Table of contents

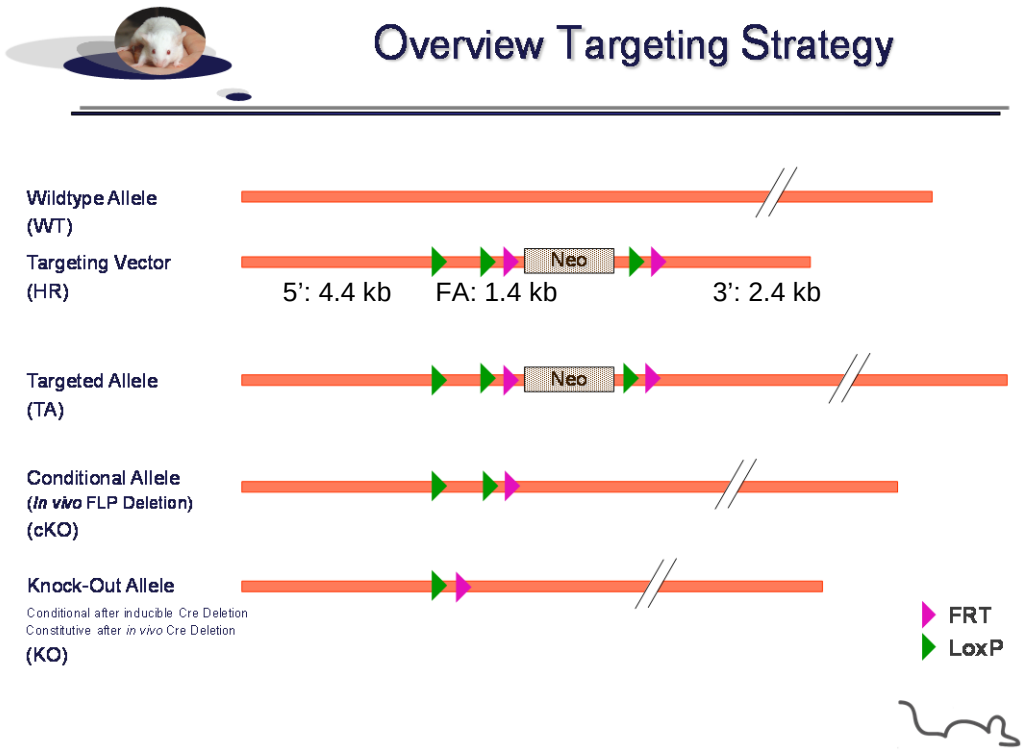
For any question, please contact:

Mouse Clinical Institute – Institut Clinique de la Souris (ICS)
1 rue Laurent Fries, BP 10142
67404 Illkirch Cedex France
Email: ics@igbmc.fr
Web site: http://www.phenomin.fr/en-us//

This protocol has been prepared by Claudia Caradec, Engineer
This protocol has been validated by Sylvie Jacquot, Ph.D., Project Manager

1. Schematic representation of the locus

1.1. Overview

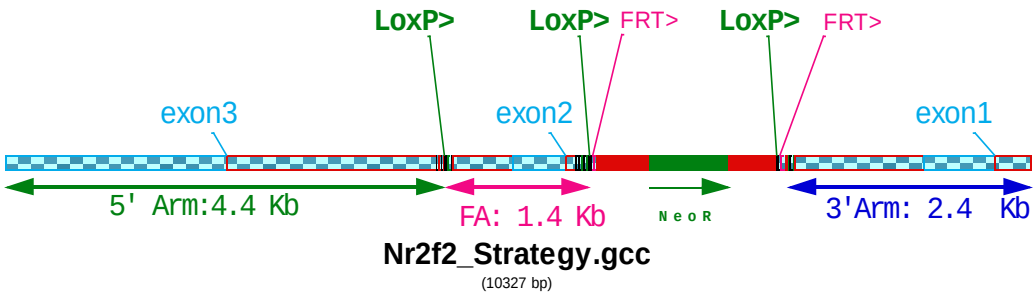


Legend:
5': 5' homology arm; FA: floxed fragment; 3': 3' homology arm
This schematic representation is not on scale

