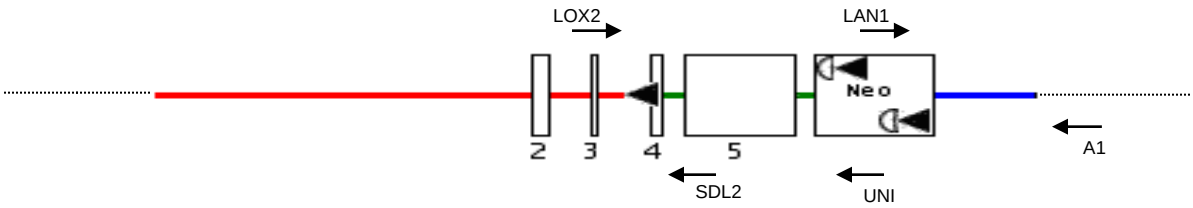
NRFT: Screening and Reconfirmation of Recombinant Clones

Sections:

1. PCR Screening Strategy

Ten micrograms of the targeting vector was linearized by NotI and then transfected by electroporation of BA1 (C57Bl/6 x 129/SvEv) (Hybrid) embryonic stem cells. After selection with G418 antibiotic, surviving clones were expanded for PCR analysis to identify recombinant ES clones.

Key: SA = Short Arm
LA = Long Arm
..... = genomic region



Primers for PCR Screening

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A1:   5′- GGA GTA GCA GAA TCT TGT CAA GTT GG -3′
LAN1: 5′- CCA GAG GCC ACT TGT GTA GC         -3′
LOX2: 5′- TGC TCT CTG TGC CTT TCC            -3′
SDL2: 5′- CAC TCC ATG TAT TTA AAG GCA GAC    -3′
UNI:  5′- AGC GCA TCG CCT TCT ATC GCC TTC    -3′
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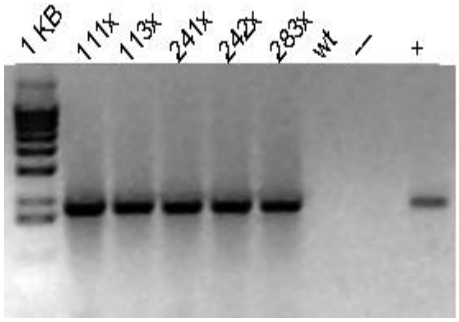
Screening primer A1 was designed downstream of the short homology arm (SA) outside the 3' region used to generate the targeting construct. PCR reactions using A1 with the LAN1 primer (located within the Neo cassette) amplify 1.88kb fragment. Clones 111, 113, 241, 242, and 283 were identified as positive and selected for expansion.

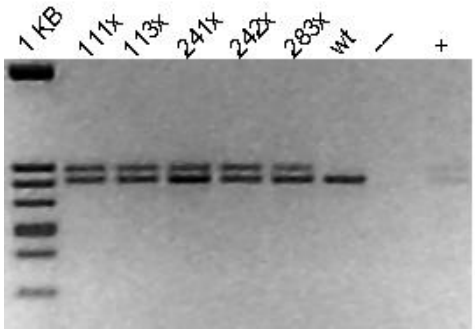
2. Reconfirmation of Expanded Clones by PCR

Clones 111, 113, 241, 242, and 283 were expanded and reconfirmed for SA integration. An "x" denotes expanded clones. DNA from an individual clone (before reconfirmation) was used as a positive control and denoted as a (+). No DNA was used as a negative control, and denoted by a (--). Wild Type DNA was used as a negative control, and denoted by a (wt).



