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EXCELLENCE IN MOUSE PHENOGENOMICS

MODEL GENERATION TECHNICAL REPORT

Zap70 conditional overexpression in Rosa / KI

Project code: Kos4489 / IR4489

Report finalized: 23/09/2024

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MODEL GENERATION TECHNICAL REPORT

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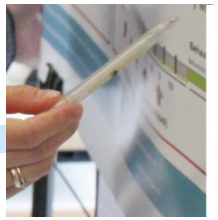
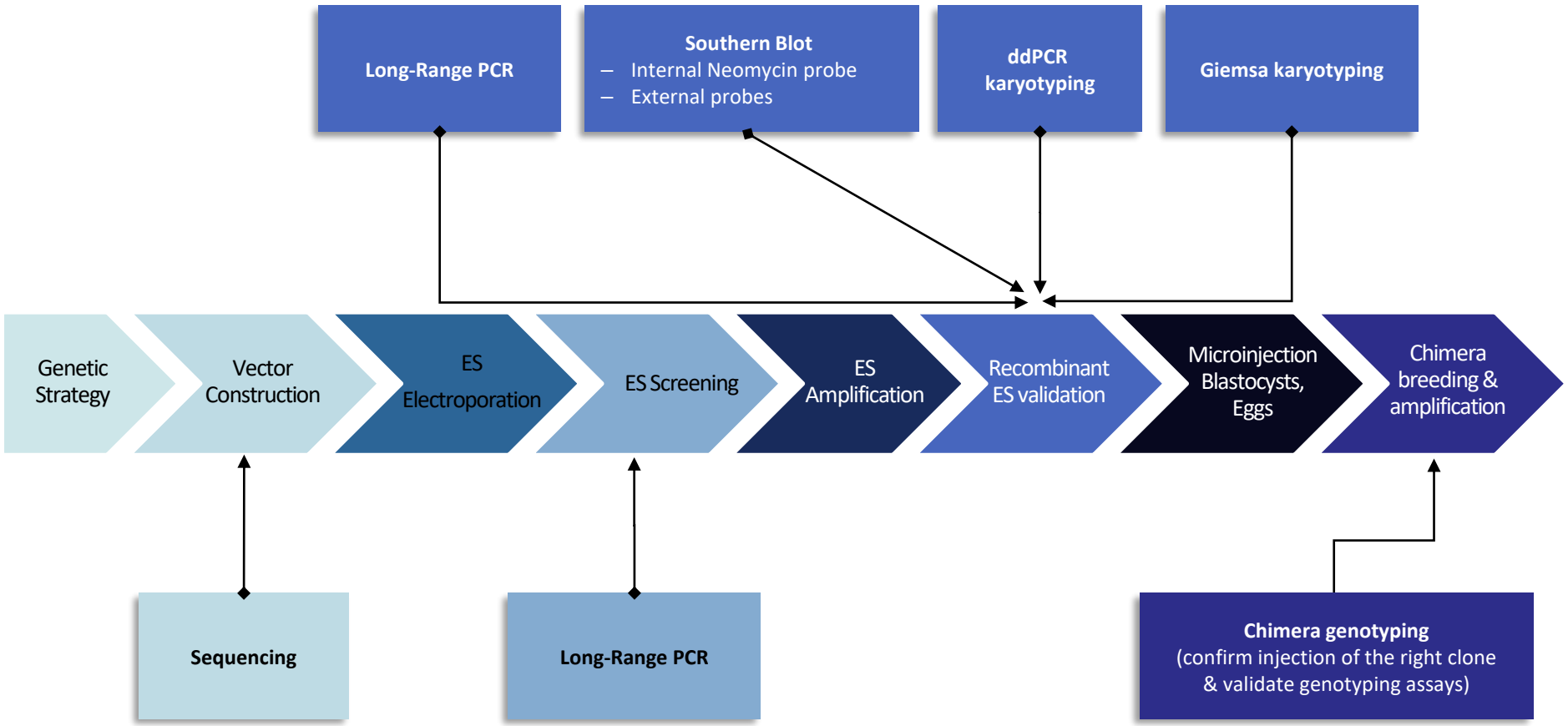
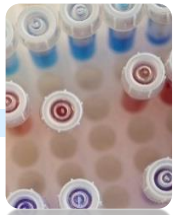
PROJECT PROCESS & QUALITY CONTROLS