1.2. Strategy chosen: flox of exon 2

Nr2f2 gene (also named COUP-TF2) is a member of the nuclear receptor family. Additional information on this gene can be accessed at:
http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=markerDetail&key=45348

Strategy used to generate the conditional knock out model

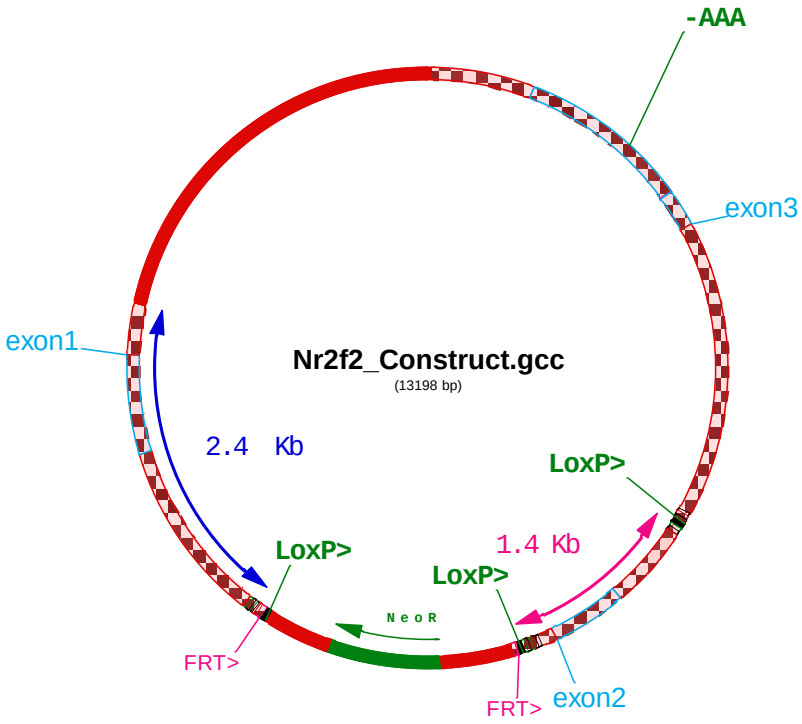


2. Construct used for homologous recombination in ES cells: Nr2f2 project

2.1. Legend

loxP sites are indicated in green ; FRT sites are indicated in purple; *Mus musculus* sequences are indicated in uppercase ; exogenous sequences are marked in lowercase.
The targeting vector was generated in 129Sv/Pas and was not fully sequenced. Regions sequenced are indicated in bolds and correspond to exon 1, 2 and 3 regions.

2.2. Map of targeting vector plasmid



The sequencing of the exons from the targeting vector shows 1 mutation.

- The missing AAA sequence is located in the 3' UTR region of Nr2f2 gene. The encoded Nr2f2 protein should thus be identical to the wild type Nr2f2 protein.

2.3. 5' homology arm (4.4 kb)

CTCCAATGGTAAAGATTCCCTTGACCTGTGGCCATGCTCTCAACAGACTGAAGAGGGAGGGGGAGGGGAAAGGCA
GGGCAACTGCAAGTTATCATGTATATACAAGAAAAACAGACCCTTCGTCGCTAGCAGCTACATCACTGTATTCAA
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AAACCATAGCTAAACCATTAACTTCAGCTCCCCCCCCACACCACACCCAAATTAAACAGGCAAATACACTAGTCT
ACCTTTGAACAAACTGATTGAGGGAGTAAACAAATGGGGCC**CCCGCTGGCTCAGCGAGAACTCACAGGGGCTCAG**
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2.4. Floxed fragment (1.4 kb)

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CCATTCTGACAGCACCCCCCACCTCCTGACGGTCTGCATTATTTTTAATGAGATTGTGATTTTA**AAAATTGCTGC**
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2.5. PGK-Neo region

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2.6. 3' homology arm (2.4 kb)

