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ES Cell Report

Quote: TKN-201123-CAA-01-TAC
Project: Mouse Uty (Plasmid 1) Constitutive Knockin

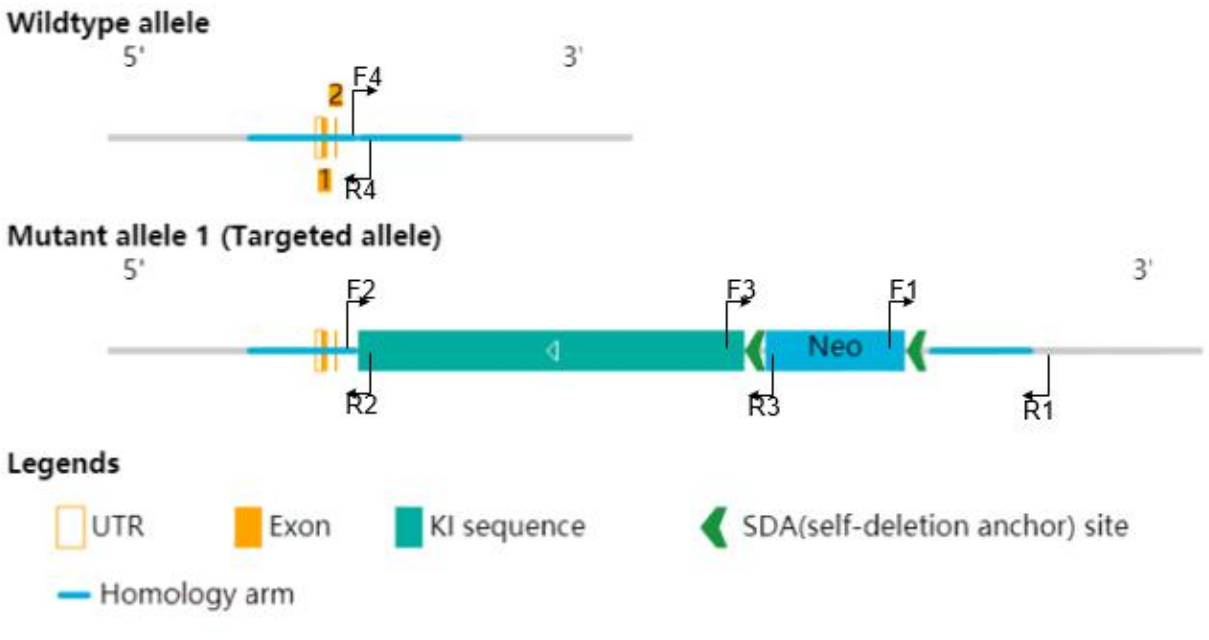
-Confidential-

1. Summary

The Uty targeting vector was transfected into C57BL/6N ES cells according to cyagen's standard electroporation procedures. The transfected ES cells were subject to G418 selection (200μg/mL) 24 hours post electroporation. 185 G418 resistant clones were picked and amplified in 96-well plates. Two copies of 96-well plates were made, one copy was frozen down and stored at -80°C and the other copy of the 96-well plates was used for DNA isolation and subsequence PCR screening for homologous recombination. The PCR screening identified forty-one potential targeted clones, from among which six were expanded and further characterized by Southern blot analysis. All of the six expanded clones were confirmed to be correctly targeted.

1.1. PCR Screening

Regions in the following diagram were selected for PCR screening.



1.1.1 3'arm PCR

Primers for 3'arm PCR:
F1: 5'-CCTGAATGAACTGCAAGACGAGG-3'
R1: 5'-GAGTTCCAGGCCAGTCTATGGTC-3'

Expected PCR Product:
Wildtype: N.A.
Targeted: 3993 bp

Reaction Mix:

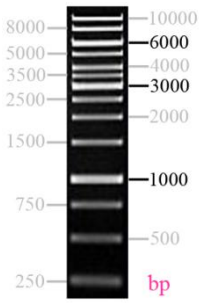
Component	x1
ES cell genomic DNA	1.5 μL
Forward primer(10 μM)	0.6 μL
Reverse primer(10 μM)	0.6 μL
dNTPs(2.5 mM)	1.8 μL
5X LongAmp Taq Reaction	3 μL
LongAmp Taq DNA Polymerase	0.9 μL
DMSO	0.5 μL
ddH_2O	6.1 μL
Total	15 μL

Cycling Condition:

