACTT  16984

Query: Sequencing data from PCR products
Sbjct: Respective targeted allele sequence
```

3. Screening for FLP Transgene

Primer set newFLP1 and newFLP2 was used to screen mice for the FLP transgene. The amplified product for primer set newFLP1 and newFLP2 is 330bp.



newFLP1 / newFLP2 (330 bp if FLP transgene present)
(*Asterisked mice are FLP present)

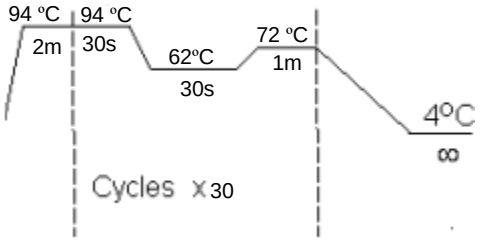


newFLP1 / newFLP2 (330 bp if FLP transgene present)
(*Asterisked mice are FLP present)

PCR Parameters for newFLP1 / newFLP2:

EconoTaq Plus Green 2x Master Mix (Lucigen catalog# 30033-1)

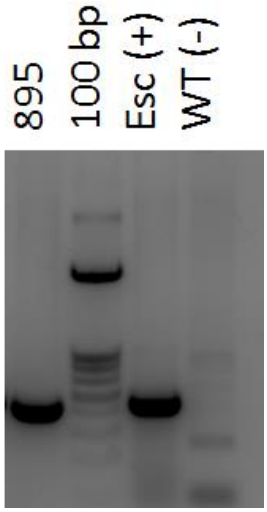
11 μL ddH_20
12.5 μL EconoTaq Plus Green 2x Master Mix
.25μL 100 μM Primer
1.0 μL DNA



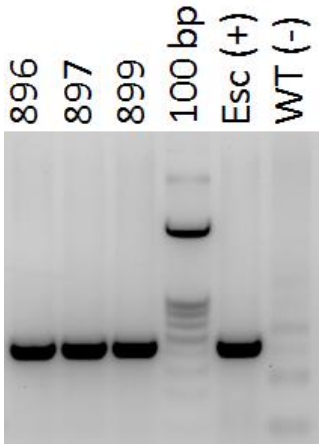
After a 2 minute hot start at 94°C the samples were run using the above conditions. The PCR product was run on a 2% gel with a 100 bp ladder as reference. Tail DNA sample from a FLP mouse was used as a positive control and is denoted by a (+) in the gel photographs.

4. Confirmation of Neo Deletion

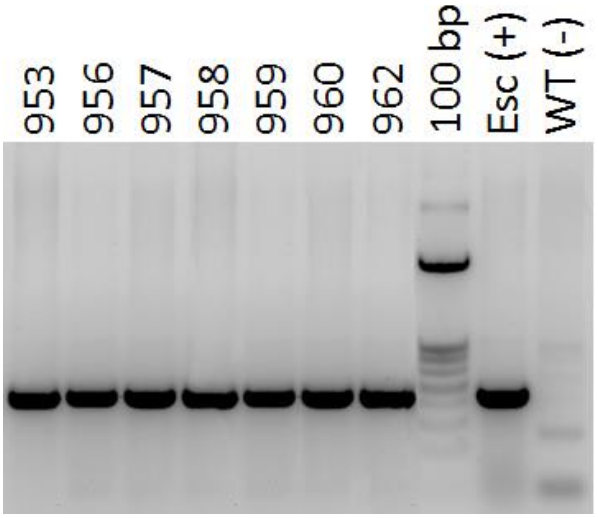
Primer set GFP SC1 and PNDEL2 was used to confirm deletion of the Neo cassette. GFP SC1 is inside the GFP cassette upstream of the remaining Neo cassette. PNDEL2 is inside the 3' UTR downstream of the Neo cassette. After Neo deletion, one FRT site remains and a PCR product with a size of 418 bp indicates Neo deletion.



