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EXCELLENCE IN MOUSE PHENOGENOMICS

MODEL GENERATION TECHNICAL REPORT

Generation of a Crlf2 (Tslpr) cKO mouse line

Project code: K515b / IR5415

Report finalized: 2024/10/25

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MODEL GENERATION TECHNICAL REPORT

1 PROJECT PROCESS & QUALITY CONTROLS

2 GENETIC STRATEGY

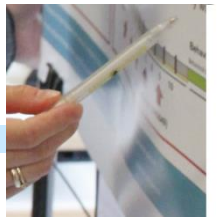
3 HOMOLOGOUS RECOMBINATION VECTOR CONSTRUCTION

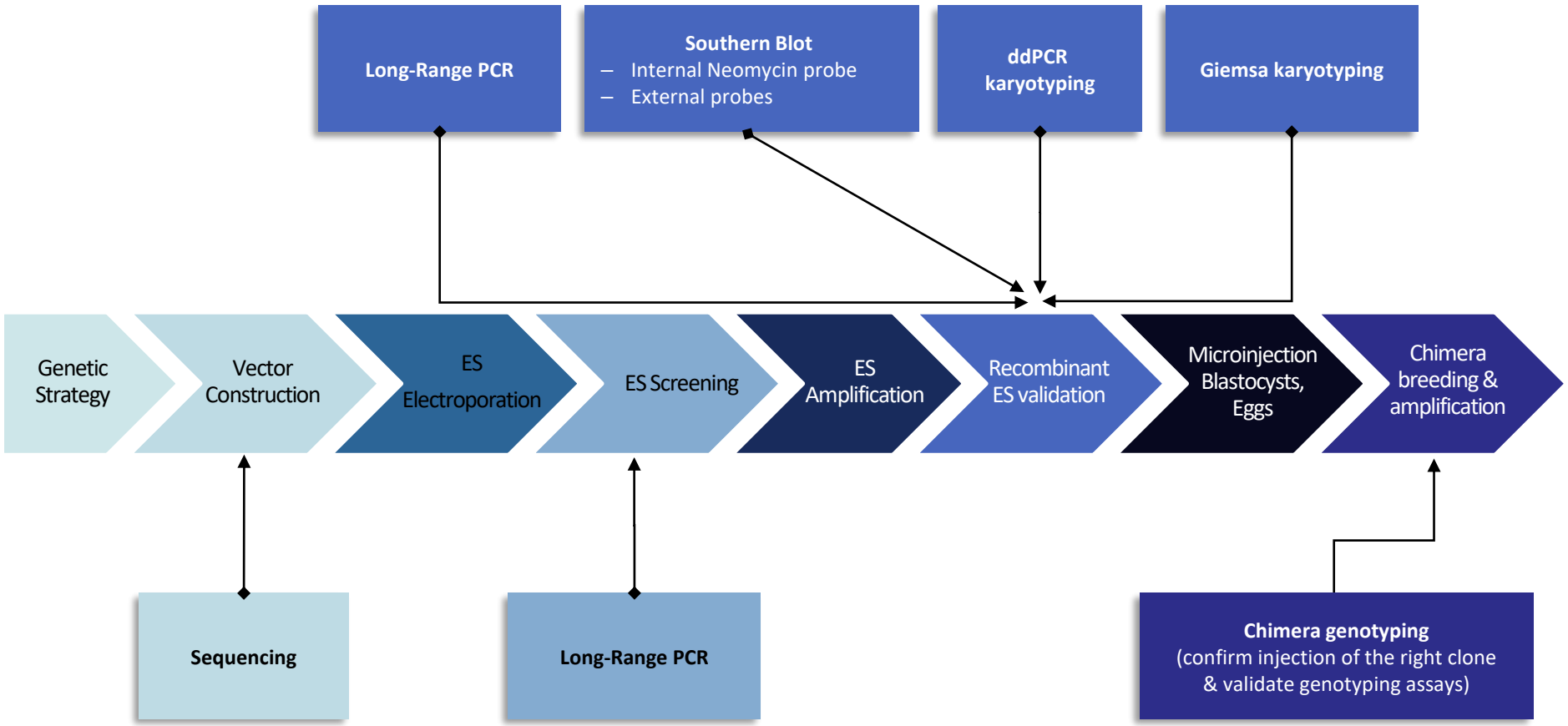
4 ES ELECTROPORATION & SCREENING OF RECOMBINANT CLONES

5 MICROINJECTION & BREEDING

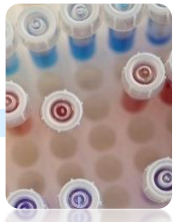
6 SEQUENCE OF THE DELIVERED ALLELE

Project process & quality controls



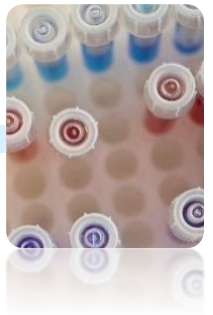


2 GENETIC STRATEGY

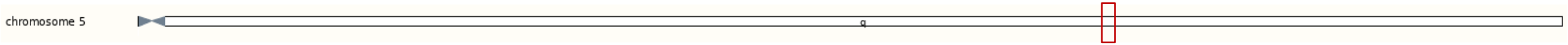


- Target locus structure
- mRNA(s) and protein(s)
- Genetic strategy
- PRO & CONS evaluation of the strategy