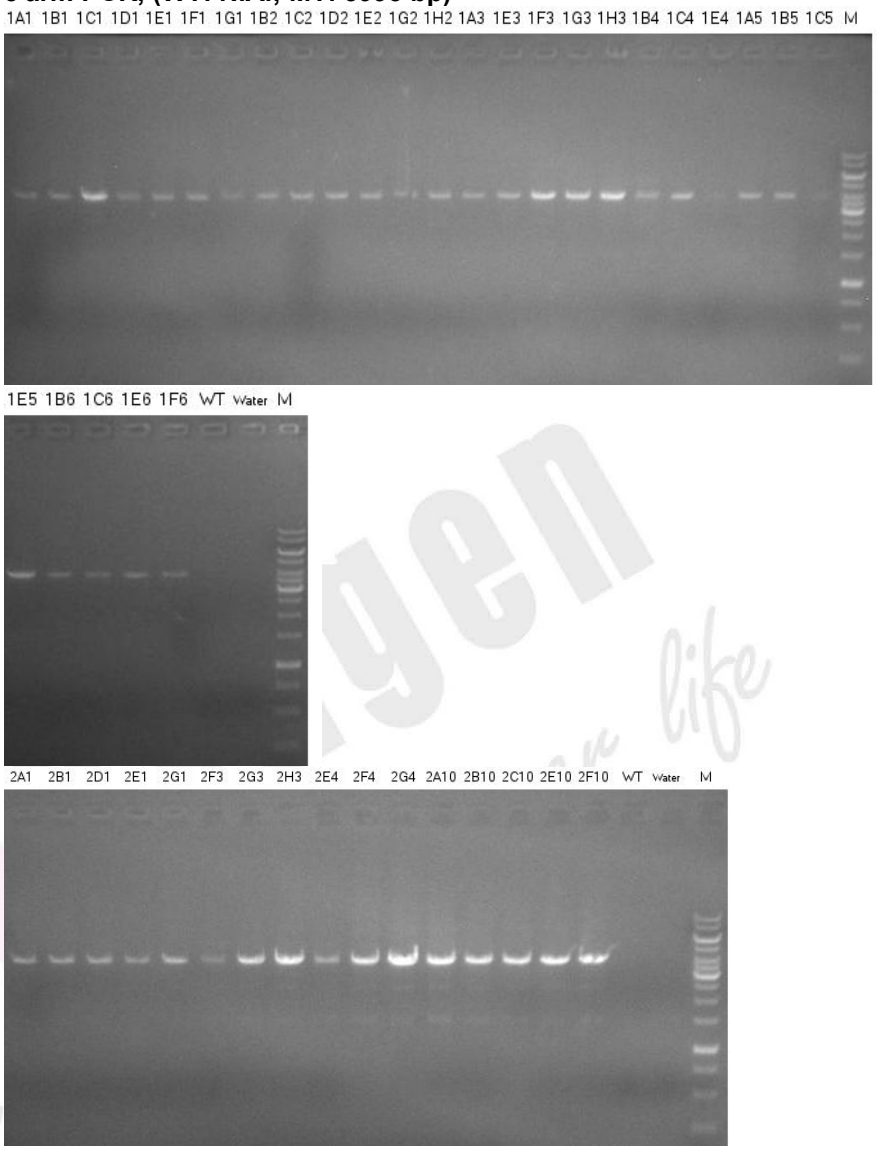
Step	Temp.	Time	Cycles
Initial denaturation	94 °C	3 min	
Denaturation	94 °C	30 s	
Annealing	60 °C	30 s	35 x
Extension	65 °C	200 s	
Additional extension	65 °C	10 min	

PCR Result

Marker



3'arm PCR, (WT: N.A.; MT: 3993 bp)



1.1.2 KI PCR

Primers for KI PCR:
F2: 5'-GGTTGTTGCAGCCTCTTGTGA-3'
R2: 5'-CGCCCCAACCTCATTGTGA-3'

Expected PCR Product:
Wildtype: N.A.
Targeted: 410 bp

Reaction Mix:

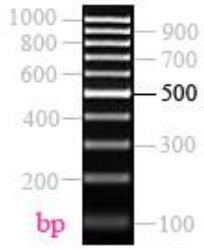
Component	x1
ES cell genomic DNA	1.5 µL
Forward primer(10 µM)	1 µL
Reverse primer(10 µM)	1 µL
P112 Taq DNA Polymerase	12.5 µL
ddH_2O	9 µL
Total	25 µL

