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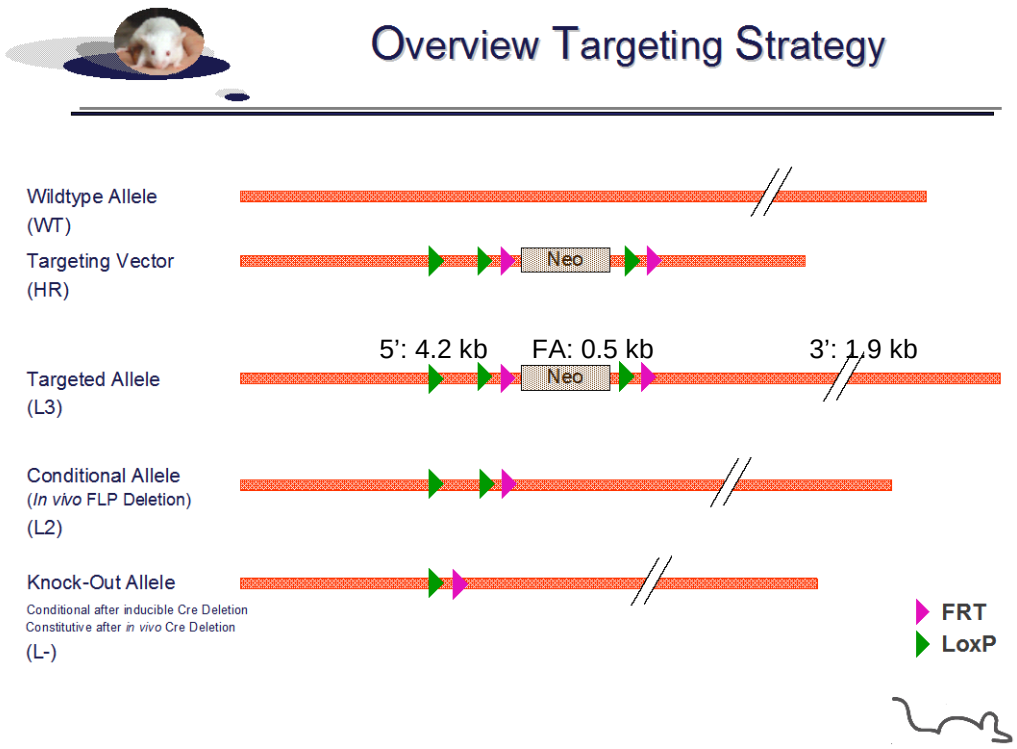
This protocol has been prepared by Claudia Caradec, Scientific Report Editor
This protocol has been validated by Sylvie Jacquot, Ph.D., Project Manager

1. Schematic representation of the locus

1.1. Overview

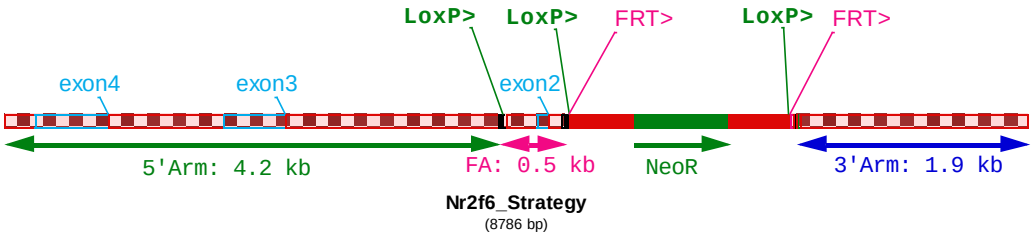


Legend:
5': 5' homology arm; FA: floxed fragment; 3': 3' homology arm
This schematic representation is not on scale

1.2. Strategy chosen: flox of exon 2

Nr2f6 gene (also named EAR2) 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=45349