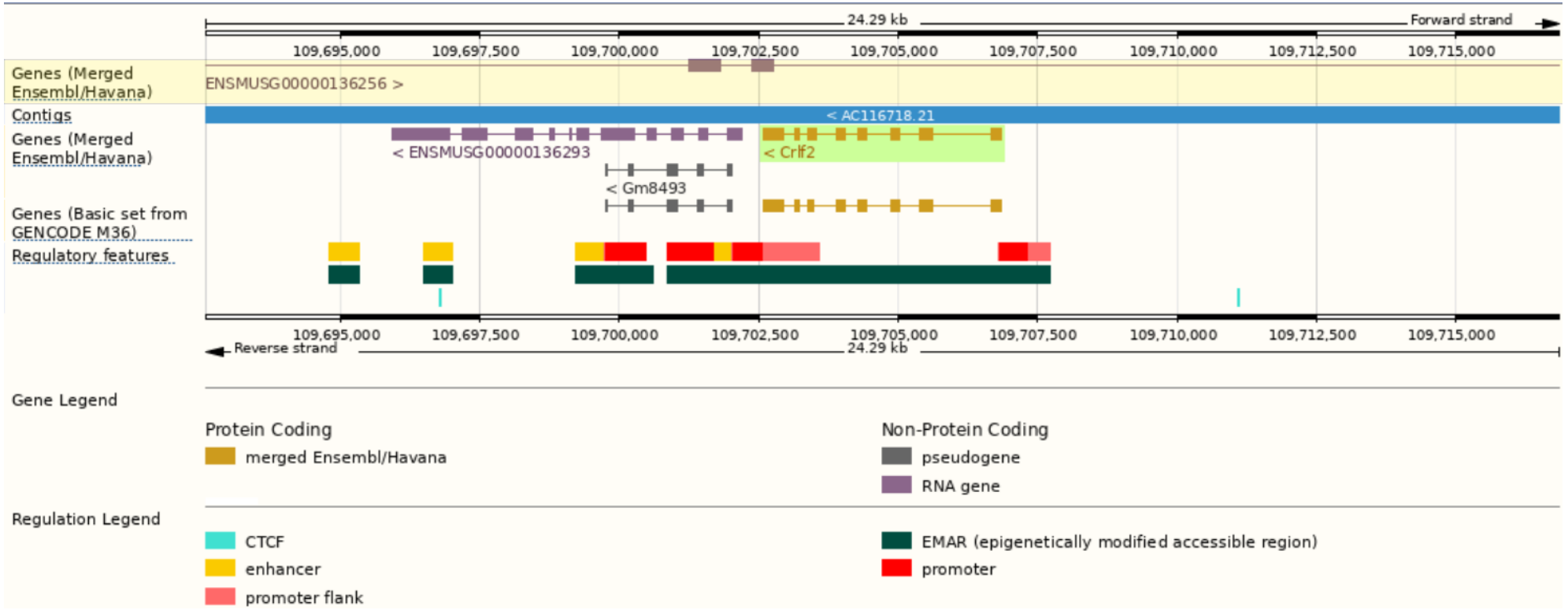
Crlf2 mouse genomic locus – structure (GRCm39)



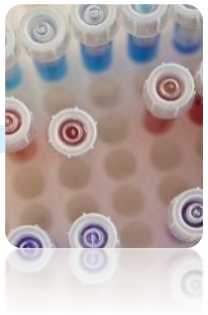
Chromosome 5: 109,702,575-109,706,859



Gene: Crlf2 ENSMUSG00000033467

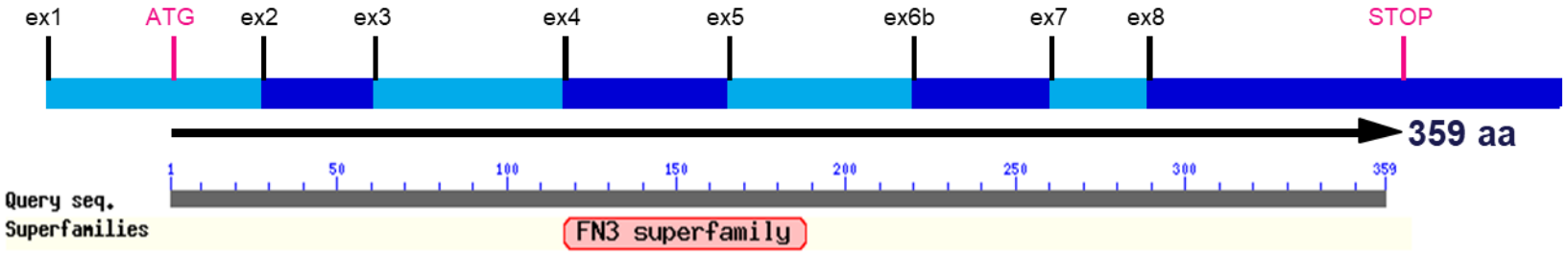


Crlf2 mRNAs and proteins

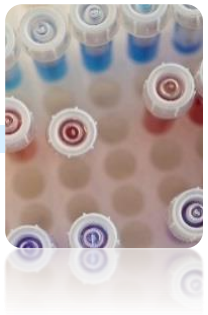


Transcript ID	Name	bp	Protein	Biotype	CCDS	UniProt Match
ENSMUST00000044579.12	Crlf2-201	1323	359aa	Protein coding	CCDS19521	A0A0R4J0F5
ENSMUST00000200284.5	Crlf2-203	1405	357aa	Protein coding	CCDS80356	A0A0G2JGP1
ENSMUST00000198960.2	Crlf2-202	812	226aa	Protein coding		A0A0G2JF13