GFP SC1 / PNDEL2 (418 bp)



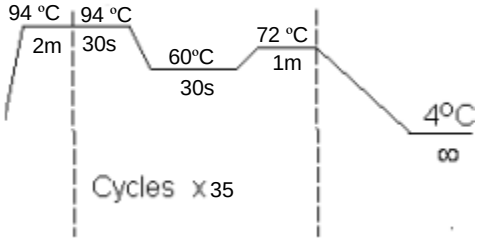
GFP SC1 / PNDEL2 (418 bp)



GFP SC1 / PNDEL2 (418 bp)

PCR Parameters for GFP SC1 / PNDEL2:

EconoTaq Plus Green 2x Master Mix (Lucigen catalog# 30033-1)



11.00 μL ddH_20
12.50 μL EconoTaq Plus Green 2x Master Mix
0.25μL 100 μM Primer
1.00 μL DNA

After a 2 minute hot start at 94°C the samples were run using the above conditions. The PCR product was run on a 2% gel with a 100 bp ladder as reference. The band representing Neo deletion was excised and sequenced in positive samples. The expanded ES clone, which was used as a positive control, is denoted by a (+) in the gel photograph above.

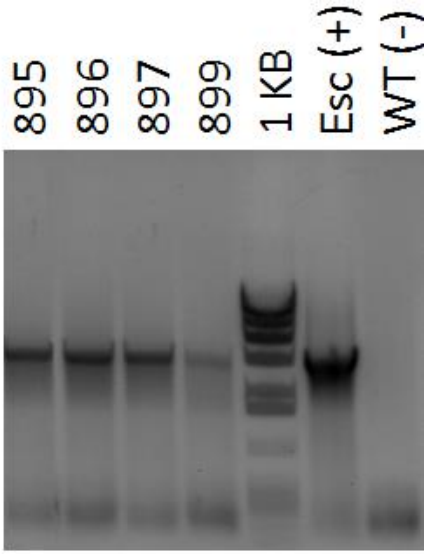
Below is sequencing of representative mouse #897 using primer PNDEL1. The sequence shows the deletion of the Neo cassette with the exception of one FRT site.

GFP sequence is in violet text, the remaining Neo cassette is shaded with the FRT site in red text, and the 3' UTR is in plain text.

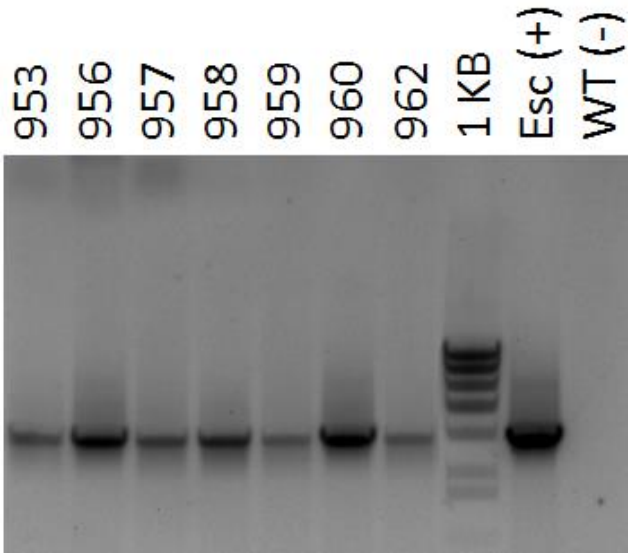
```
Query  22     AGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCA  81
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16860  AGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCA  16919