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2.7. Vector backbone sequence

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ctcactaaagggaacaaaagctggagctcgcggccgcggcgcgcc

3. ES cell lines targeted and validation data:

3.1. ES cell lines targeted

The targeting vector was electroporated in P1 ES cells [MCI-129Sv/Pas background]

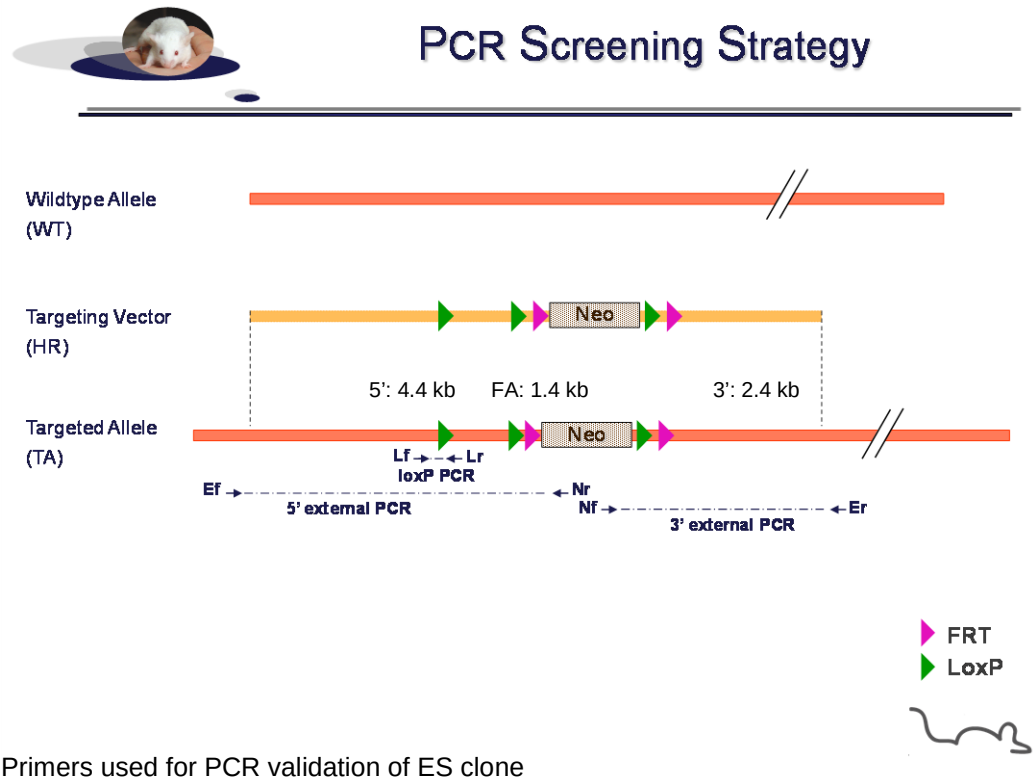
Number of clones screened: 372
Number of positive: 1

Reference of clone used to generate the mouse line:

- clone **K194-152**

3.2. PCR data on positive clone

3.2.1.PCR screening strategy

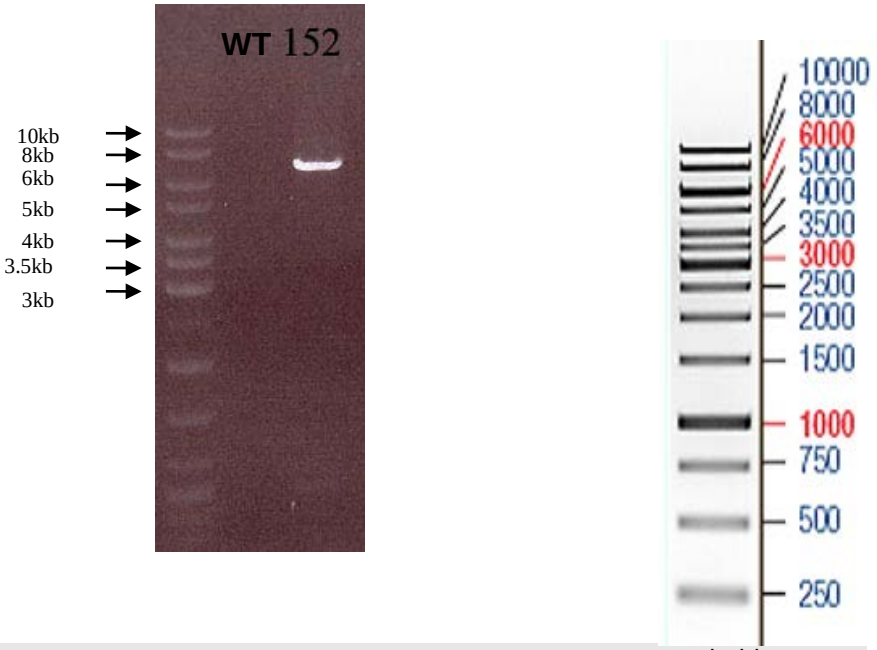


Primers used for PCR validation of ES clone

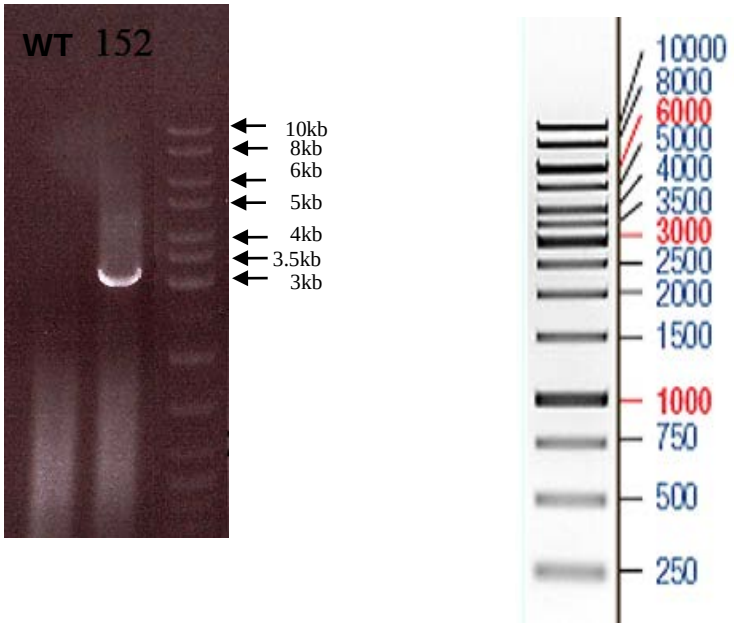
PCR	Primer Name	Primer sequences	PCR product size
5' external	Ef	CCCAGCAGTGAGCTCTTCCCAAACC	6.80 kb
	Nr	GCGGCCGGAGAACCTGCGTGCAATC	
loxP	Lf	AGACCCTCAGACCCAGCCCAAGTGG	0.25 kb WT 0.30 kb cKO
	Lr	CTCCAGGCAGCTCCAACCTGAGAGC	
3' external	Nf	AGGGGCTCGCGCCAGCCGAACTGTT	3.40 kb
	Er	TCCCAGTCCTCCTGATTTCGATGGC	