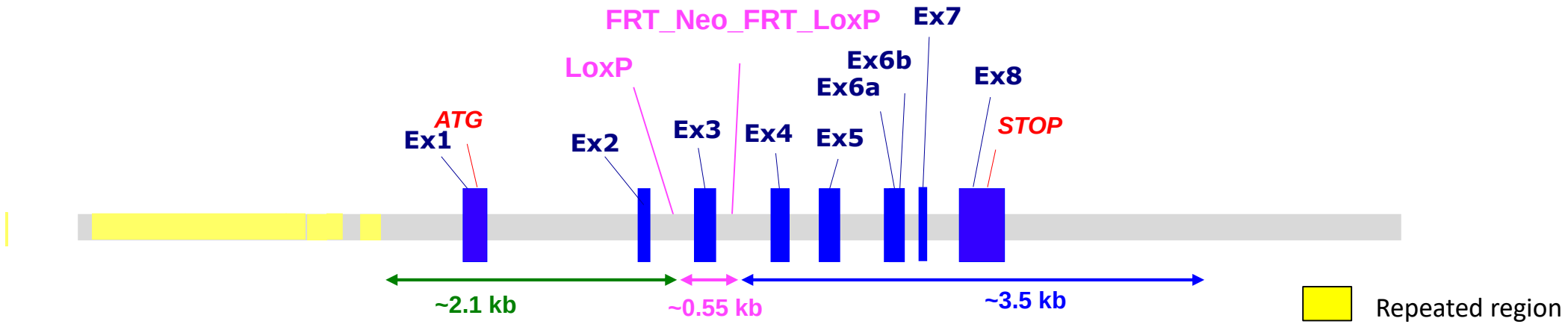
Crlf2-201



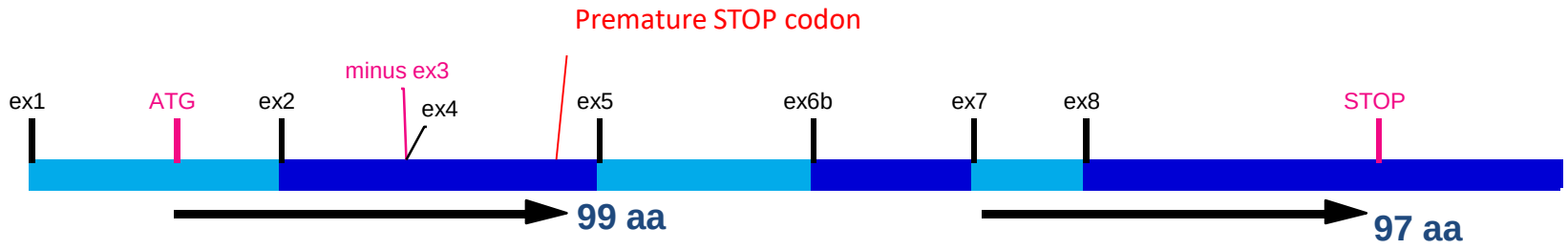
Approach undertaken: flox exon 3



Targeted locus

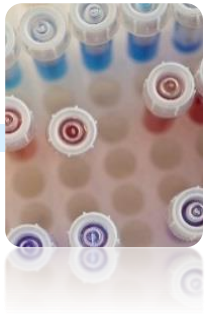


mRNA and protein expected after Cre mediated excision



No putative conserved domains have been detected

PROs& CONs evaluation of the strategy



Pros

- ✓ Small floxed fragment

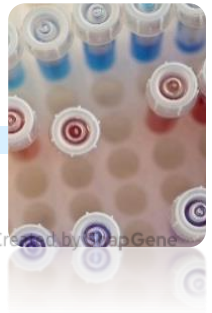
Cons

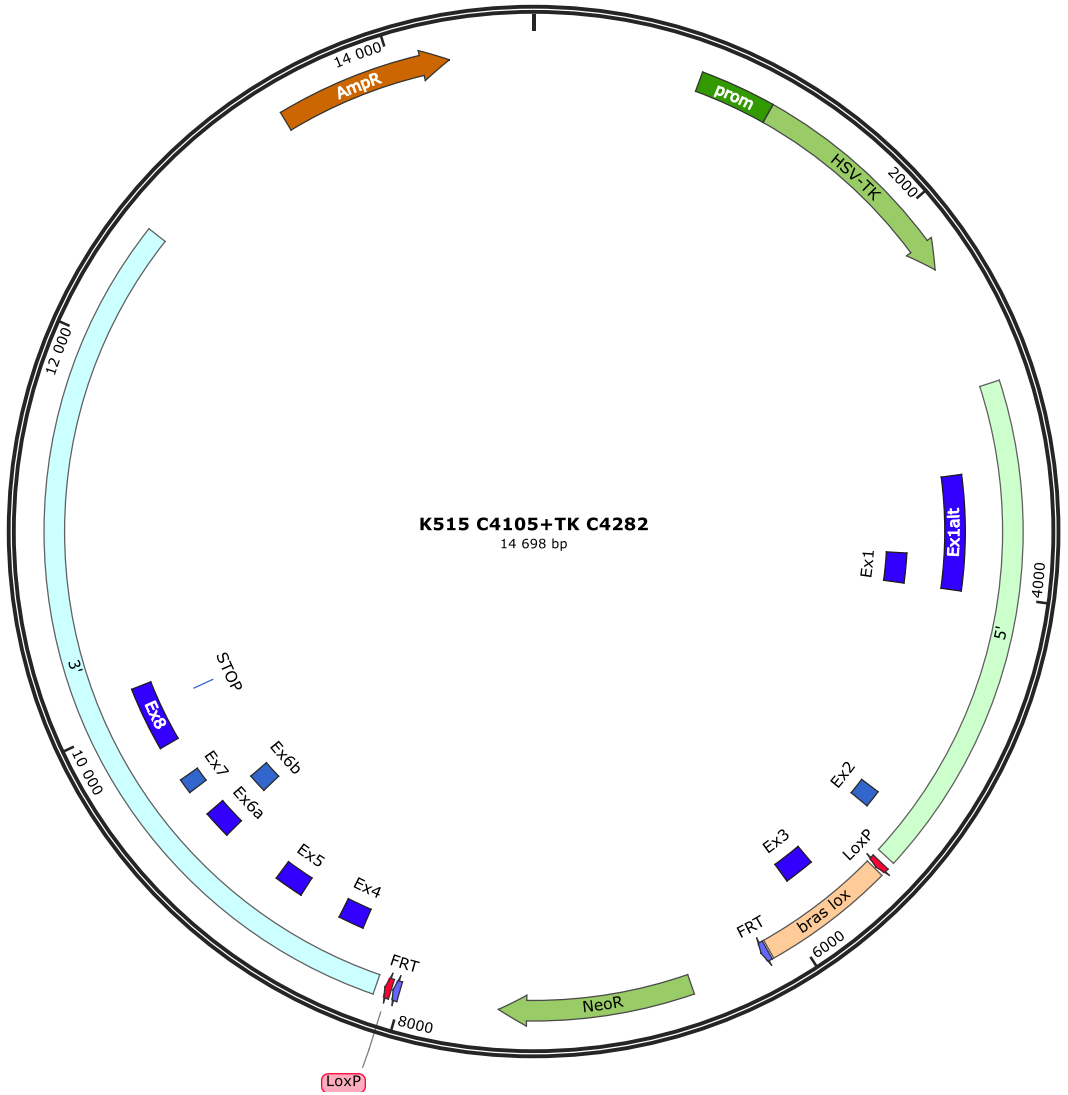
- ✓ Insertion of the 5'LoxP site into small intron (intron 2, 350bp): could interfere with the splicing mechanism of the conditional (WT) allele
- ✓ A protein of 99 aa corresponding to the 61 first N-term aa from the TSLP rec protein plus 98 out of frame aa might be expressed after Cre mediated excision (if RNA decay does not occur). This protein would not contain any putative conserved domains.
- ✓ A protein of max 97 aa might be expressed after Cre mediated excision if reinitiation occurs at the in frame ATG present in exon 7. This protein would not contain any putative conserved domains.
- ✓ Presence of repeats in the 5' region: necessity to reduce the size of the 5' homology arm.