Cycling Condition:

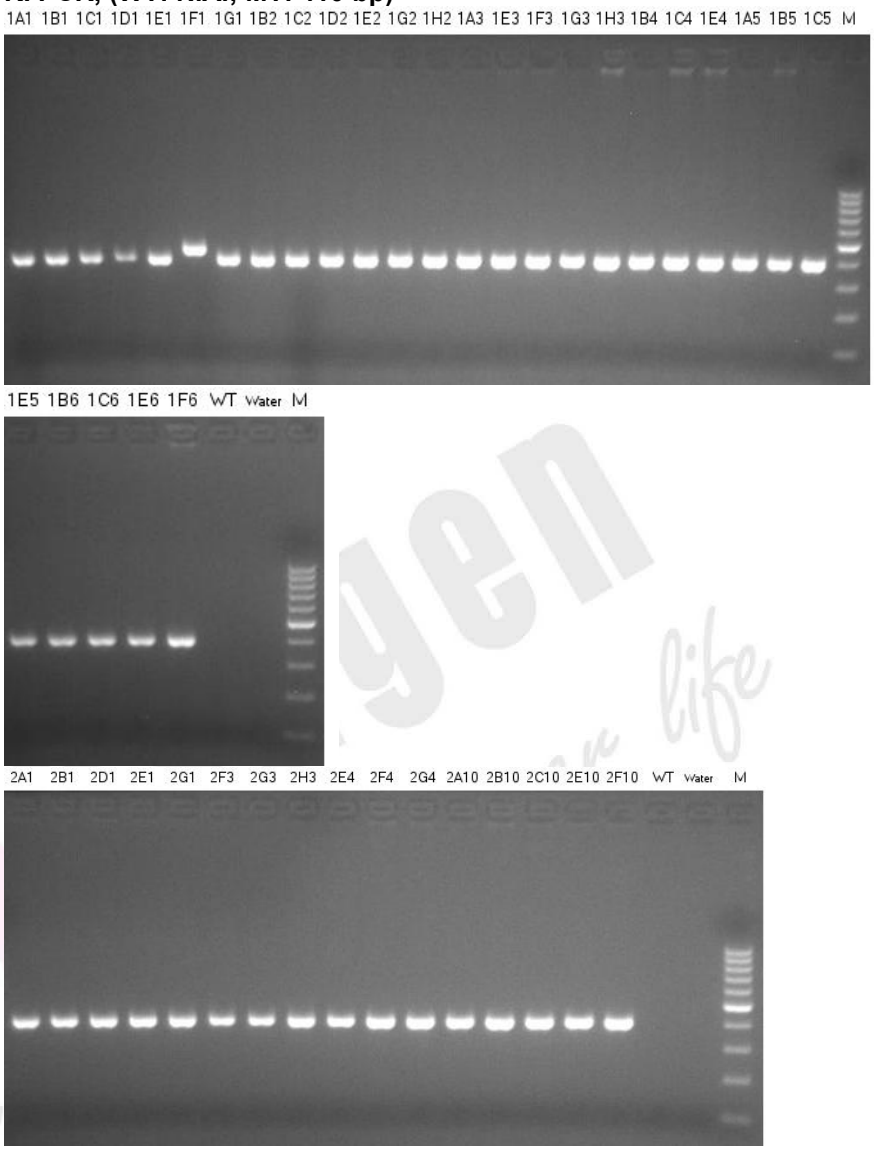
Step	Temp.	Time	Cycles
Initial denaturation	94 °C	3 min	
Denaturation	94 °C	30 s	
Annealing	62 °C	35 s	35 x
Extension	72 °C	35 s	
Additional extension	72 °C	5 min	
Storage temperature	25 °C		

PCR Result

Marker



KI PCR, (WT: N.A.; MT: 410 bp)



1.1.3 Neo-L PCR

Primers for Neo-L PCR:
F3: 5'-CTTAGACATGCTCCCAGCCGA-3'
R3: 5'-GGCCCACAACAGCACCATTG-3'

Expected PCR Product:
Wildtype: N.A.
Targeted: 493 bp

Reaction Mix:

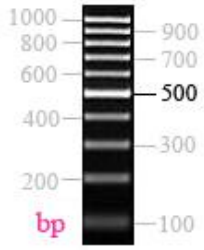
Component	x1
ES cell genomic DNA	1.5 μL
Forward primer(10 μM)	1 μL
Reverse primer(10 μM)	1 μL
P112 Taq DNA Polymerase	12.5 μL
ddH_2O	9 μL
Total	25 μL

Cycling Condition:

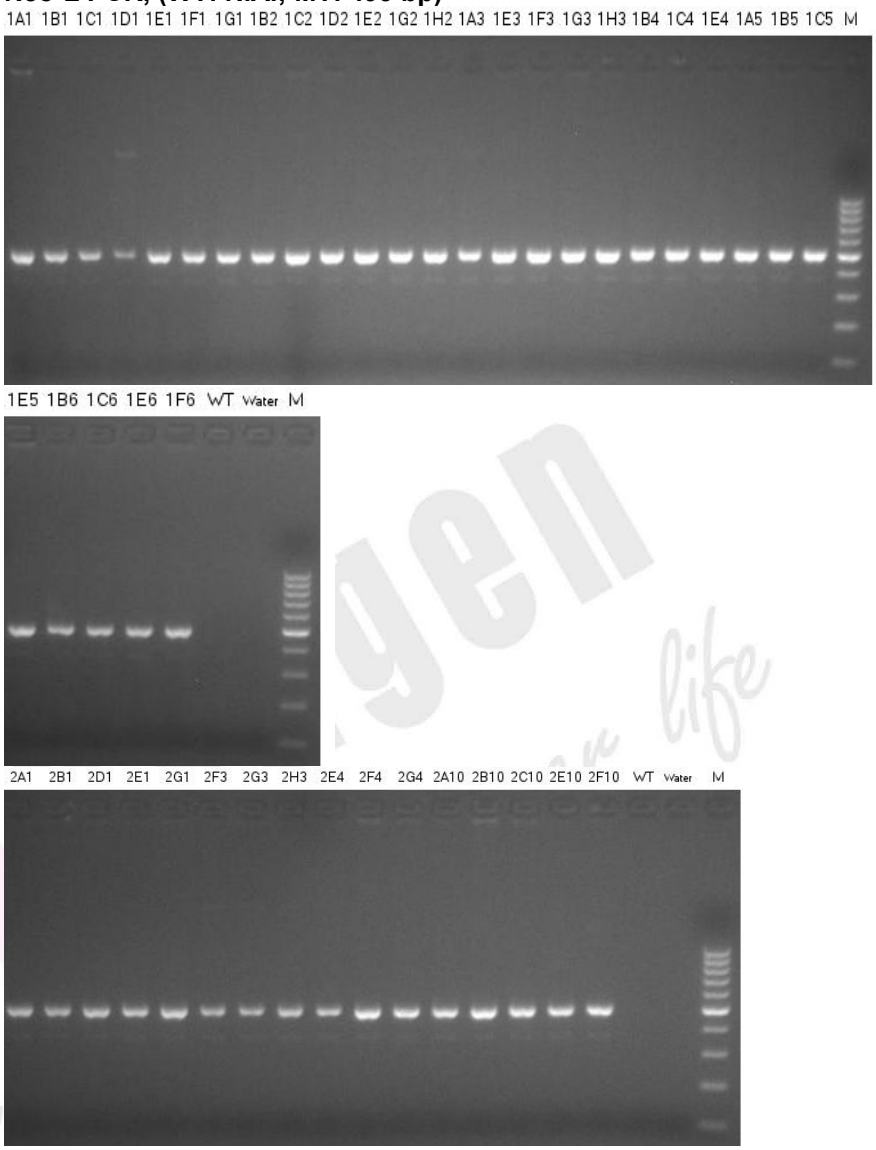
Step	Temp.	Time	Cycles
Initial denaturation	94 °C	3 min	
Denaturation	94 °C	30 s	
Annealing	62 °C	35 s	35 x
Extension	72 °C	35 s	
Additional extension	72 °C	5 min	
Storage temperature	25 °C		

PCR Result

Marker



Neo-L PCR, (WT: N.A.; MT: 493 bp)



1.1.4 Wildtype PCR

Primers for Wildtype PCR:
F4: 5'-GGTTGTTGCAGCCTCTTGTGAT-3'
R4: 5'-TGGTAAAACTACCCTGTTTCCTTTC-3'

Expected PCR Product:
Wildtype: 348 bp
Targeted: N.A.