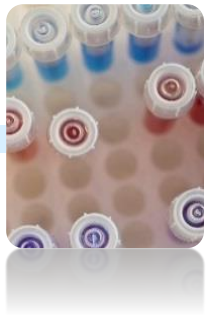


2 GENETIC STRATEGY

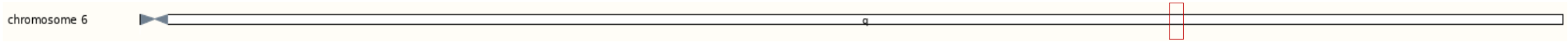


- Target locus structure
- Zap70 coding sequence

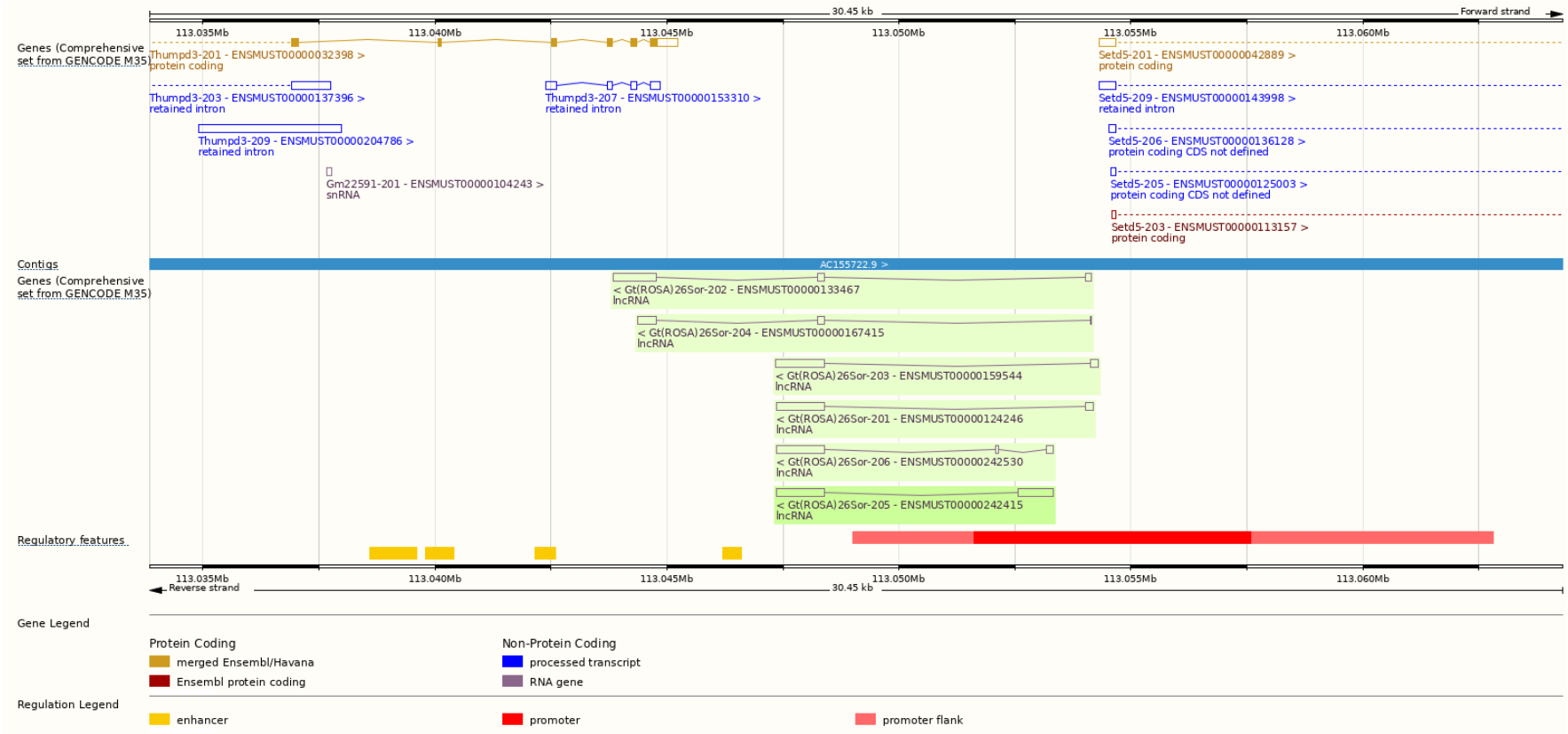
Rosa mouse genomic locus – structure



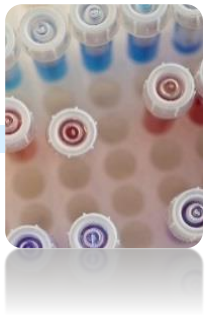
Location : Chromosome 6: 113,047,359-113,053,323



Ensembl Gene ID: Gt(ROSA)26Sor ENSMUSG00000086429



Zap70 mRNA sequence



Transcript: ENSMUST00000027291.7 Zap70-201

Coding sequence :