Strategy used to generate the conditional knock out model



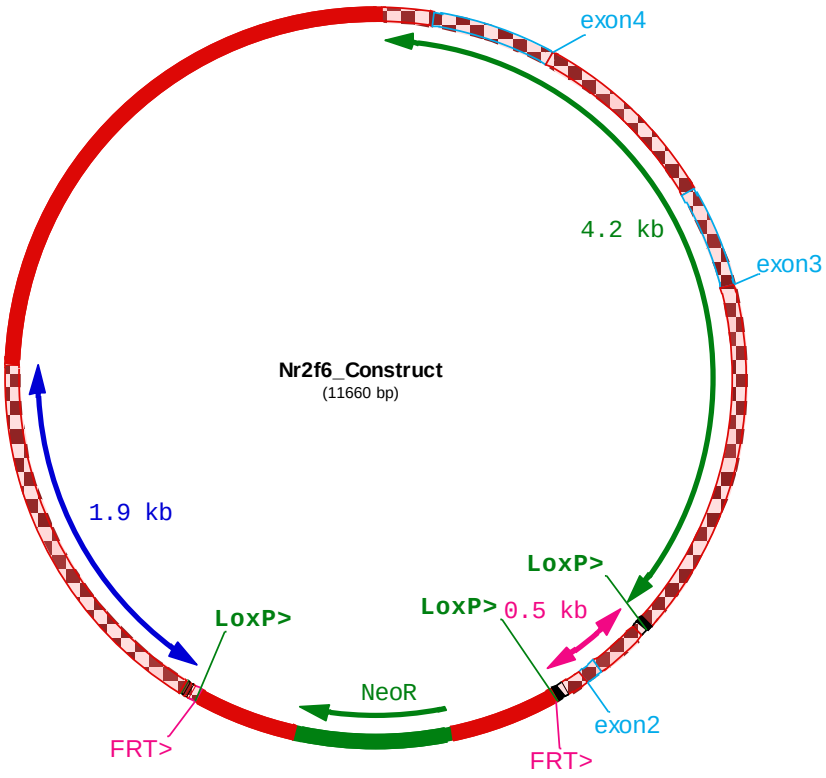
Nr2f6_Strategy
(8786 bp)

2. Construct used for homologous recombination in ES cells: Nr2f6 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.

2.2. Map of targeting vector plasmid



2.3. 5' homology arm (4.2 kb)

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AGGCGAGTCTCCCACCCTACTGGGACCTACAGAGCACAGACTAGACCCTGGGGTTACTTTCCCAATACTGCAGAA
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2.4. Floxed fragment (0.5 kb)

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GGCCAGGGTTCCCAGTGATGTGCCTGCAAACCCCTGGGAAATACCAGTGGGGGATCCCTGACCTGTGATGGGAGC
CCCAAGAGTACATGGTGAGGACTCAGTTCCCTAGCTTGGAAGGAGCAcaccggtg**ataacttcgtataatgtatg
ctatacgaagttat**

2.5. PGK-Neo region

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2.6. 3' homology arm (1.9 kb)

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2.7. Vector backbone sequence

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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]

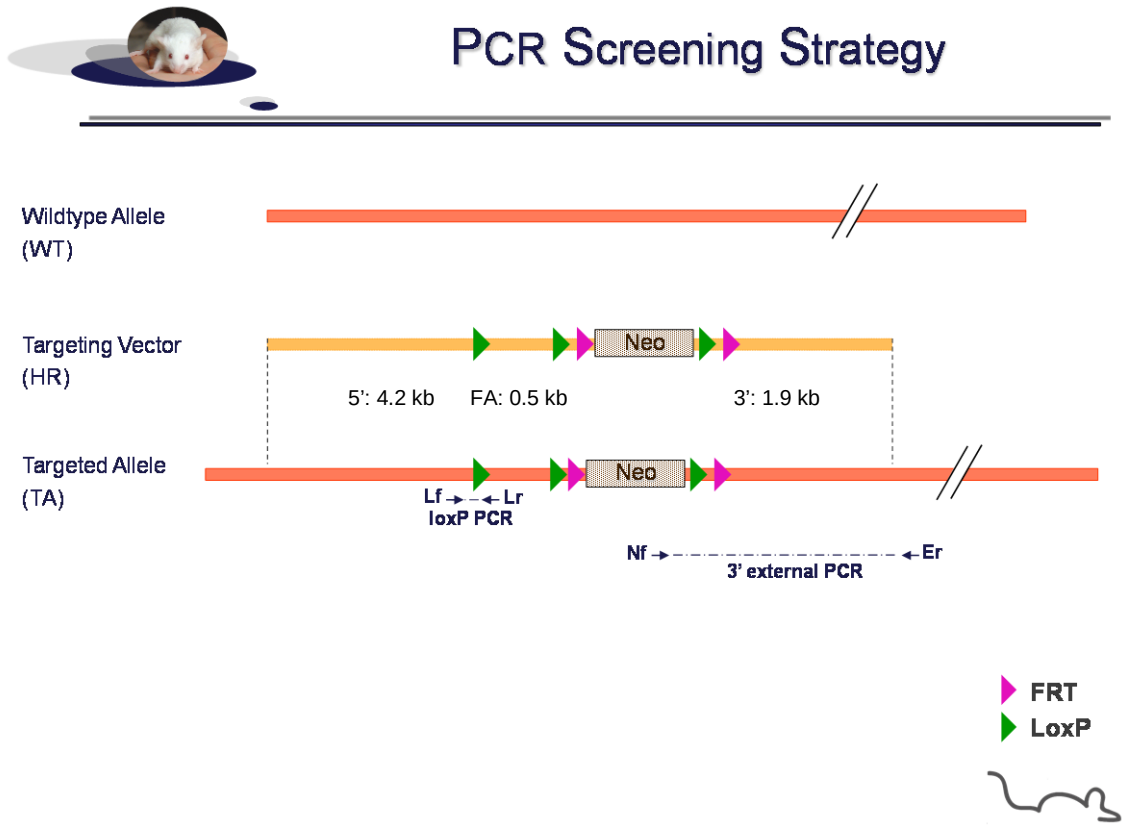
Number of clones screened: 400
Number of positive: 4