Reaction Mix:

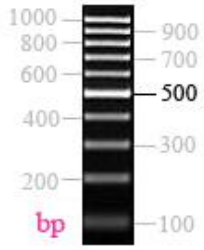
Component	x1
ES cell genomic DNA	1.5 μL
Forward primer(10 μM)	1 μL
Reverse primer(10 μM)	1 μL
P112 Taq DNA Polymerase	12.5 μL
ddH_2O	9 μL
Total	25 μL

Cycling Condition:

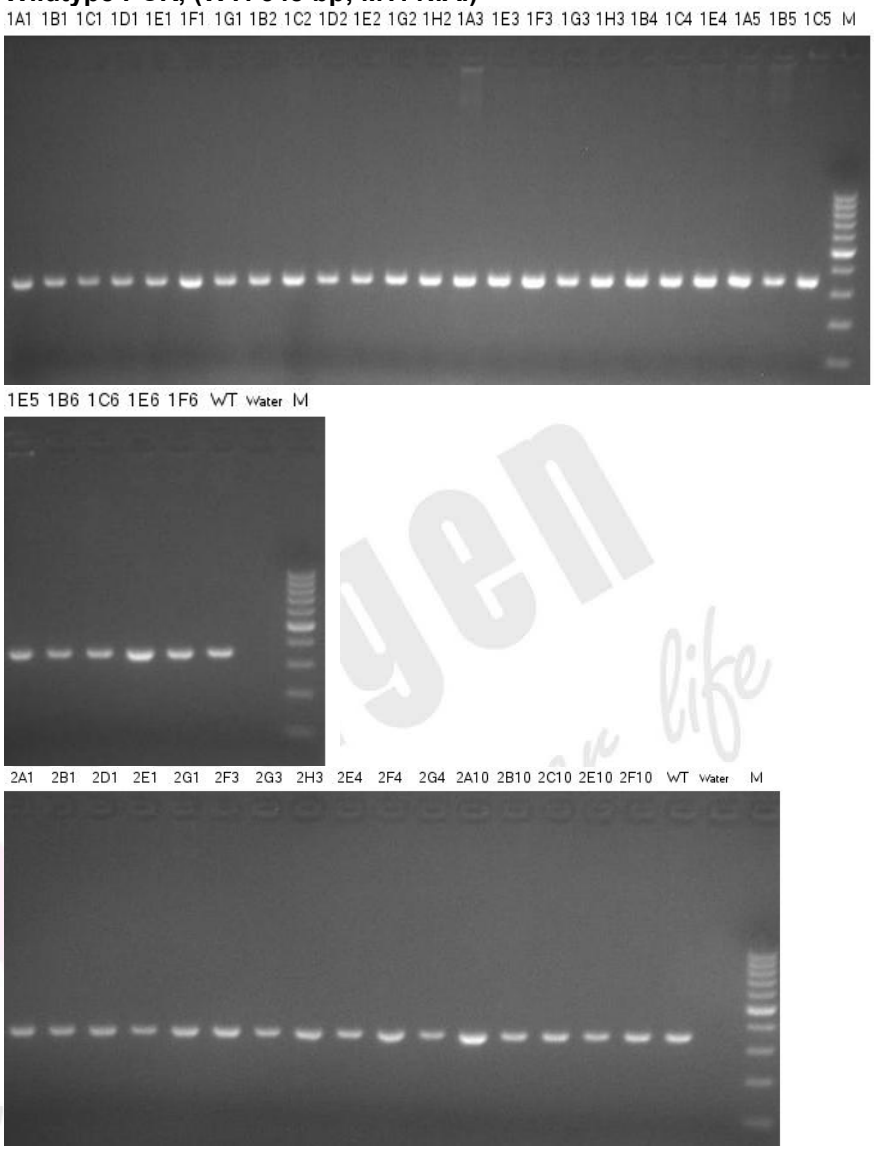
Step	Temp.	Time	Cycles
Initial denaturation	94 °C	3 min	
Denaturation	94 °C	30 s	
Annealing	62 °C	35 s	35 x
Extension	72 °C	35 s	
Additional extension	72 °C	5 min	
Storage temperature	25 °C		

PCR Result

Marker



Wildtype PCR, (WT: 348 bp; MT: N.A.)