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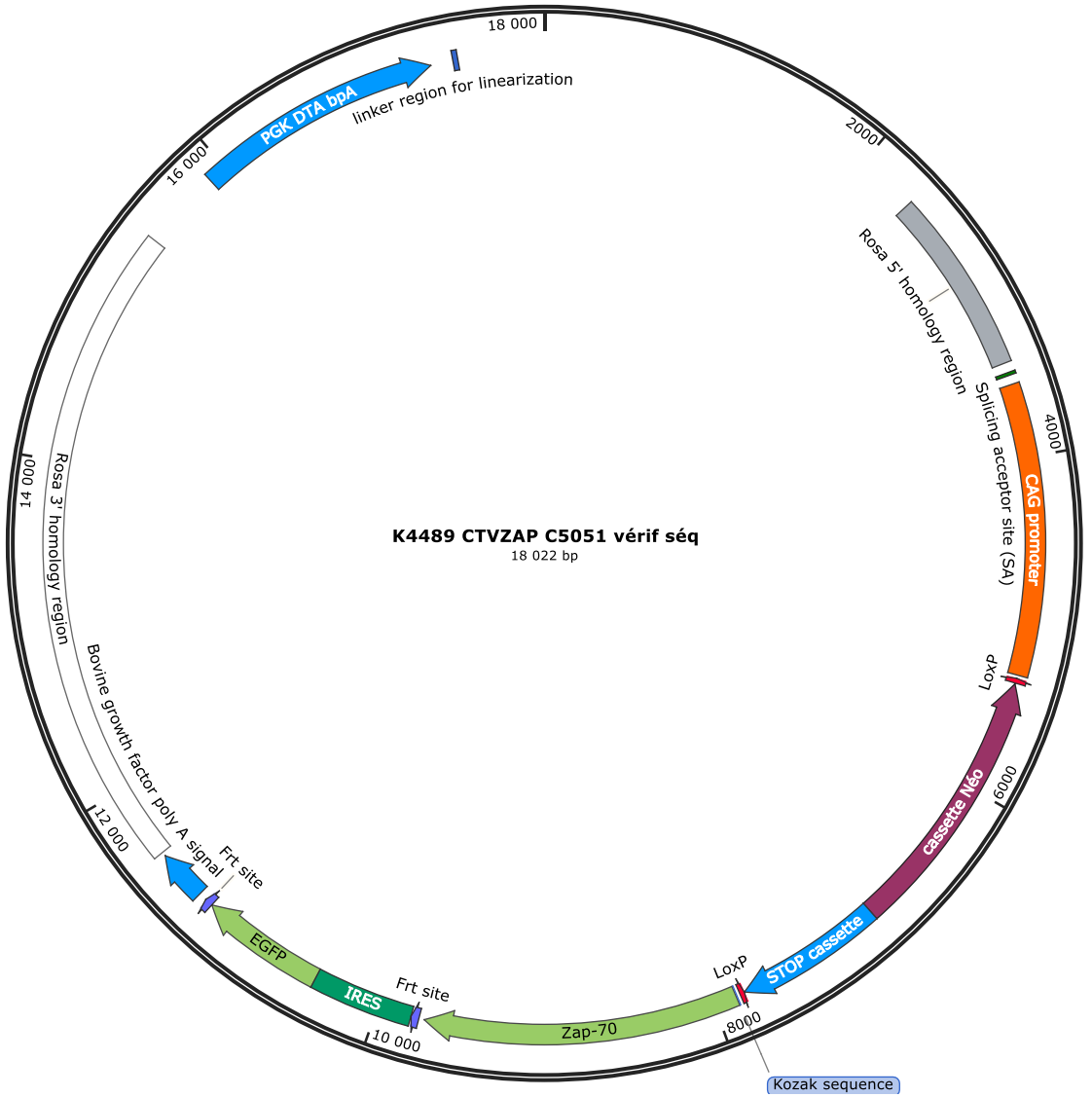
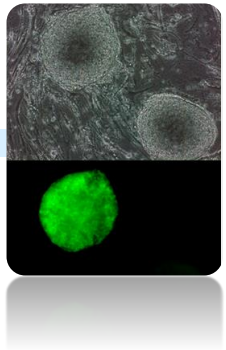
3 HOMOLOGOUS RECOMBINATION - VECTOR CONSTRUCTION

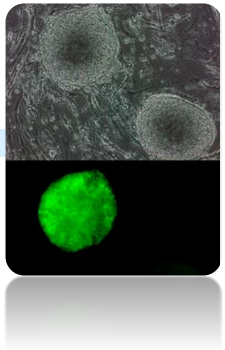


4 ES cell electroporation & Screening of recombinant clones



- Electroporation and screening process
- Long range PCR screening – strategy
- Long-Range 5’ PCR screening – results
- Recombinant ES validation by Long Range PCR
- Recombinant ES clones validation by Southern Blot – internal probe
- Recombinant ES clones validation by Southern Blot – External probe
- Aneuploidy screening in ES recombinant clones

Electroporation and screening process



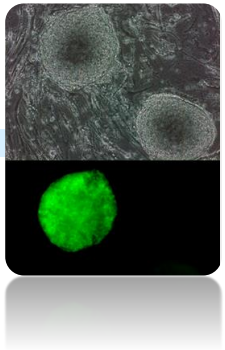
The targeting vector was electroporated in the ICS proprietary C57BL/6NTac TB1 cell line.

Transfected ES clones were submitted to neomycin selection (G418) and 32 resistant ES clones were isolated. The clones were then submitted to the screening process allowing secured identification of those harbouring the expected recombination events at both ends of targeting vector.

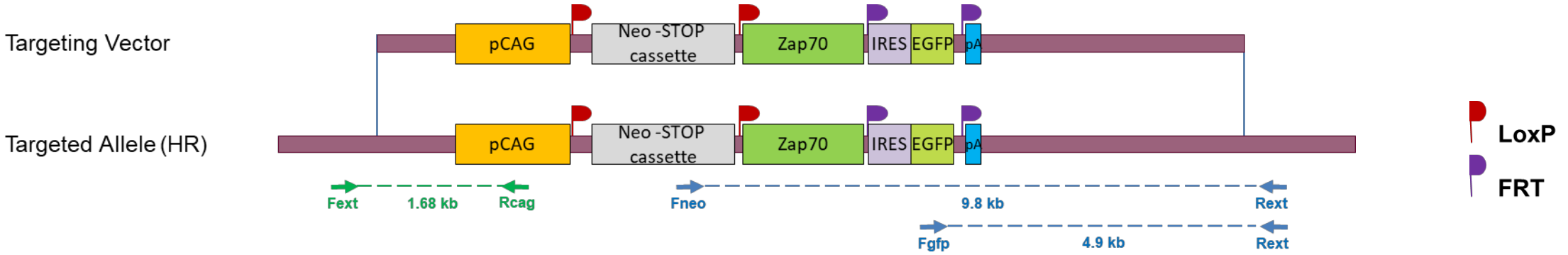
Screening process steps:

1. Identification of candidate recombinant clones by initial 3’ Long-Range PCR
2. Nine of 3’ PCR positive clones are confirmed for 5’ recombination event by Long-Range PCR
3. Positive clones in step2 are further validated by Southern blot analysis using internal and external probes
4. The karyotype of at least 2 validated clones is verified using Giemsa staining

Long range PCR screening – strategy

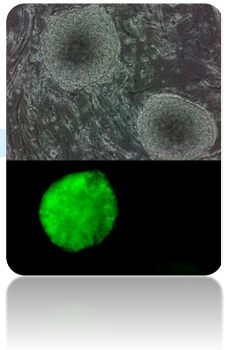


Schematic 5’ and 3’ PCR screening strategy

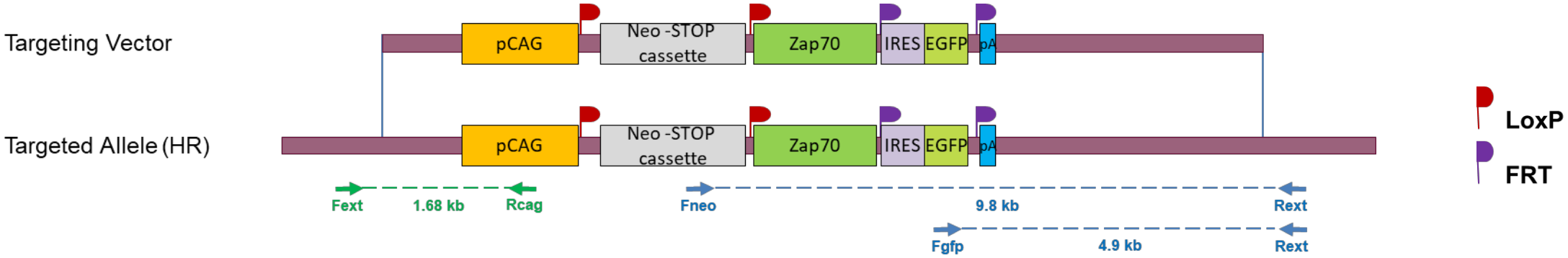


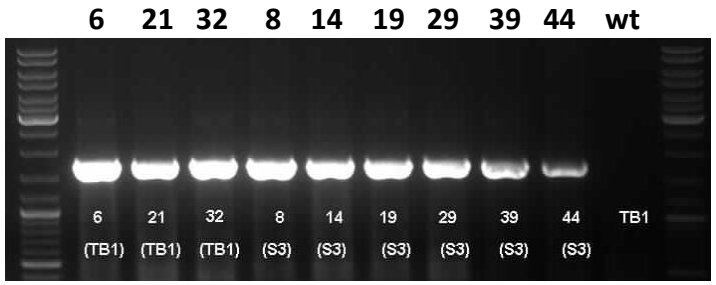
PCR	Primer Name	Primer sequences	PCR product size
5' PCR	Fext	GGTAGGGGATCGGGACTCTGGCGGG	1.68 kb
	Rcag	GGAGAGTGAAGCAGAACGTGGGGCT	
3’ PCR	Fneo	GCGGCCGGAGAACCTGCGTGCAATC	9.8 kb
	Rext	CTCAGTGGCTCAACAACACTTGGTC	
3’ PCR	Fgfp	CCCGTGCTGCTGCCCGACAACCACT	4.9 kb
	Rext	CTCAGTGGCTCAACAACACTTGGTC	

Recombinant ES validation by Long Range PCR

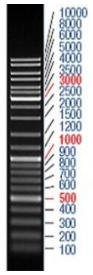


Confirmation and Validation of candidate recombinant ES clones by 5’ and 3’ PCRs

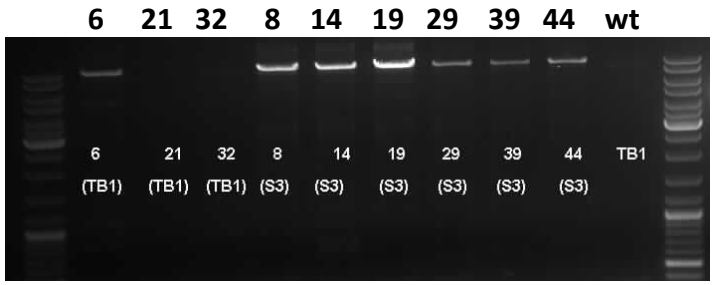




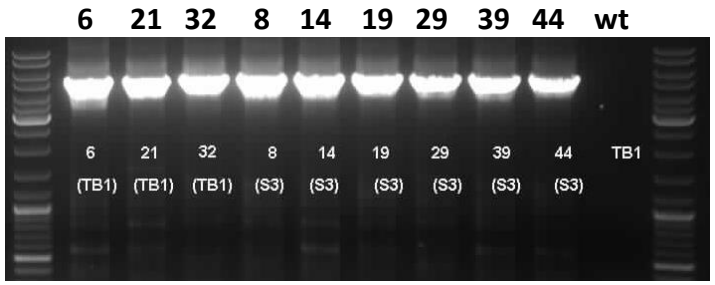
PCR Fext – Rcag : 1.68 kb



Ladder pattern



PCR Fneo – Rext : 9.8 kb



PCR Fgfp – Rext : 4.9 kb

Nine candidate clones identified by 3’ PCR screening were further analysed by 5’ Long-Range PCR screening. Six clones (clones #8, #14, #19, #21, #29, and #32) were further analysed.