3.2.1.Picture of PCR on positive clone

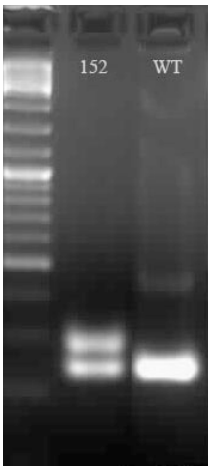
5' external PCR ladder



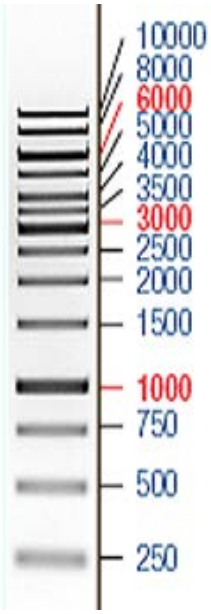
3' external PCR ladder



LoxP PCR



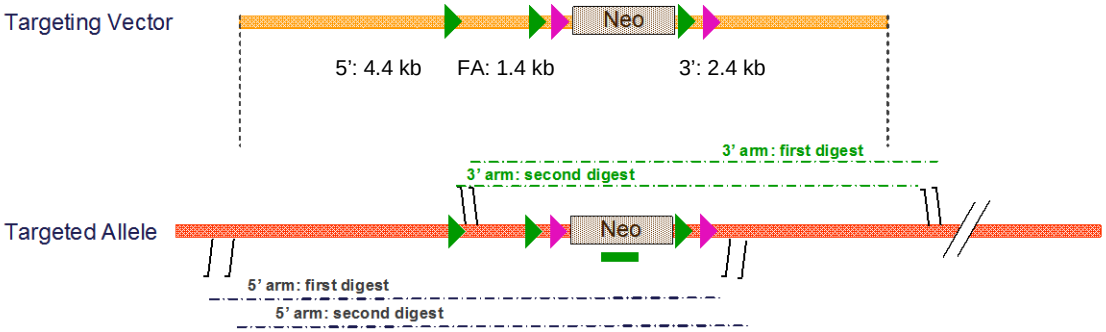
ladder



3.3. Southern data on positive clone

3.3.1.Neo Southern strategy

Southern Screening Strategy



Neo probe

FRT

LoxP

Digestions used to validate the 5' and 3' insertion

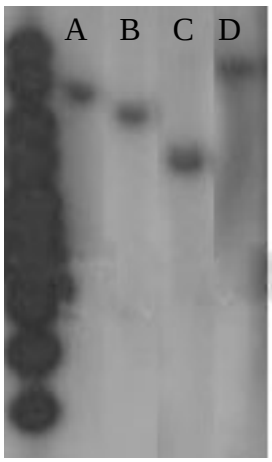
Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
Neo	5' arm first digest	Sac I	/	8.5
	5' second digest	BamHI	/	7.3
	3' arm first digest	Hinc II	/	5.7
	3' arm second digest	Hind III	/	10.5

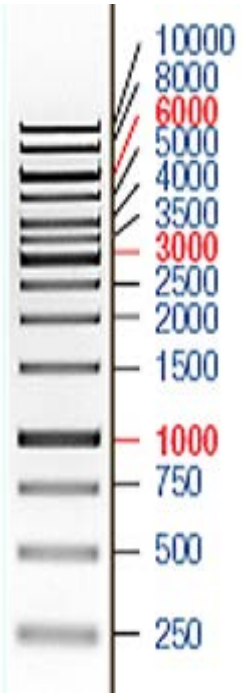
Four different digests are used to validate correct HR event. Two digests validate the 5' insertion, 2 other digests validate the 3' insertion