Reference of clone used to generate the mouse line:

- clone **K178P1-265**

3.2. PCR data on positive clone

3.2.1.PCR screening strategy

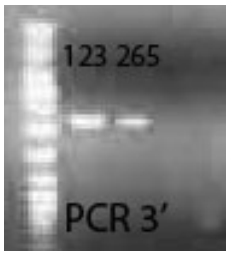


Primers used for PCR validation of ES clone

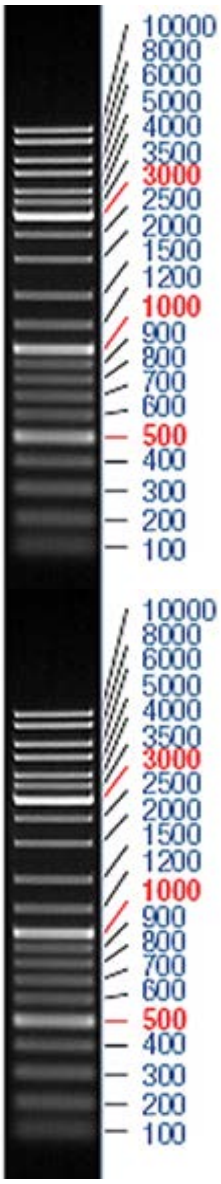
PCR	Primer Name	Primer sequences	PCR product size
loxP	Lf	TGCACTTGACTGTAAGGATCTGAGG	0.22 kb WT 0.27 kb TA
	Lr	TCCTTGATGACCTCTCCCGATCTCC	
3' external	Nf	AGGGGCTCGCGCCAGCCGAACTGTT	3.0 kb
	Er	GGGGCCCGTCACGCGTGGTGCCTCG	