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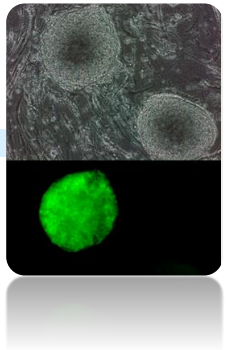
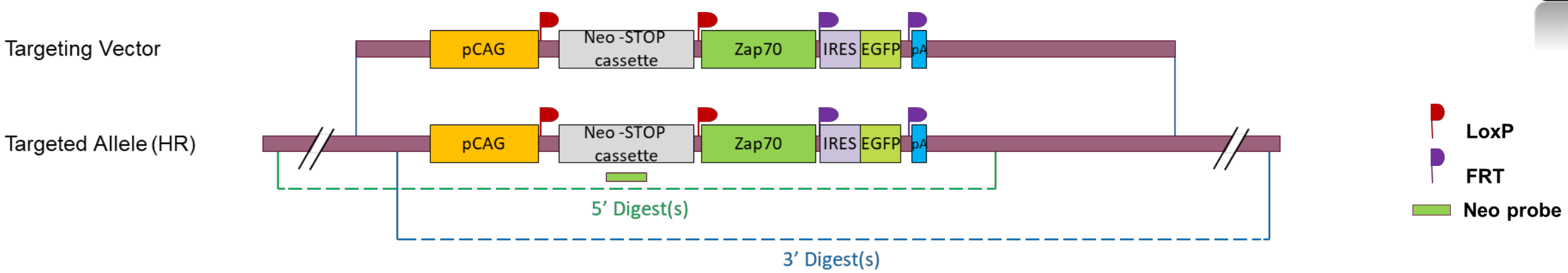
Recombinant ES clones validation by Southern Blot – Internal probe



Schematic Southern Blot validation strategy

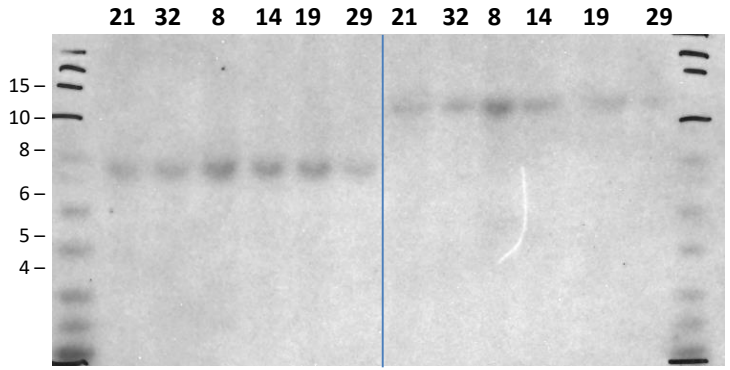
Digests on the scheme illustrate the position of the chosen restriction sites relative to the probe. They don't show the exact position of the restriction sites.



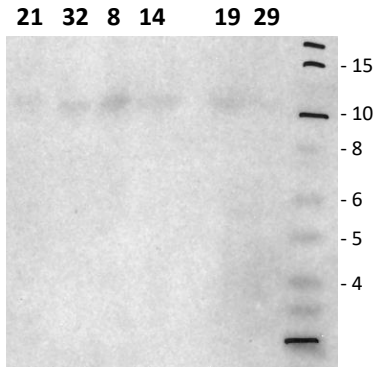
Digestions used to validate the 5' and 3' insertion

Probe		Genomic DNA digest	Targeted Allele (kb)
Neo	5' digest	BstEII	7.7
		EcoNI	11.9
	3' digest	PacI	10.8

Southern blot - Neo 5'



Southern blot - Neo 3'



