D50R genotyping protocol

DNA extraction from tails

- Add 0.5ml tail buffer containing proteinase K to tube with tail.

Tail buffer: 100mM tris pH8
5mM EDTA
0.2% SDS
200mM NaCl
100µg/ml Proteinase K

- Incubate o/n at 50C
- Flick to mix. Centrifuge at 13K (bench top centrifuge) for 20mins
- Remove supernatant to fresh tube
- Add 0.5ml of phenol:chlorophorm:isoamylalcohol
- Flick to mix, centrifuge at 13K for 10min
- Transfer aqueous top layer to new tube
- Add 0.5ml of isopropanol, flick to mix and precipitate DNA
- Centrifuge for 30mins at 13K
- Remove wash (check to see pellet in tube and try not to suck up pellet)
- Add 0.5ml 70% ethanol, flick to wash
- Centrifuge at 13K for 30mins
- Remove supernatant and air dry for 10mins
- Add 40µl of TE
- Keep in fridge use 1 or 2µl in PCR

PCR

The standard PCR reaction mix was set up as shown in Table 2.1. DNA amplification was carried out in a PTC-100 thermal cycler (MJ Research, Inc) using the standard conditions shown in Table 2.2.

Reagent	Volume
10x buffer (Bioline)	5 µl
Primer 1 (100ng/µl)	2.5 µl
Primer 2 (100ng/µl)	2.5 µl
dNTPs (10mM, Amersham)	1 µl
taq (Bioline,5U/µl)	0.5 µl
DNA (1µg genomic DNA)	1 µl
H_2O	37.5 µl

Table 2.1. Standard PCR reaction mix.

PCR primers

Primer 1	**intron 4aR1**	**ACTATGAAGTCCAGAGTGCC**
Primer 2	**intron 2F7**	**GCATTGAATCCCCAGCATCA**

Step	Condition
1	95°C for 5 minutes
2	95°C for 1 minute
3	61°C for 1 minute
4	72°C for 3 minutes
5	Go to step 2 x30
6	72°C for 10 minutes

Table 2.2. Standard PCR conditions

Following PCR, 25ul PCR reaction was digested by StuI restriction enzyme digest using standard conditions at 37C for 2 hrs before analysis by agarose gel electrophoresis on a 1.2% gel.

The expected wild type (WT) allele PCR product size was 1470 bp whilst the mutant PCR product sizes were 1133 bp and 334 bp after StuI digestion

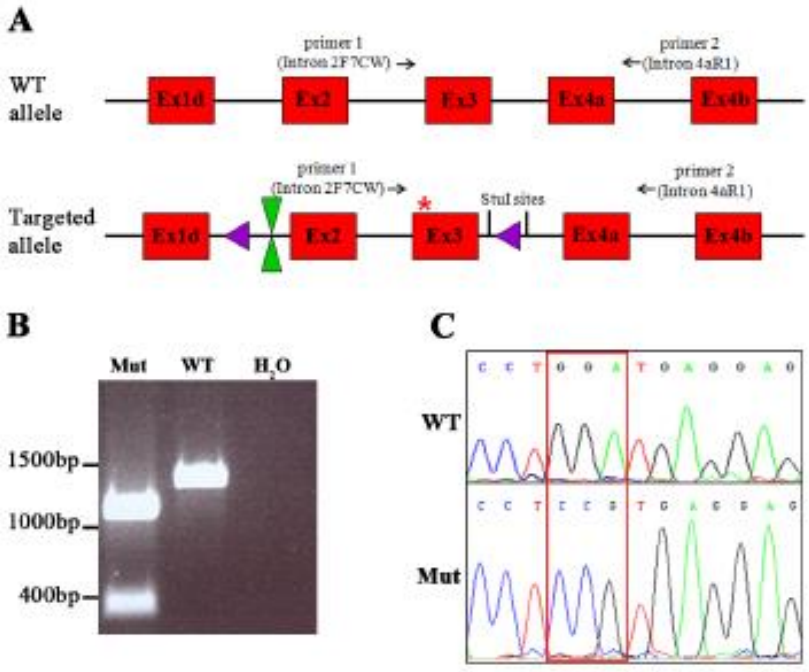

Figure 5.3. Confirmation of the Nbr1^{D50R} knock-in mouse genotype by genomic PCR and sequencing.
A. Schematic diagram showing the location of the PCR primers and StuI restriction enzyme sites used for genotyping.
B. Agarose gel electrophoresis separation of PCR product. The PCR product spans from intron 2 to intron 4a and the expected WT PCR product size is 1470bp. The mutant allele contains two introduced StuI restriction sites either side of the second LoxP site, therefore, on restriction enzyme digest, the mutant PCR product is cut into two sized bands of 1133bp and 334bp. The LoxP site is 40bp so cannot be visualised on this gel.
C. Electropherogram showing the correct mutation is present in the D50R knock-in mouse compared with WT (red box).
WT: Wild type; Mut: Nbr1^{D50R} mutant.