1.2. Result

Samples 1A1, 1B1, 1C1, 1E1, 1G1, 1B2, 1C2, 1D2, 1E2, 1G2, 1H2, 1A3, 1E3, 1F3, 1G3, 1H3, 1B4, 1C4, 1A5, 1B5, 1E5, 1B6, 1C6, 1E6, 1F6, 2A1, 2B1, 2D1, 2E1, 2G1, 2F3, 2G3, 2H3, 2E4, 2F4, 2G4, 2A10, 2B10, 2C10, 2E10 and 2F10 were identified positive by PCR and confirmed as potentially targeted ES clones.

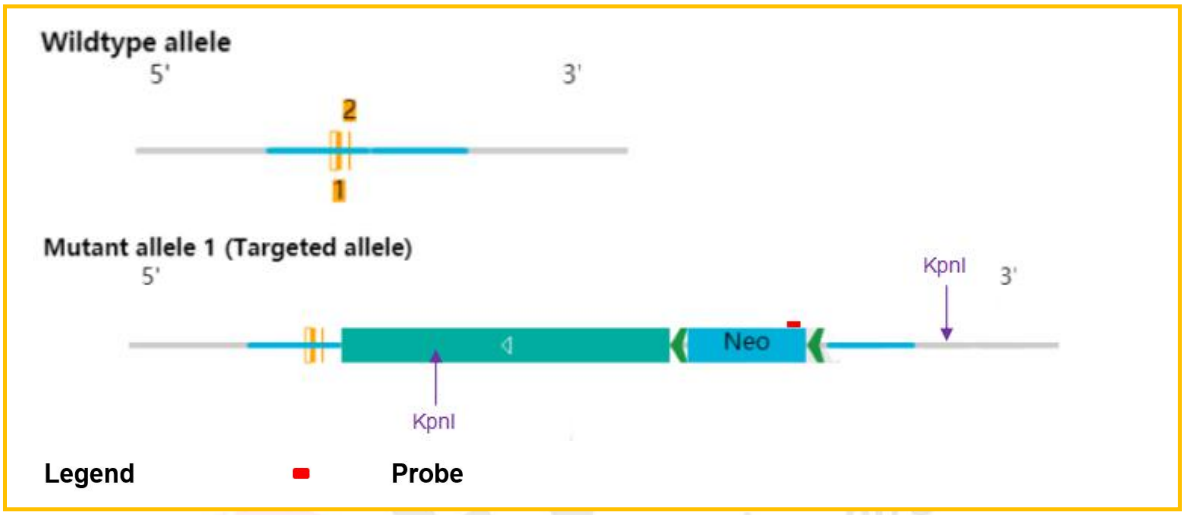
1.3. Southern Blot Analysis

The positive clones (1B4, 1C2, 1D2, 1E3, 1G2 and 1H3) from PCR screening was expanded and further characterized by Southern blot analysis. The Southern strategy is shown in the diagram below.

1.3.1 Neo or puro probe

The genomic DNA was digested with KpnI, and hybridized using a Neo probe. The Neo probe is expected to detect the following DNA fragment from targeted allele in the Southern analysis: ~17.54 kb (with KpnI digestion).

Diagram:
Regions in the following diagram were selected for Southern blot.



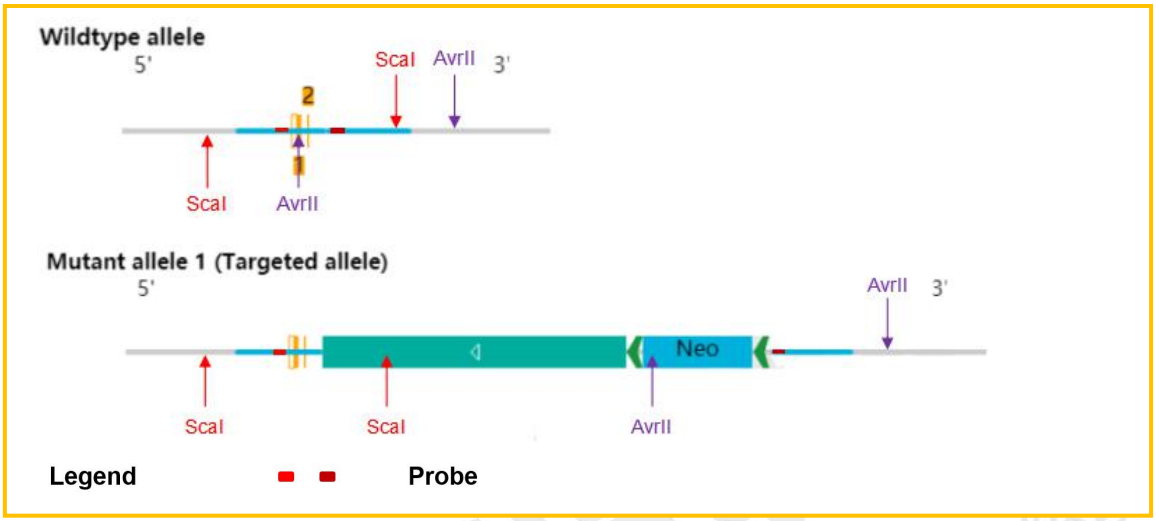
Expected Fragment Sizes for Southern Blot:
Neo Probe (containing 3'arm)-17.54 kb-KpnI