Query  82     TGGACGAGCTGTACAAGTAACGTACGTTCGTGGGATTGTGTCCGTGTCGCGAAGTTCCTA  141
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16920  TGGACGAGCTGTACAAGTAACGTACGTTCGTGGGATTGTGTCCGTGTCGCGAAGTTCCTA  16979

Query  142    TACTTTCTAGAGAATAGGAACTTCGTTGGTACCGTACGCTAGCTGAGCCAGGATCCAGAA  201
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16980  TACTTTCTAGAGAATAGGAACTTCGTTGGTACCGTACGCTAGCTGAGCCAGGATCCAGAA  17039

Query  202    CTCCAGGCTGCTCCAACTGCTGACACCACCTGTCTTTCTACTGGAAACAGCCCGAGTCAG  261
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17040  CTCCAGGCTGCTCCAACTGCTGACACCACCTGTCTTTCTACTGGAAACAGCCCGAGTCAG  17099

Query  262    AAGTCCGTTCAGTTCACTCCCTCGA  286
              |||||||||||||||||||||||||
Sbjct  17100  AAGTCCGTTCAGTTCACTCCCTCGA  17124

Query: Sequencing data from PCR products
Sbjct: Respective targeted allele sequence
```

5. Confirmation of Short Homology Arm Integration

Tail DNA samples from positive mice were amplified with primers NEOGT and A1. NEOGT is located inside the remaining Neo sequence and A1 is located upstream of the short homology arm, outside the region used to create the targeting construct. NEOGT / A1 amplifies a fragment of 2.83 kb in length.



NEOGT / A1 (2.83 kb)

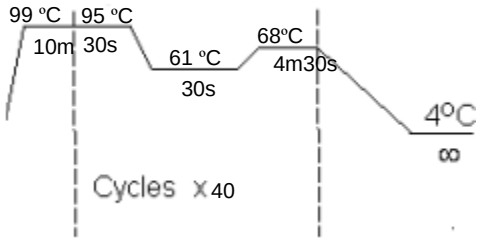


NEOGT / A1 (2.83 kb)

PCR Parameters for NEOGT / A1:

Expand High Fidelity PCR System (Roche catalog # 04 738 276 001)

17.50 µL ddH_20
2.50 µL 200.0 µM dNTP
2.50 µL PCR Buffer with 15mM $MgCl_2$
1.00 µL DMSO
0.25 µL 100 µM Each Primer
1.00 µL 1.5 µL DNA



After a 10 minute hot start at 99°C, 0.125 μL of Taq polymerase was added to each PCR sample followed by a layer of 2 drops mineral oil. The PCR product was run on a 0.8% gel with a 1 KB ladder as reference. The expanded ES clone, which was used as a positive control, is denoted by a (+) in the gel photograph above.

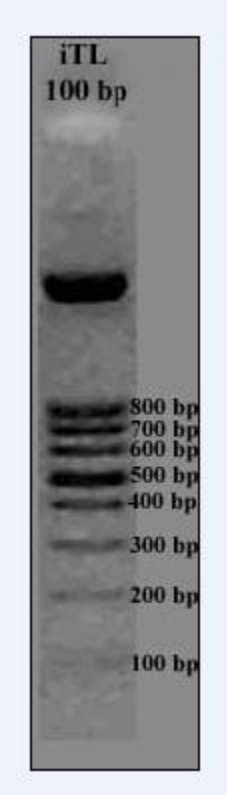
6. Germline Neo Deleted Mouse Information

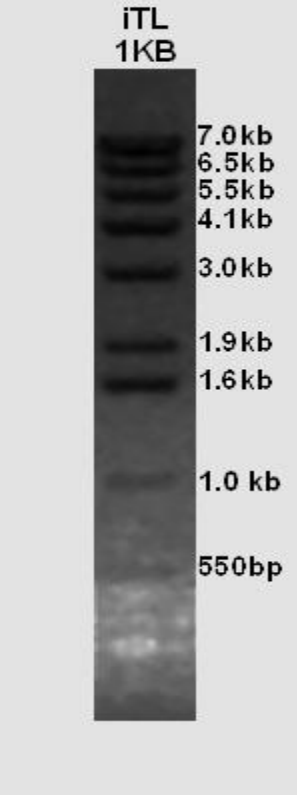
The following heterozygous mice were confirmed for Germline Neo Deletion.

Mouse #	Sex	DOB	Clone #	Parent Info
895**	M	10/28/2020	131	CH X C57BL/6N WT
896*	M	10/28/2020	131	CH X C57BL/6N WT
897	M	10/28/2020	131	CH X C57BL/6N WT
899*	M	10/28/2020	131	CH X C57BL/6N WT
953	M	11/01/2020	131	CH X C57BL/6N WT
956*	F	11/01/2020	131	CH X C57BL/6N WT
957*	F	11/01/2020	131	CH X C57BL/6N WT
958	F	11/01/2020	131	CH X C57BL/6N WT
959*	M	11/01/2020	131	CH X C57BL/6N WT
960*	F	11/01/2020	131	CH X C57BL/6N WT
962	F	11/01/2020	131	CH X C57BL/6N WT

***Asterisked mice are recommended to be mated with Wildtype to remove FLP Transgene**
****Asterisked mouse #895 will be retained at iTL facilty as backup.**

7. Reference


iTL
100 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp


iTL
1KB
7.0kb
6.5kb
5.5kb
4.1kb
3.0kb
1.9kb
1.6kb
1.0 kb
550bp