The selection cassette (FRT-Neo-FRT) will be removed by breeding male chimera with a Flp deleter line which shows maternal contribution (*Birling et al.*, 2012)

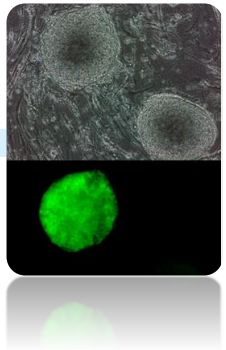
Highly-efficient, fluorescent, locus directed cre and FlpO deleter mice on a pure C57BL/6N genetic background.
Birling MC, Dierich A, Jacquot S, Hérault Y, Pavlovic G. Genesis. 2012 Jun;50(6):482-9. doi: 10.1002/dvg.20826.

3 HOMOLOGOUS RECOMBINATION - VECTOR CONSTRUCTION



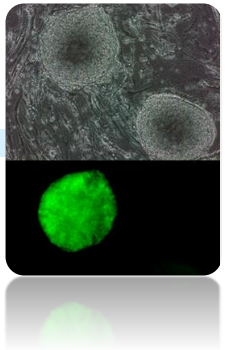


ES cell electroporation & Screening of recombinant clones



- 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 whole process of ES cells validation is described in Erbs *et al.*.*

The circular targeting construct was co-electroporated with a circular CRISPR plasmid expressing the WT SpCas9 and the TTTTAAACGCCTGAAAGAGT guide RNA in the proprietary C57BL/6NCrl S3 cell line.

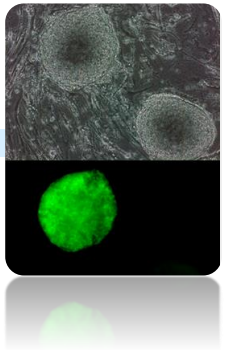
Transfected ES clones were submitted to neomycin selection (G418) and 158 resistant ES clones were isolated. The clones were then submitted to the screening process allowing secured identification of those harbouring the expected recombination events at both ends of targeting vector.