1.3.2 5'arm or 3'arm probe

The genomic DNA was digested with either ScaI or AvrII, and hybridized using a 5'arm or 3'arm probe. The 5'arm or 3'arm probe is expected to detect the following DNA fragment from targeted allele in the Southern analysis: ~8.83 kb-WT, 11.48 kb-MT (with ScaI digestion) and ~7.68 kb-WT, 10.08 kb-MT (with AvrII digestion).

Diagram:

Regions in the following diagram were selected for Southern blot.



Expected Fragment Sizes for Southern Blot:

5'arm Probe-ScaI: 8.83 kb-WT, 11.48 kb-MT

3'arm Probe-AvrII: 7.68 kb-WT, 10.08 kb-MT

Primers for 5'arm Probe:

5'arm-Probe-F: 5'-CAGGGATAACCTTTGTGAGGGACTGTT-3'

5'arm-Probe-R: 5'-AAGTTCCCAGAATCGGTGCTCCTT-3'

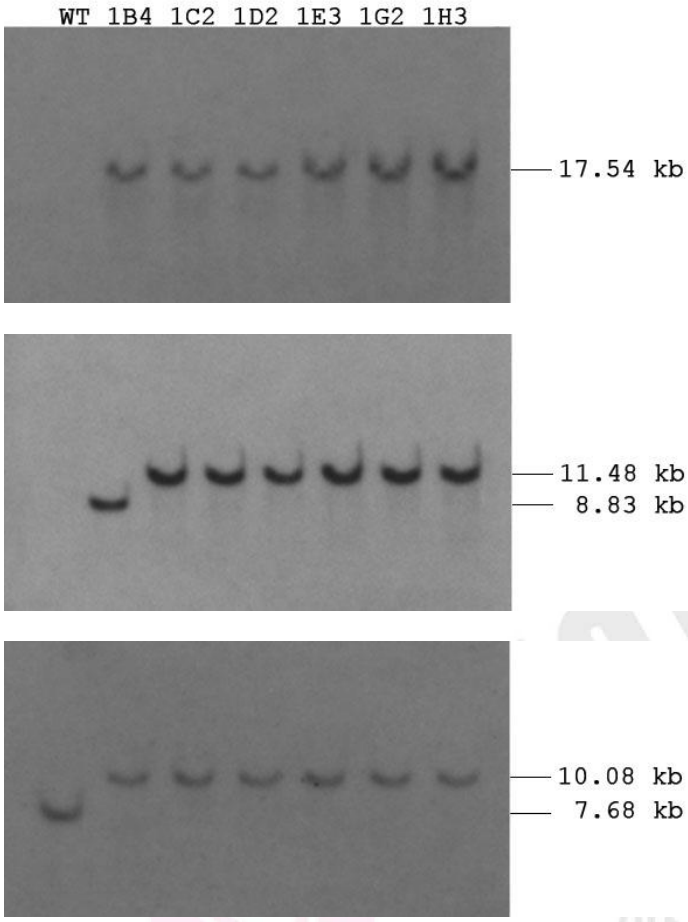
Primers for 3'arm Probe:

3'arm-Probe-F: 5'-GAAATAGAGGGAGGAGAGGGGAAATG-3'

3'arm-Probe-R: 5'-GGGAATACTTTTCATTAACCTGTACTTGCT-3'

1.4. Result

All of the six ES clones (1B4, 1C2, 1D2, 1E3, 1G2 and 1H3) were confirmed correct by Southern blot analysis.



Neo_3'arm (17.54 kb; KpnI)

5'arm (8.83 kb-WT, 11.48 kb-MT; ScaI)

3'arm (7.68 kb-WT, 10.08 kb-MT; AvrII)