3.2.2.Picture of PCR on positive clone

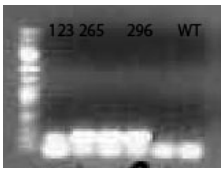
3' external PCR



ladder

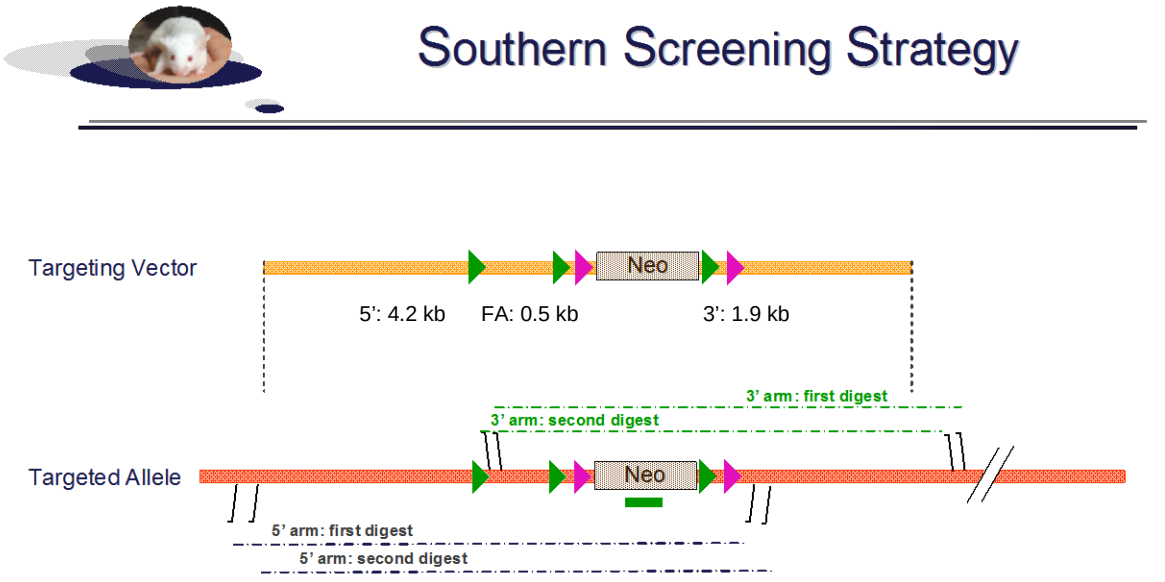


LoxP PCR



3.3. Southern data on positive clone

3.3.1.Neo Southern strategy



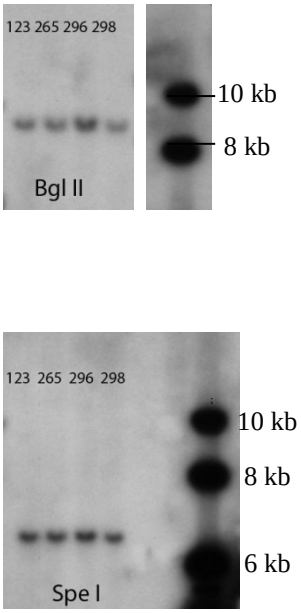
Digestions used to validate the 5' and 3' insertion

Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
Neo	5' arm first digest	Bgl II	/	8.5
	3' arm first digest	Spe I	/	6.3

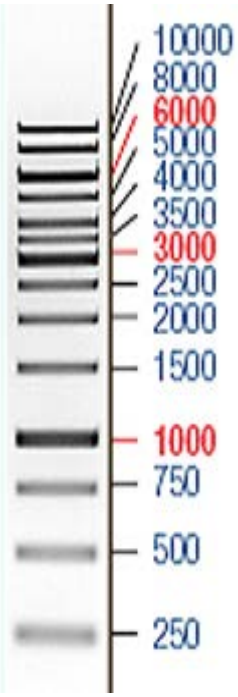
Two different digests are used to validate correct HR event. One digest validates the 5' insertion one other digest validates the 3' insertion

3.3.2.Picture of Neo Southern

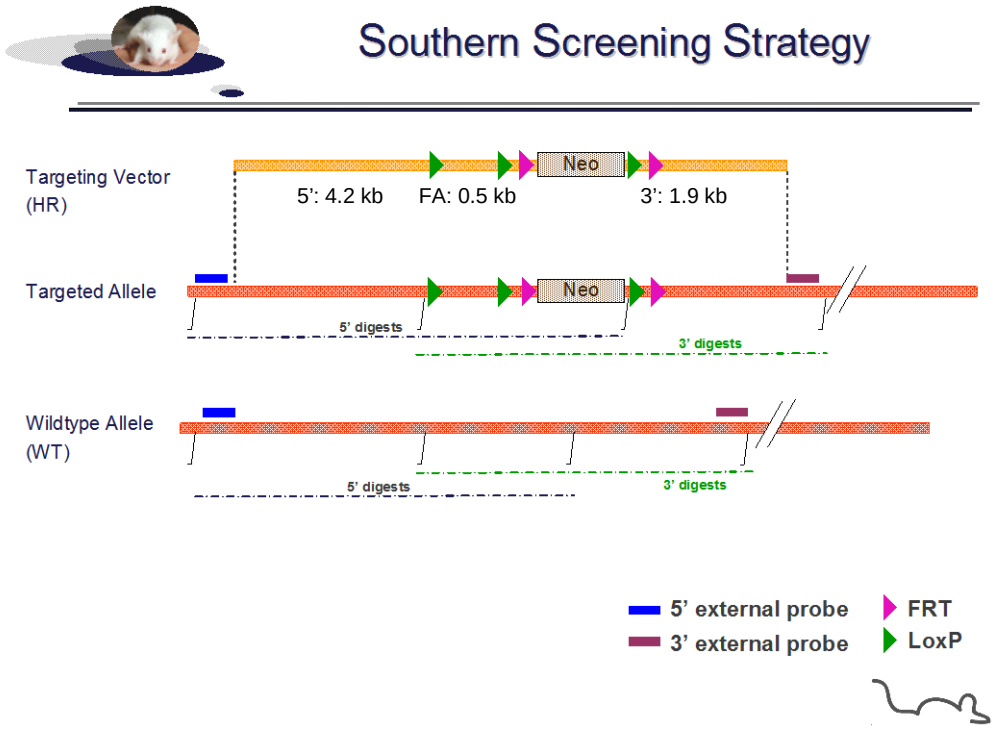
Neo southern blot: 5' and 3' arm validation