NRFT LOX2/SDL2 (1.88 KB)

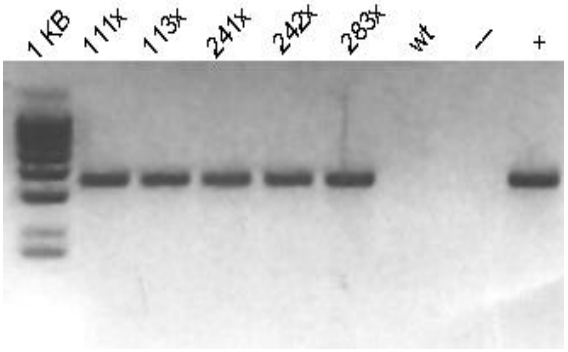
A PCR was performed on clones 111, 113, 241, 242, and 283 to detect presence of the third LoxP site using the LOX2 and SDL2 primers. This reaction amplifies a wild type product 726 bp in size. The presence of a second PCR product 68 bp greater than the wild type product indicates a positive LoxP PCR.



NRFT LOX2 / SDL2
wt = 726 bp; LoxP pos = 793 bp

3. Confirmation of Third LoxP Retention by DNA Sequencing

Confirmation of third LoxP retention was performed by PCR using the LOX2 and UNI primers. This reaction produces a product 3.61 kb in size.



NRFT LOX2/UNI (3.61 KB)

Sequencing was performed on purified PCR DNA to confirm presence of the third LoxP cassette using the SDL2 primer. The sequence from a confirmed clone is shown below.

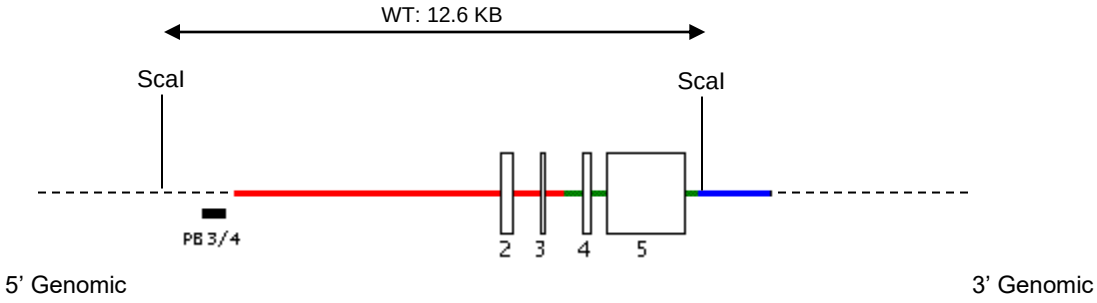
ATCTGTACCCATAATATCTAGCAGAATGCTTCAAACAACAGATTAAGATTTTTTTAATCCAAATGCTGATT
AATAGTTGTTCTTTACTAGTCAAAATATTTTATATACCTATATCCAGTTTCCACGGTGGCGACGAATTCAT
AACTTCGTATAATGTATGCTATACGAAGTTATGGTACCTGGCCAAGTACTGGGAAAATTATTCTGATTCCT
GTTTCCAGCTATACAGTAAAATGGACTTGTAGTTTACAGGCCTTATTTTTTTTTTTTTAAAGATTTATTTA

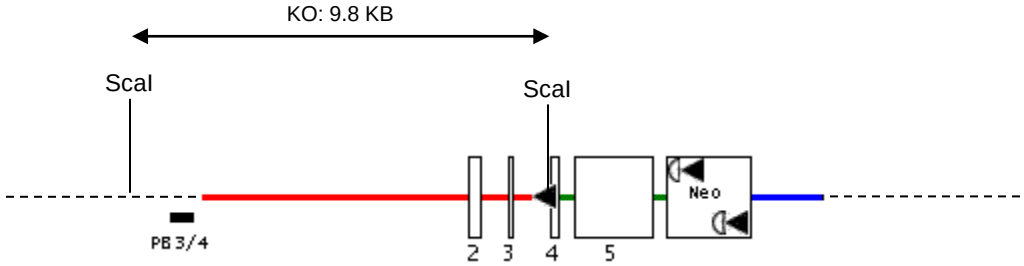
The cassette containing third LoxP and engineered restriction enzymes is shaded.

Clones 111, 113, 241, 242, and 283 were confirmed by PCR and were further analyzed by Southern blot.