Screening process steps:

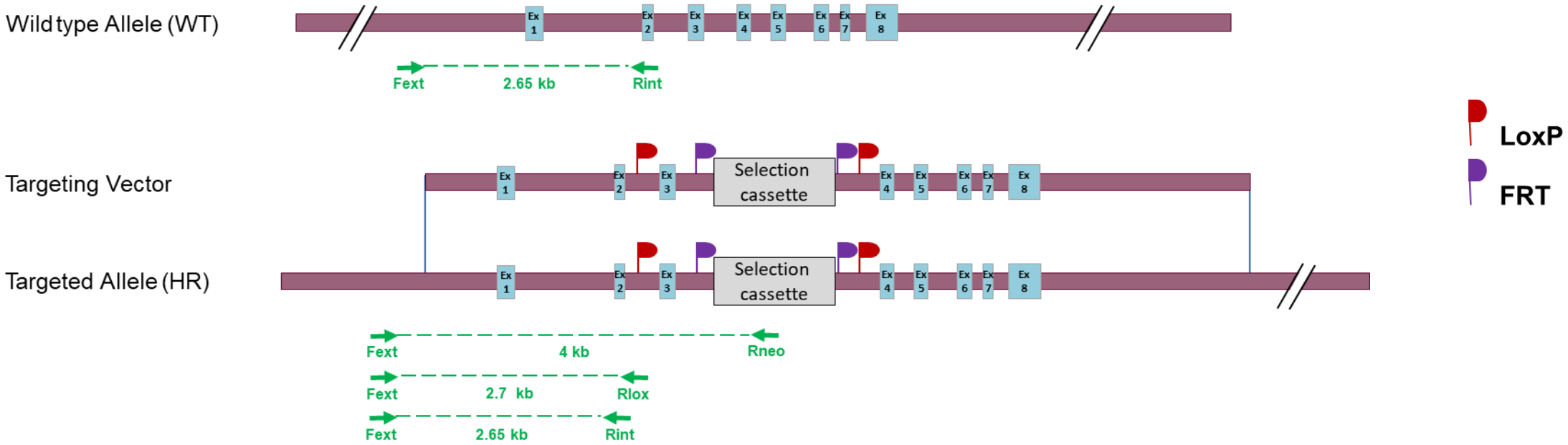
1. Identification of candidate recombinant clones by initial 5' Long-Range PCR
2. Nine of 5' PCR positive clones are confirmed for 5' recombination event by Long-Range PCR
3. Positive clones in step2 are further validated by Southern blot analysis using internal and external probes
4. The karyotype of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

*Erbs V, Lorentz R, Eisenman B, Schaeffer L, Luppi L, Lindner L, Hérault Y, Pavlovic G, Wattenhofer-Donzé M, Birling MC. **Increased On-Target Rate and Risk of Concatemerization after CRISPR-Enhanced Targeting in ES Cells.** Genes (Basel). 2023 Feb 3;14(2):401. **doi: 10.3390/genes14020401**.

Long range PCR screening – strategy

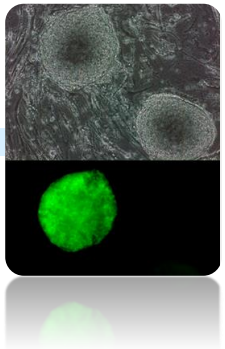


Schematic 5’ and 3’ PCR screening strategy

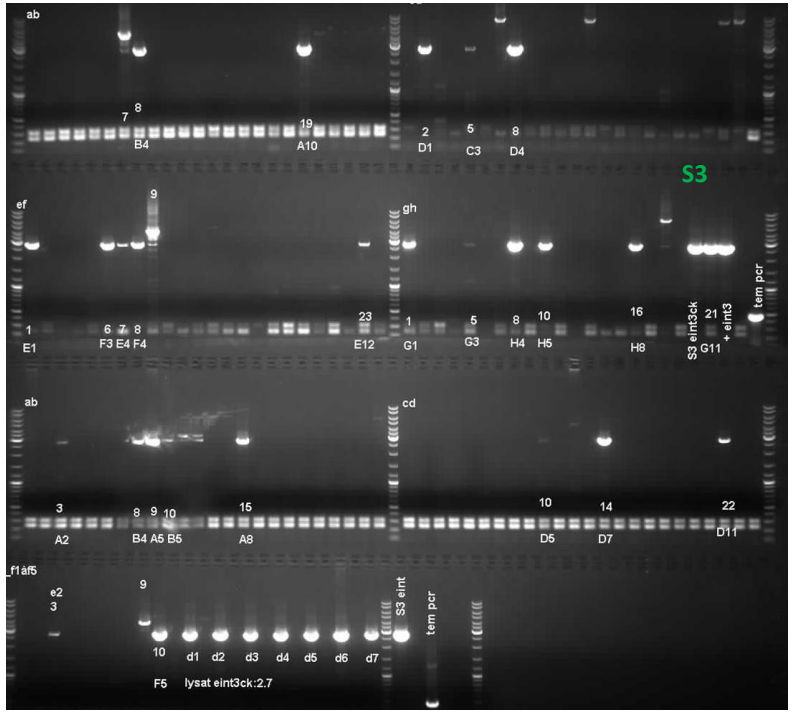


PCR	Primer Name	Primer sequences	PCR product size
5' PCR	Fext	GCCCTGTTTGTAAGTTCAAATTTGTA	2.7 kb
	Rlox	GTTATCTGCAGGTCGACCTTAAGCT	
5’ PCR	Fext	GCCCTGTTTGTAAGTTCAAATTTGTA	2.65 kb
	Rint	AGGCGTTTAAAAGAATGAACAGCAA	
5’ PCR	Fext	GCCCTGTTTGTAAGTTCAAATTTGTA	4 kb
	Rneo	GCGGCCGGAGAACCTGCGTGCAATC	