ladder



3.3.3.External probes Southern strategy



Digestions used to validate with 5' and 3' probes

Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
5' external	5' digest	Bgl II	13.3	8.5
3' external	3' digest	Spe I	17.5	6.3

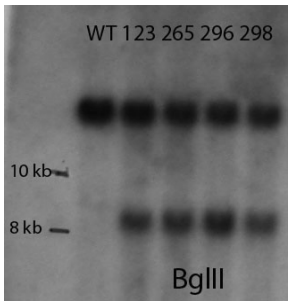
Primers for probe synthesis:

5' probe
GGCTAAGCTCCAGCAACTGGAGGCC
AGCGAGCCTGCTTATCCAGGAAGCC

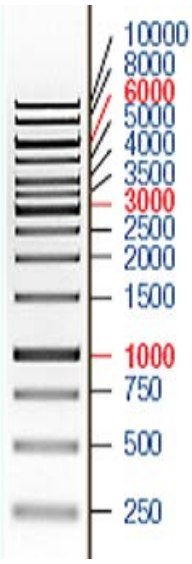
3' probe
CGGATCGATCCGCGCGGCCGCAGAC
GAGTTTCTTCAAGCGCAGCATCCGC

3.3.4.Picture of Southern with 5' and 3' probes

5' external probe

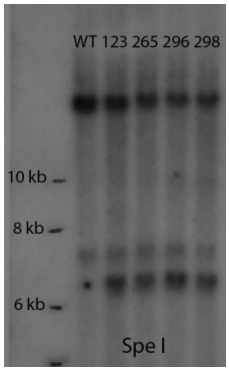


Ladder

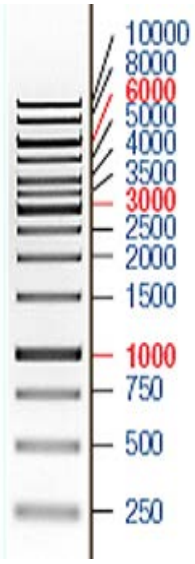


3' external probe

Non specific band →



Ladder

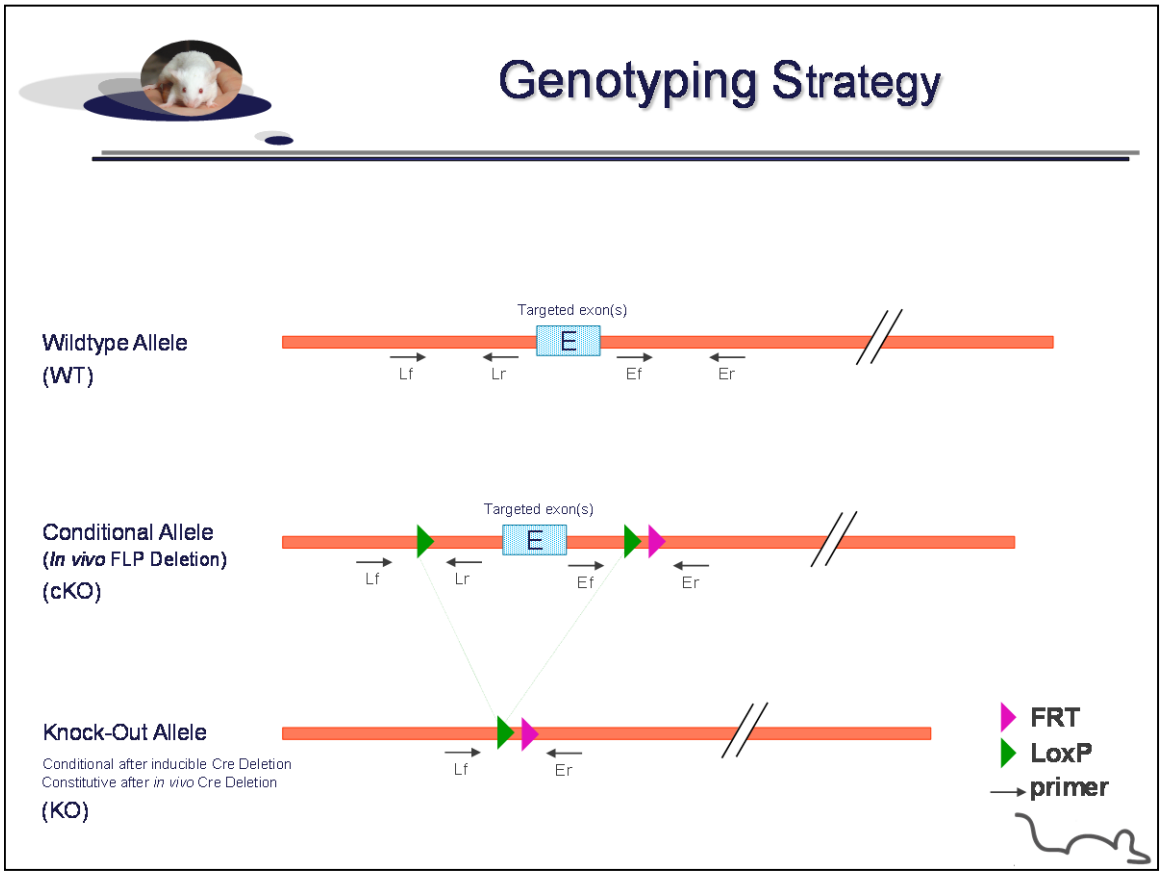


4. Genotyping protocol and data on conditional and knock-out animals