4. External Long Arm Schematic and Southern Blot Strategy

Secondary confirmation of positive clones identified by PCR was performed by Southern Blotting analysis. DNA was digested with ScaI, and electrophoretically separated on a 0.8% agarose gel. After transfer to a nylon membrane, the digested DNA was hybridized with a probe targeted against the 5' external region. DNA from C57Bl/6 (B6), 129/SvEv (129), and BA1 (C57Bl/6 x 129/SvEv) (Hybrid) mouse strains were used as wild type controls. The expected sizes are indicated on the schematic below.





5. External Long Arm Probe Sequences and Southern Blot Results

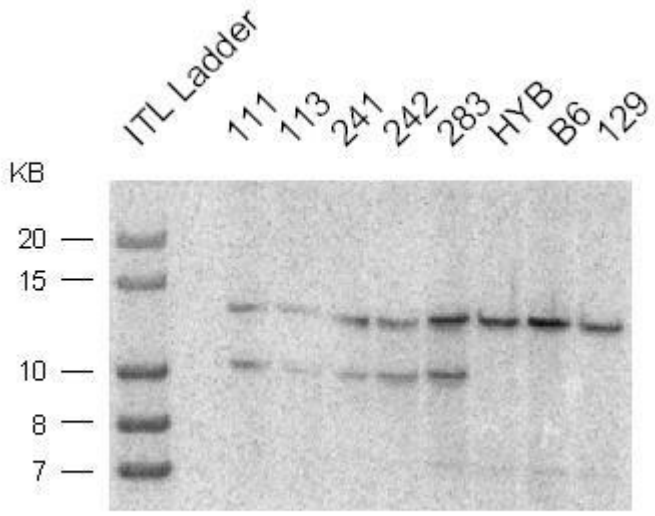
PB3/4 Probe Primers:

PB3 5′- ACA GCT ATG CCT CTG GAG AAA GTC –3′
PB4 5′- CAG GTA CAC TAT TGC TGT TTA TG –3′

PB3/4 Probe Sequence (521 bps)

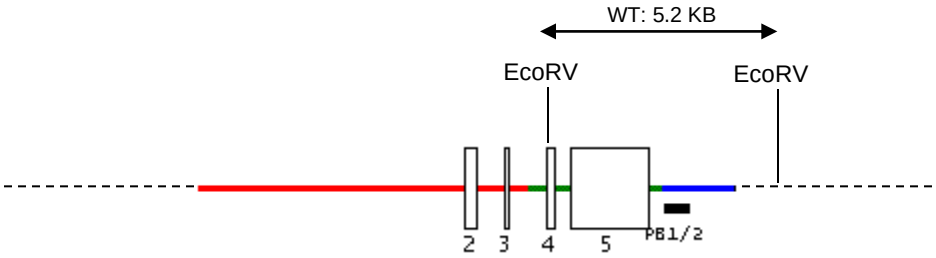
ACAGCTATGCCTCTGGAGAAAGTCGGGCTTCTGACTGCCTTCACTTTCCAGCTTTGCGTAGTCTTGCATCA
TGTTGCAGGTTATGGGCTGCGTAATGGGTGAGAGTTCCAGTAAGCTGGCCGGGTCCTATTTCTTAGATTGA
GTGAGTCATTCCTAAGAACCTTGCTTGAGAACAGGAAGCAGCCCATGGACGTTTCTGTGCATGGTAATGTA
GCCTGCCAGGCTGAGGAAGCAGACACTTTCTAGCTCAGACCAAGTGGAGGATGTCTCCAGGCCTCTGGAGT
GGTGCTTTCGTGACCGGGGCTTTAACCTTGCTGAGTGATTTGTATTTGGTACGAATTCAGAAATTTTTATG
TGGTGAAAATCTCTAGTTGTTGATTATTGAGATAGGATTTCTGTAGGAGGAAAACTTGGGTTGGCTGGTGT
TGAGTATTAATAGTCTGCCAAGTATATATGAGTTGTTTAACTAGGTCTTTCTAATATTGCTGAGGTAACAC
ACATAAACAGCAATAGTGTACCTG