Neo probe sequence

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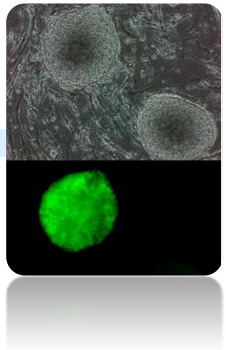
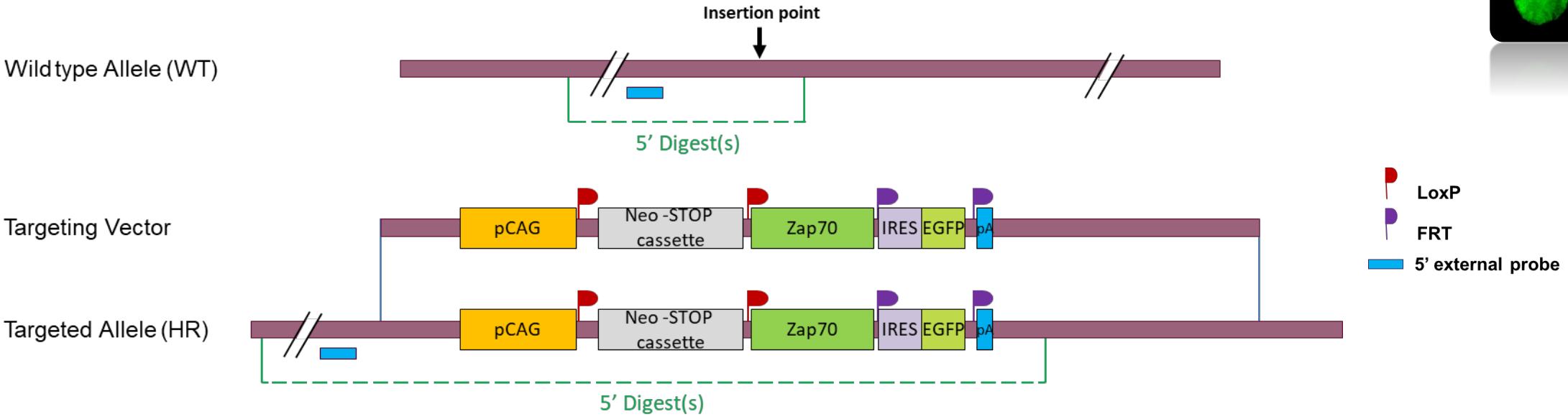
CCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAG
CCGCGCTGCCTCGTCCTGCAG

Recombinant ES clones validation by Southern Blot – External probe

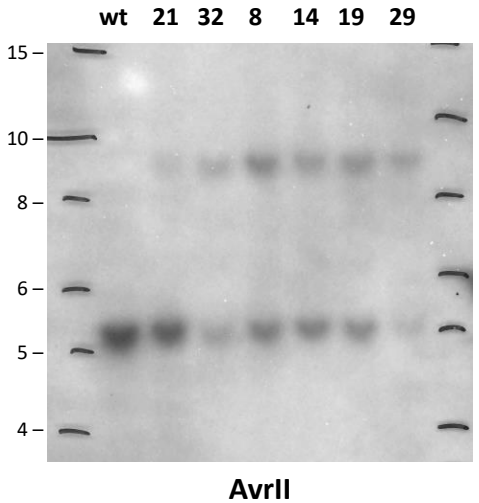


Schematic Southern Blot validation strategy

Digests on the scheme illustrate the position of the chosen restriction sites relative to the probe. They don't show the exact position of the restriction sites.



Southern blot – 5' probe



5' PROBE SEQUENCE

TATGTGTATTTTGAGAGCAGGGTTGGGAGGCCTCTCCTGA
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GAGGGAACGCAGGGAGACTGAGGTGACCCTTCTTTCCCCC
GGGGCCCGGTCGTGTGGTTCGGTGTCTCTTTTCTGTTGGA
CCCTTACCTTGACCCAGGC

Digestions used to validate the 5' and 3' insertion

Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
5' external probe	5' first digest	AvrII	5.3	8.8

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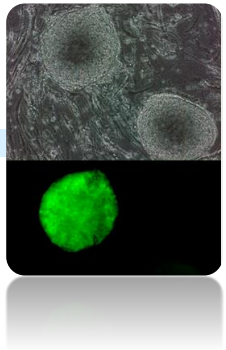
Aneuploidy screening in ES recombinant clones

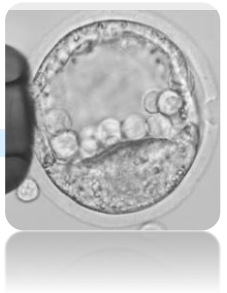


Selected recombinant ES cells clones were karyotyped by Giemsa metaphase staining. Results of aneuploidy analysis are presented in the table below.

Clone ID	ddPCR	Giemsa
#21	Pass	Not done
#32	**Pass**	**Pass**

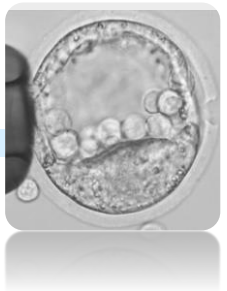
[1] Codner, G.F., Lindner, L., Caulder, A., Wattenhofer-Donzé, M., Radage, A., Mertz, A., Eisenmann, B., Mianné, J., Evans, E.P., Beechey, C.V., Fray, M.D., Birling, M.-C., Hérault, Y., Pavlovic, G., Teboul, L
Aneuploidy screening of embryonic stem cell clones by metaphase karyotyping and droplet digital polymerase chain reaction.
BMC Cell Biology 2016 **doi:10.1186/s12860-016-0108-6**

5 MICROINJECTION & BREEDING



- Microinjection
- Breeding to F1 generation

Microinjection



- The ES cells used in the injection experiment were originally derived from a C57BL/6NTac mouse strain (which have black coat colour). These cells were injected into blastocysts derived from an BALB/cN strain, which have a white coat colour. The resulting offspring are thus chimeras of two different cell types (ES cell-derived cells and host blastocyst-derived cells) and the degree of chimerism was monitored by the percentage of light and dark patches on these animals.
- Recipient blastocysts were isolated from mated BALB/cN females (Health status SPF Specific Pathogens Free).
- Recombinant ES clones #32 validated in previous project phase was injected into blastocysts to generate chimeric males. The results are presented in the table below.