3.3.2.Picture of Neo Southern

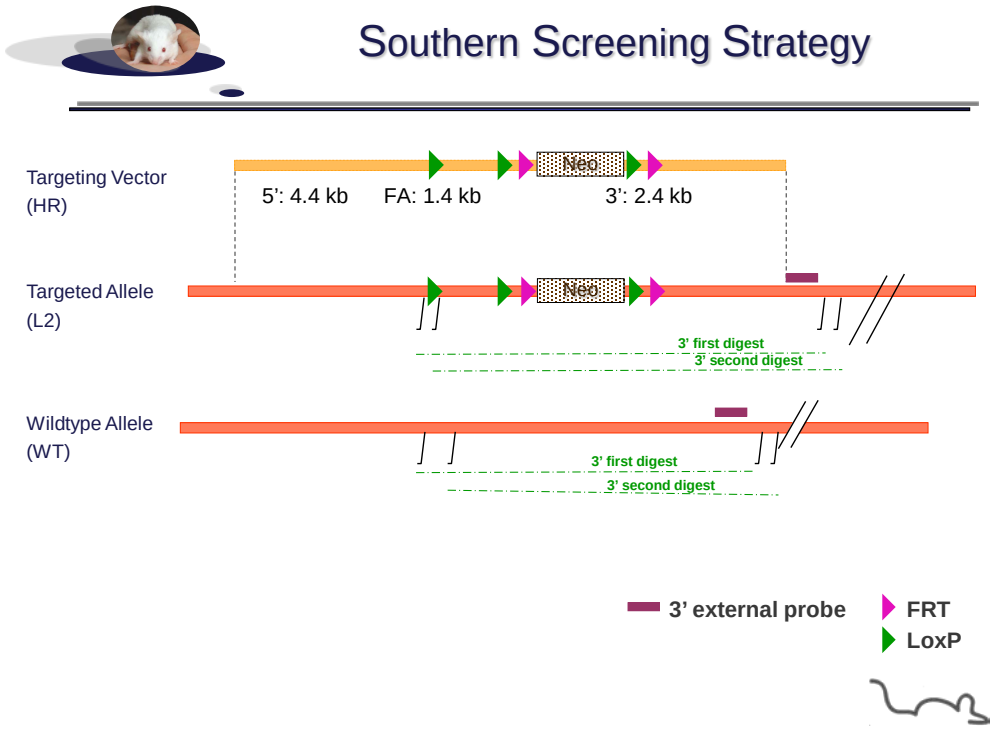
Neo southern blot: 5' and 3'arm validation ladder

A: SacI 8.5Kb
B: BamHI 7.3Kb
C: HincII 5.7Kb
D: HindIII 10.5Kb





3.3.3.External probes Southern



Digestions used to validate with 3' probe

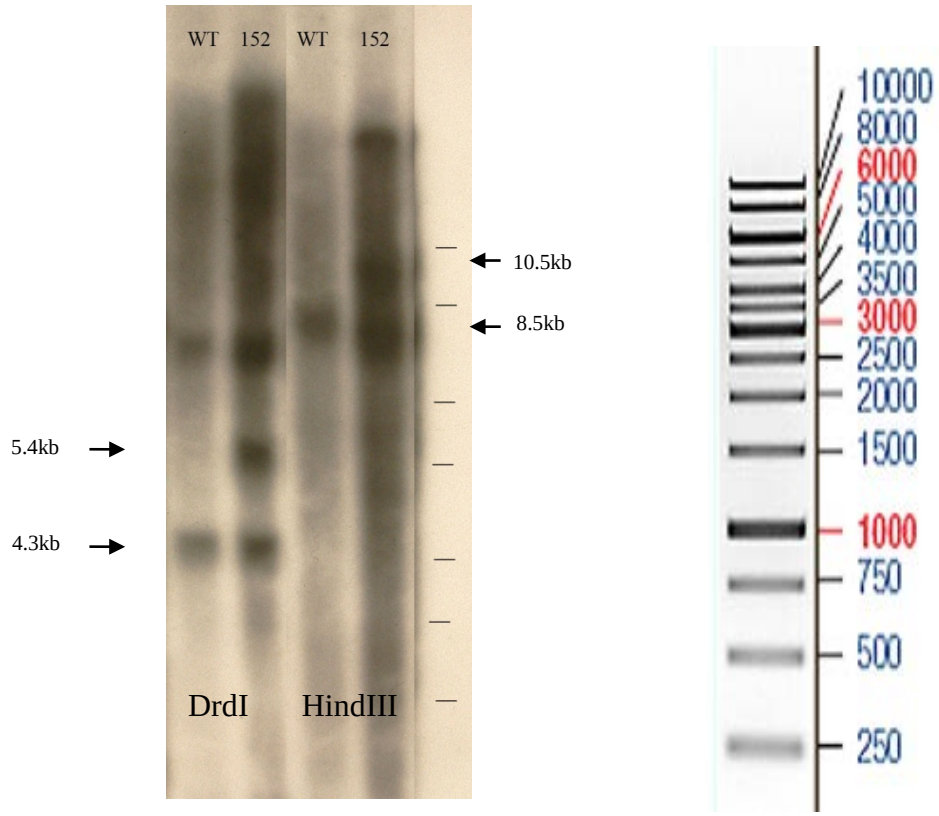
Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
3' external	first digest	Drd I	4.3	5.4
	second digest	Hind III	8.5	10.5