Long-Range 5’ PCR screening – results

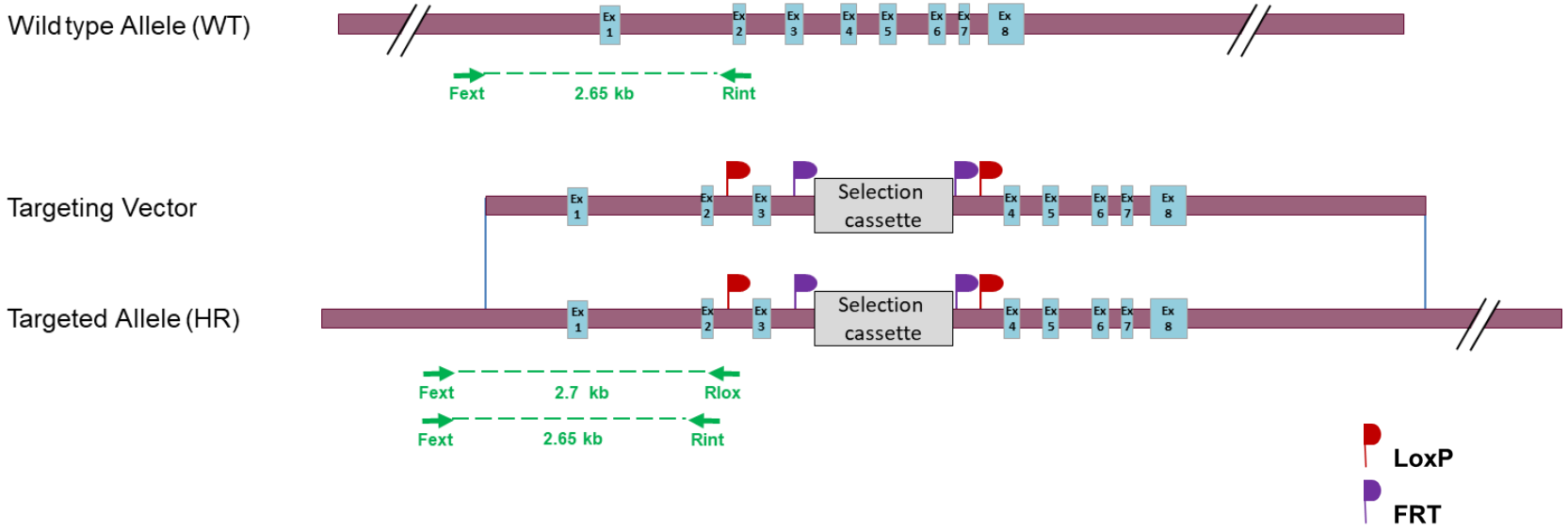


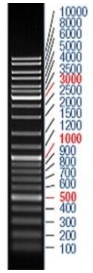
PCR Fext – Rlox : 2.7 kb



S3 : Control DNA

PCR Fext – Rint : 2.65 kb





Ladder pattern

Nine candidate clones out of the positive clones were selected for 5’ Long-Range PCR and Southern blot validation.

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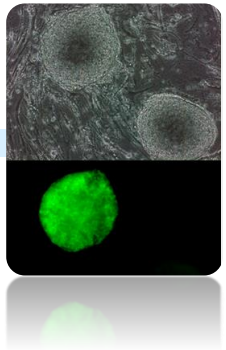
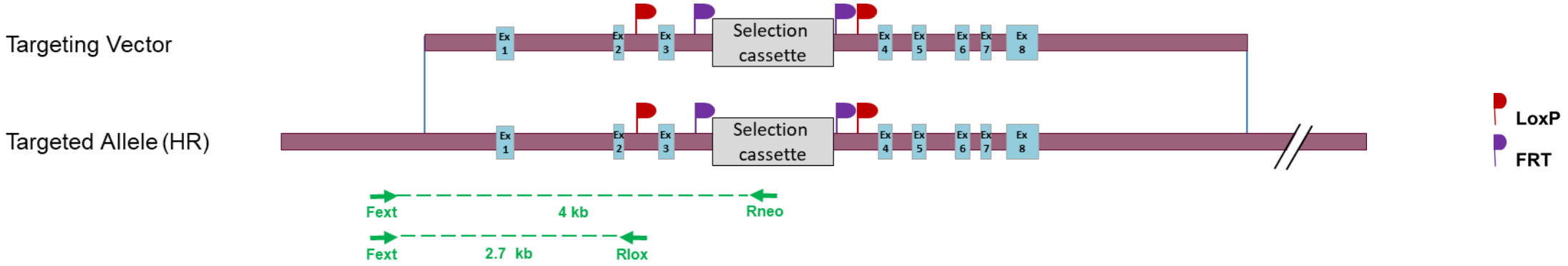
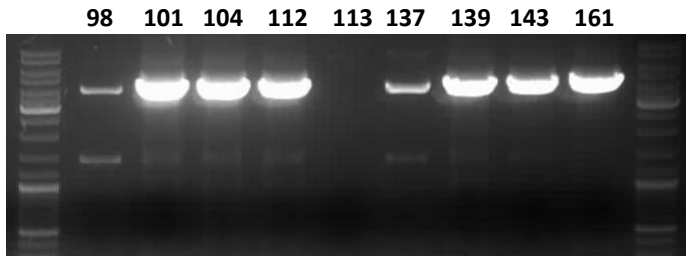
Recombinant ES validation by Long Range PCR