Clone ID	Number of chimeric males identified according to chimerism rate (Number of chimeric males bred to F1 generation)			
	5 - 40%	45% - 55%	60-100%	Total
#32	0	3	5	8

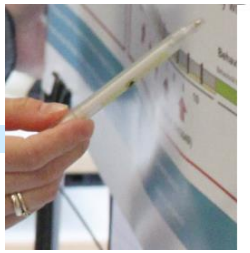
Breeding to F1 generation

- Four highly chimeric males generated in the previous phase by blastocyst injection of the ES clones were mated with wild-type C57BL/6NCrl females (health status SPF – Specific Pathogen Free) to investigate whether the recombined ES cells have contributed to the germ layer.

- Germ line transmission was obtained the : 13/08/2014

- Allele nomenclature (following MGI guidelines) : **GT(ROSA)26Sor$^{tm6(Zap70, IRES-EGFP)Ics}$**

6 SEQUENCE OF THE DELIVERED ALLELE



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ISO 9001 · NFX 50-900

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LoxP pCAG FRT Neo - STOP cassette Zap70 EGFP bGH pA

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REPORT REDACTION & VALIDATION

Protocol finalized on 2023/09/23

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Genotyping protocol

ZAP70 conditional overexpression in Rosa

IR00004489 / K4489

(ICS internal reference)

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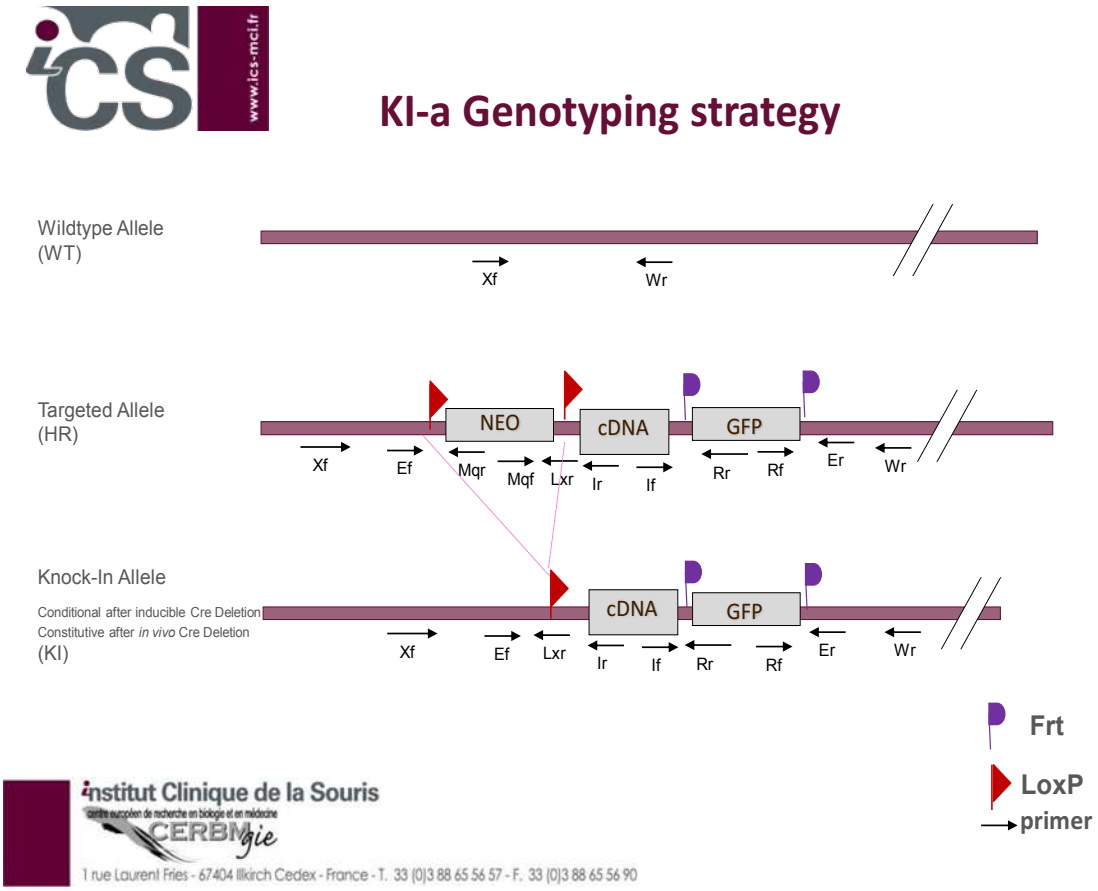
1. Genotyping protocol and data

This section describes the condition used at the Mouse Clinical Institute (ICS) to genotype your **ZAP70 condtional overexpression in Rosa** Knockin (KI) project.