Results

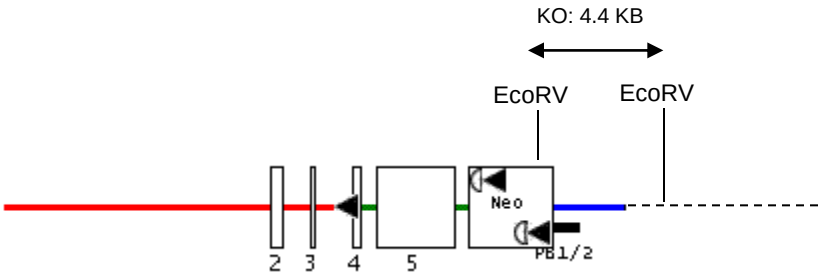


6. Internal Short Arm Schematic and Southern Blot Strategy

Positive clones were further confirmed by Southern Blotting analysis using an internal probe. DNA was digested with EcoRV, and electrophoretically separated on a 0.8% agarose gel. After transfer to a nylon membrane, the digested DNA was hybridized with a probe targeted against the 3' internal region. DNA from C57Bl/6 (B6), 129/SvEv (129), and BA1 (C57Bl/6 x 129/SvEv) (Hybrid) mouse strains were used as wild type controls. The expected sizes are indicated on the schematic below.



5' Genomic 3' Genomic



7. Internal Short Arm Probe Sequences and Results

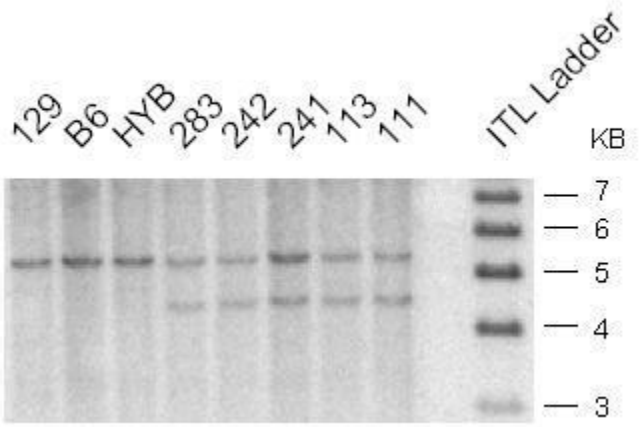
PB1/2 Probe Primers:

PB1 5′- CCT CCC TGT GTT CTT AGA AGT GGC -3′
PB2 5′- TAT GGA ACA ACT GGT CTC TCT CGT C -3′

PB1/2 Probe Sequence (537 bps)

CCTCCCTGTGTTCTTAGAAGTGGCATTCTAAGAGGGCTAGGAAGCATACTAAGGAAGGGGGGGGGGGATTG
AGAAAACCAGTTTTTAAAAAAAGCTGGTCGTAATGGCTTACATCTTCAATTGCTGAGCTCTGTAGACAAGG
CTACCTTTGAACTCTGGTGCAGAGTCTGGCAAATCTGTGAGTTGCCCAGCCTGGTCTACATAGAGAGTTCC
AGGACAATCAGAGCTATGTAGAAAGAGACCCTGGGTCAACAAGTCCAAATAAACACTAGTGAAAATCATCA
CAACGTATAGGAGCTTGCAACATCTGAAGGTATAGGTTGATATGCTAATCAGCTCCACAGAGCACAGCTGA
GCAGGCTGCACAGAGGCTGGAAGTGATGGGAAGAAGAGCGTTATGAAAAGCACAGGCATGGGCAGTCTTAA
AAAGGGGGGATCTGAGGTTGAAGTGGGCTATGAGACTGGTCCTCTGGTCAGTGAACCTGAACCCGAGCAGC
CCAGGTGAGGGGTGGGACGAGAGAGACCAGTTGTTCCATA

Results



Clones 111, 113, 241, 242, 283 were confirmed as correctly targeted and recommended for injection.

8. References

Below are references for the1 kb and 100 bp ladders.