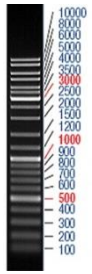


Confirmation and Validation of candidate recombinant ES clones by 5’ and 3’ PCRs

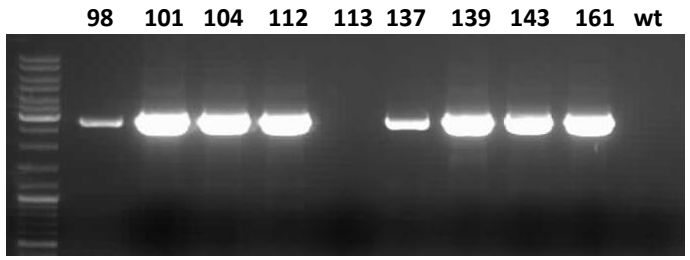




PCR Fext – Rneo : 4 kb



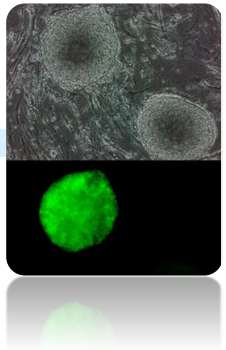
Ladder pattern



PCR Fext – Rlox : 2.7 kb

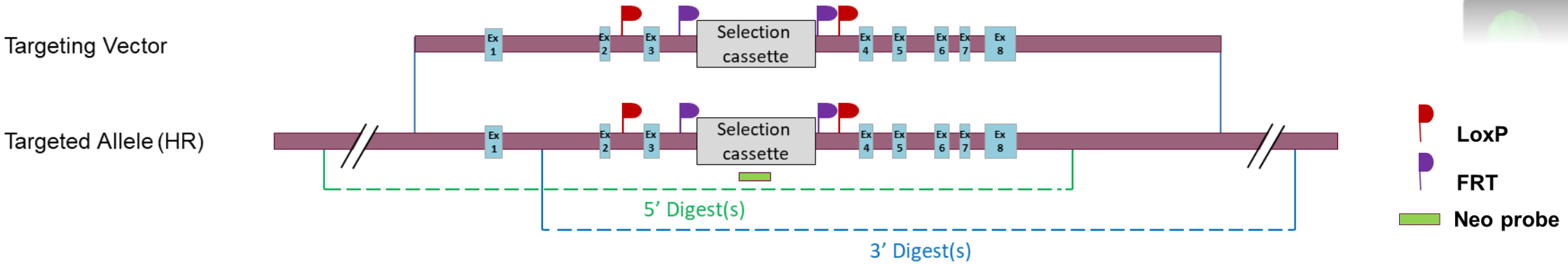
Nine candidate clones identified by 5’ PCR screening were further analysed by 5’ Long-Range PCR screening.
The next slides focus on clone #161 that was at the origin of the mouse line (and was analysed with other clones not show in this slide)

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.



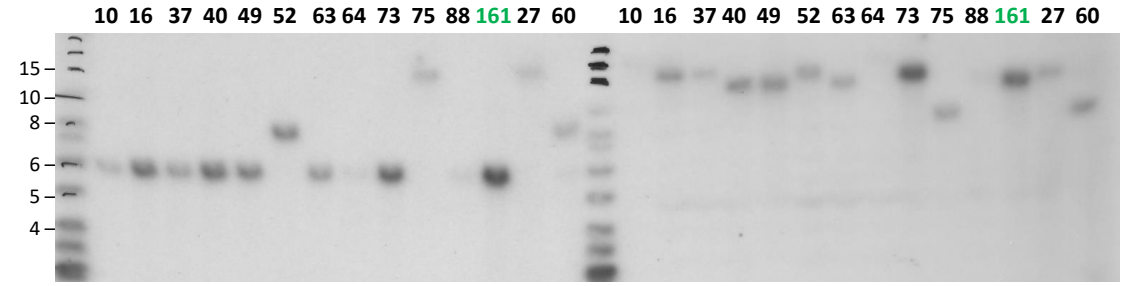
Digestions used to validate the 5' and 3' insertion

Probe		Genomic DNA digest	Targeted Allele (kb)
Neo	5' digest	BamHI	6.2
		AflIII	13.6
	3' digest	HindIII	5.9