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	7847	GCGGCTCTAGAGCCTCTGCTAAC
If	7850	CGTATGCGGAACTATTACTACAGCCTG
If[2]	7851	GAGTGTCCTCCTGAGATGTATGCAC
Ir	7848	GAAGCGCACGTCGTGCACCAAC
Ir[2]	7849	CGCGAGATGCTGCCATAGAAGAATG
Er	7852	CACCTTCCAGGGTCAAGGAAGG
Er[2]	7853	GAATGACACCTACTCAGACAATGCGATG
Lxr	7855	CGAAGTTATTTAATTAACTCGAGG
Mqf	2687	CTGCATTCTAGTTGTGGTTTGTC
Mqr	240	CCTTCTTGACGAGTTCTTCTGAGGG
Rf	2778	CCTGAGCAAAGACCCCAACG
Rr	7854	CCTTATTCCAAGCGGCTTCGGC
Wr	7013	CGCCCACACACCAGGTTAGCCTTTA
Xf	7012	TAAGGGAGCTGCAGTGGAGTAGGCG

[2]: for a selected position, a second primer was designed

PCR fragments expected size (bp):

Region analyzed	Primers used	Position on the primer *(see the map above)*	Targeted allele (HR)	KI allele	WildType allele
WildType allele specific PCR (5' part of the targeted locus)	7012-7013	Xf / Wr	8363	5858	234
Excision of the selection marker	7847-7848	Ef / Ir	2849	344	---
5' part of the selection marker	7847-240	Ef / Mqr	238	---	---
3' part of the selection marker	2687-7849	Mqf / Ir[2]	181	---	---
Excision of the reporter	7850-7852	If / Er	1687	---	---
5' part of the reporter	7851-7854	If[2] / Rr	321	321	---
3' part of reporter	2778-7853	Rf / Er[2]	367	367	---
LoxP specific PCR	7847-7855	Ef / Lxr	130	130	---

*: this PCR product will not be observed using our PCR gen otyping conditions (see description below)
**: this PCR is only verified if mice are generated
---: no Amplicon should be obtained

1.2. PCR protocol

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

Reagents:	Volume:
- FastStart PCR Master (Roche)	7.5µl
- DNA (50ng/µl)	1.5µl
- 5' primer (100 µM)	0.06µl
- 3' primer (100 µM)	0.06µl
- Sterile H_2O	up to 15 µl

Cycling conditions:

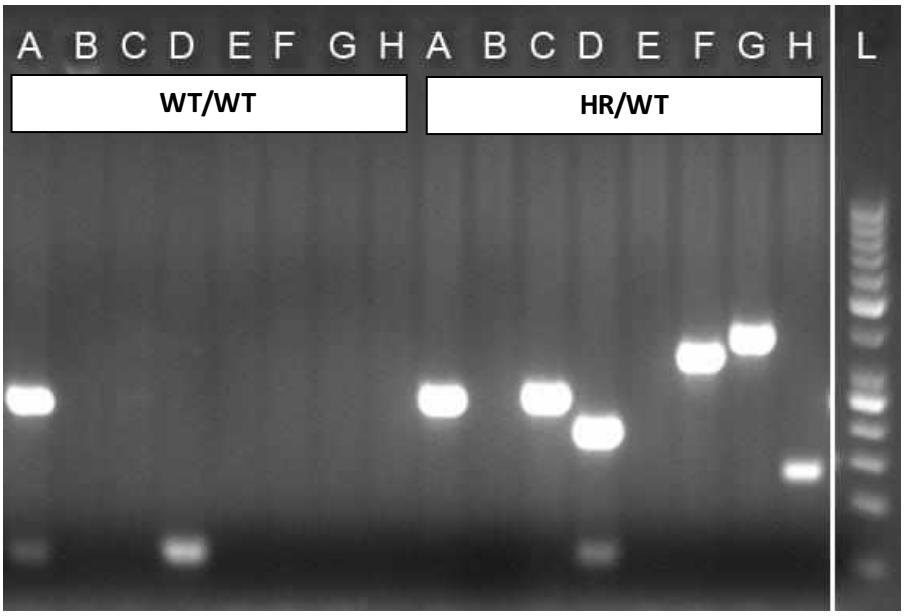
Temp	Time	#Cycles
95°C	4min	1
94°C	30s	
62°C	30s	34
72°C	1min	
72°C	7min	1
20°C	5min	1

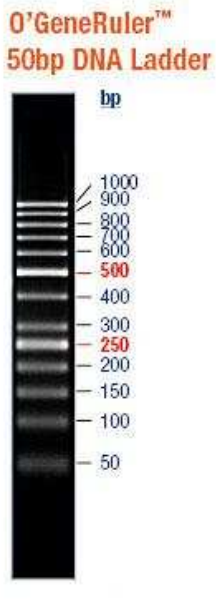
NB: These PCR conditions have been optimized for high-throughput genotyping. Adaptation to small-scale may be required.

1.3. Picture of genotyping with various alleles

Analysis of PCR products pattern was done by gel electrophoresis 2% agarose (SB buffer).

Representative genotyping picture





A: WildType allele specific PCR (5' part of the targeted locus)
B: Excision of the selection marker
C: 5' part of the selection marker
D: 3' part of the selection marker
E: Excision of the reporter
F: 5' part of the reporter
G: 3' part of the reporter
H: LoxP specific PCR
L: 0'GeneRuler 50bp DNA Ladder

2. Cre and Flp genotyping method

You will find the genotyping protocol in the publication:

Highly-efficient, fluorescent, locus directed cre and FlpO deleter mice on a pure C57BL/6N genetic background.
Birling MC, Dierich A, Jacquot S, Hérault Y, Pavlovic G.
Genesis. 2012 Jun;50(6):482-9. doi: 10.1002/dvg.20826. Epub 2012 Mar 20.