Primers for probe synthesis:

3' probe
ACACACCGCGGAGAGAAAAGAGCAG
GCTGTCTCGACGCCGCTCGCGCTAG

3.3.4.Picture of Southern with external 3' probe

3' external probe Ladder



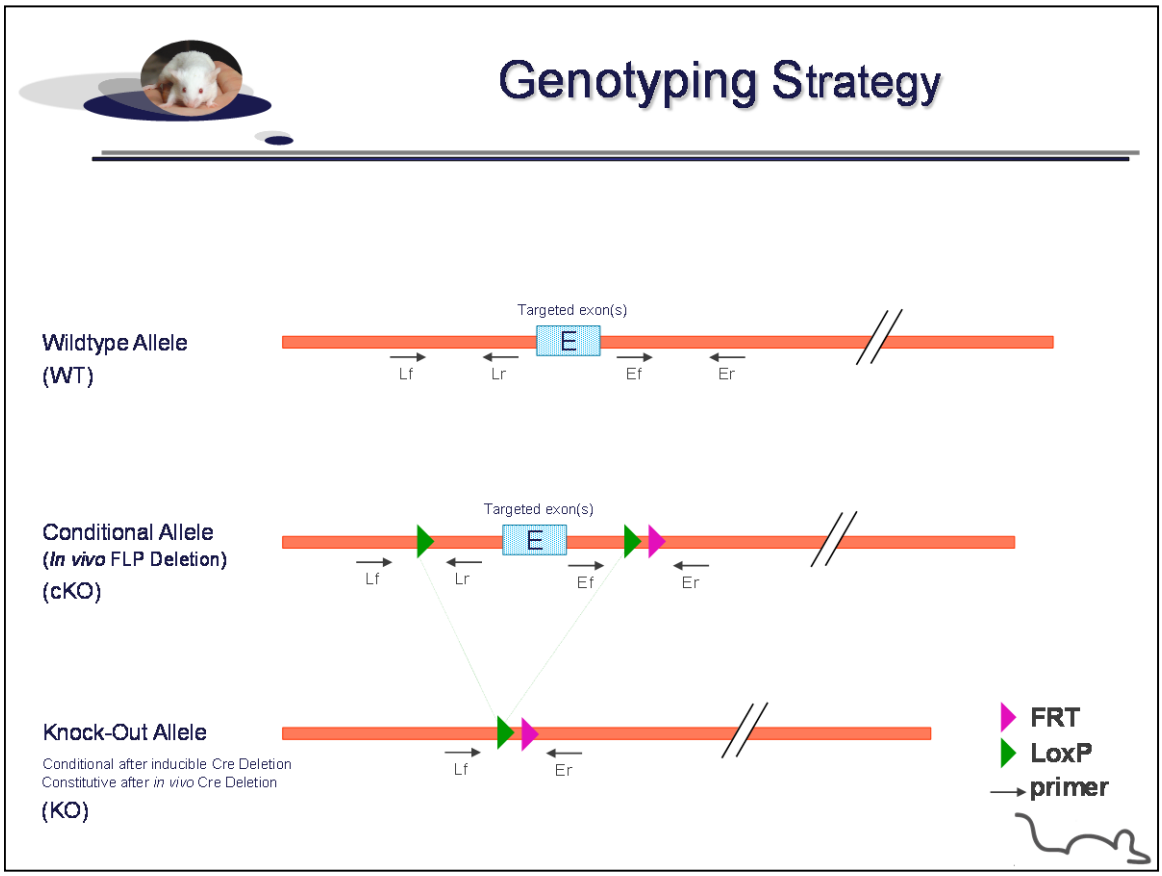
4. Data on conditional and knock-out animals

Both conditional and knock-out mouse models were backcrossed in C57BL/6J background.