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

4.1. 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	1292	TTCCCACTGATGTGCCTGCAAACC
Er	1294	GTCTGTTCCATTGTGTCCTCTACCAG
Lf	1290	TCTGTCTCCCCAGGTCTCATGG
Lr	1291	TAGGATACCAGATGCCTGGTAGTGG

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	1290-1291	Lf / Lr	333	---	283
Excision of the selection marker(Neo excision)	1292-1294	Ef / Er	335	---	197
Excision of the floxed exon(s), i.e. knock out	1290-1294	Lf / Er	846*	274	621

* 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 µM)	0.125µl
-3' primer (100 µM)	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.

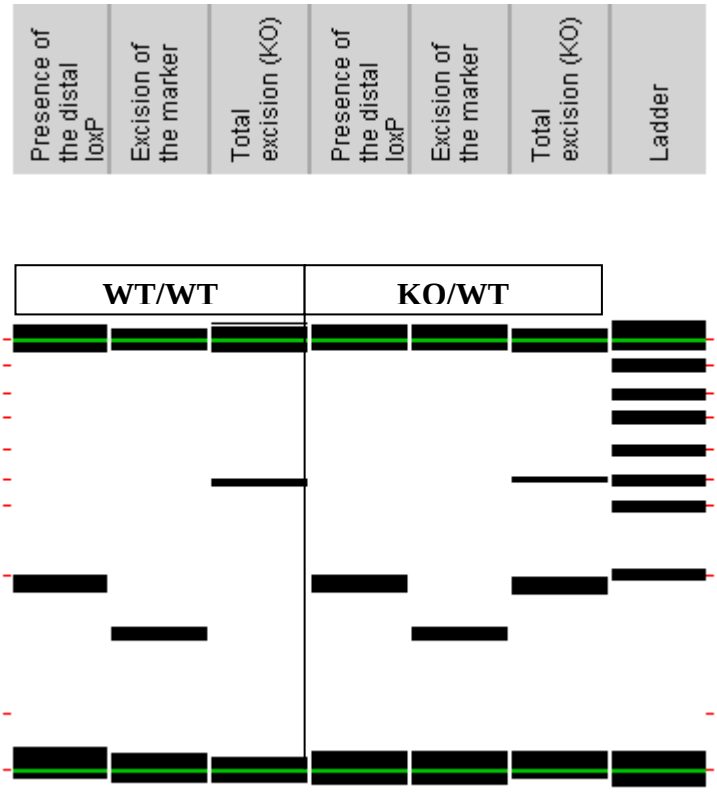
4.3. Picture of genotyping with various alleles

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

Data not shown

- 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.