4.1. Genotyping protocol and data

4.1.1. Genotyping strategy

The map below describes the position of the primers used for genotyping for each possible allele.



Sequence of primers used for genotyping

Position	Primers	Sequence
Ef	2349	GCAACCACTCCTCACAAATCACTTCC
Er	2351	CCAGACCAAGACTCTGTCTCTCG
Lf	2346	GCACACACTTTGGGTTGCTTCAAAATTGC
Lr	2347	CTGCGAAGTCAGTAGGAGTTGGG

PCR fragments expected size (bp):

Region analyzed	**Primers used**	Position on the primer (see the map above)	Conditional allele (cKO)	Knock-Out allele (KO)	WT allele (WT)
Presence of the distal loxP	2346-2347	Lf / Lr	294	---	244
Excision of the selection marker(Neo excision)	2349-2351	Ef / Er	535	---	396
Excision of the floxed exon(s), i.e. knock out	2346-2351	Lf / Er	2000*	531	1811*

* This PCR product will not be observed using our PCR genotyping conditions (see description below)
--- No Amplicon should be obtained

4.2. PCR protocol

This section describes the composition of the mix and cycling conditions used for genotyping.

Reagents:		Volume:
-10x Buffer (Roche)		2.5µl
-dNTPs 10mM (Amersham Biosciences)		0.5µl
-Taq DNA Polymerase (Roche)	0.2µl	
-DNA (50ng/µl)	3µl	
-5' primer (100 □M)		0.125µl
-3' primer (100 □M)		0.125µl
-Sterile H2O		up to 25 µl

Cycling conditions:

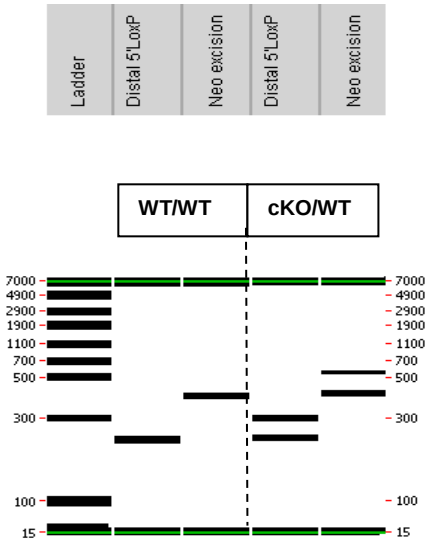
Temp	Time	#Cycles
94°C	3min	1
94°C	1min	
62°C	1min	2
72°C	1min	
94°C	30s	
62°C	30s	30
72°C	30s	
72°C	3min	1
4°C	∞	

NB: These PCR conditions have been optimized for high-throughput genotyping. Adaptation to small-scale may be required.

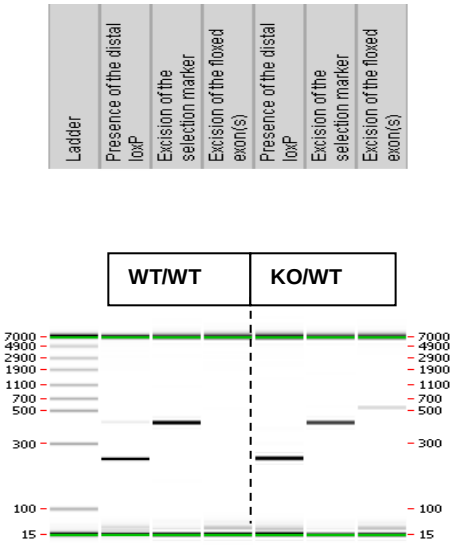
4.3. Picture of genotyping with various alleles

Analysis of PCR products pattern was not done by gel electrophoresis but using LabChip® 90 microfluidic apparatus. PCR products were run on the HT DNA 5K LabChip® 90 Assay Kit.

- Picture of genotyping with conditional knock-out (cKO) allele



- Picture of genotyping with knock-out (KO) allele



Note that as this technology is more sensitive than gel analysis, non specific signals and/or primer dimers may be visible on the picture.