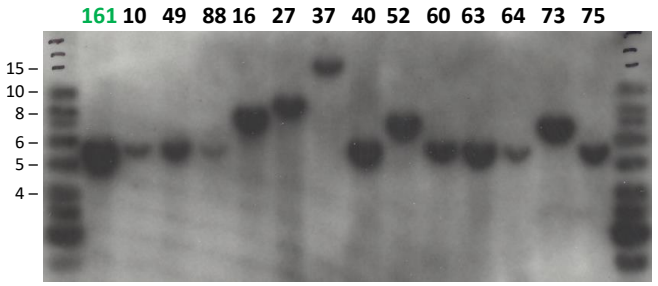
Neo probe sequence

AGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCG
AATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAG
CCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCA
ACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGCCAC
AGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATAT
TCGGCAAGCAGGCATCGCCATGGGTCACGACGAGATCCTCGC
CGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTG
GCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGA
CAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGAT
GTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCG
TATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCT
CGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCA
CTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAA
CGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCC
ACGATAGCCGCGCTGCCTCGTCCTGCAG

Southern blot - Neo 5'



Southern blot - Neo 3'



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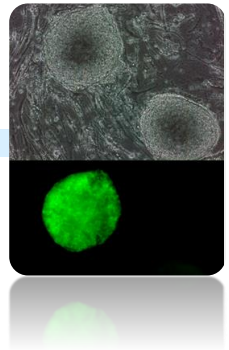
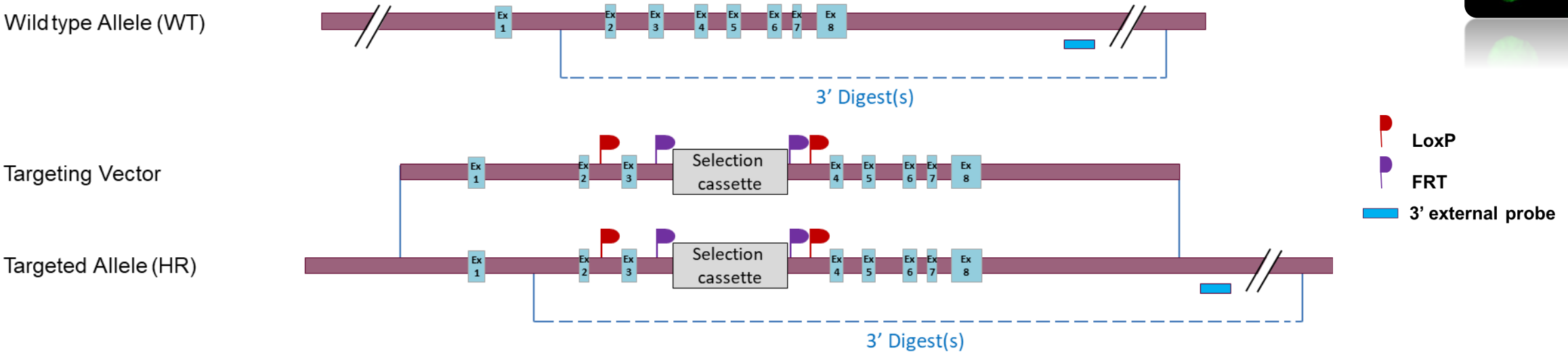
Recombinant ES clones validation by Southern Blot – External probe



Schematic Southern Blot validation strategy

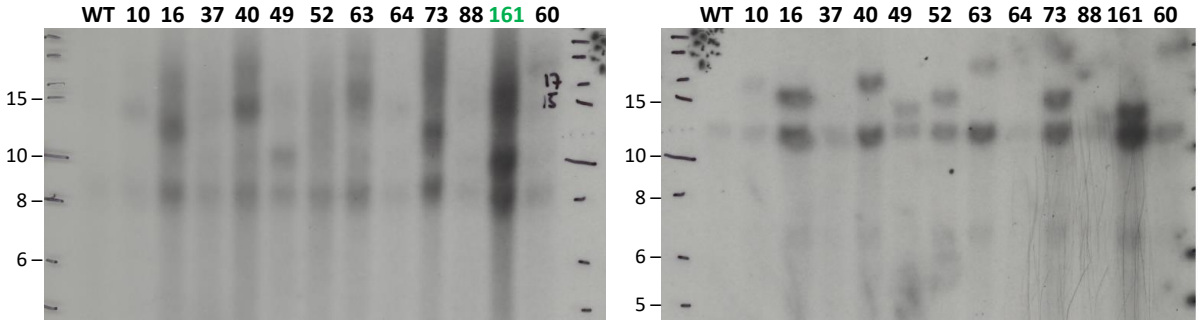
Digests on the scheme illustrate the position of the chosen restriction sites relative to the probe. They don't show the exact position of the restriction sites.



Digestions used to validate the 5' and 3' insertion

Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
3' external probe	3' first digest	NdeI	8.5	10.6
	3' second digest	BclI	12	14

Southern blot – 3' probe

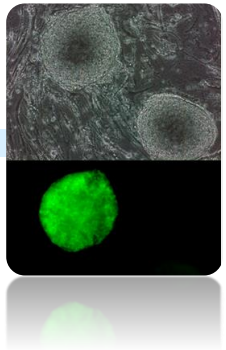


NdeI : 8.5 / 10.6 **BclI : 12 / 14**

3' probe sequence

GCGTGGTGGTCGAGCTCGAGTTCTATGAGGAGCAGGCCTGGCACATGGCCCCCCAAGGCATGGAGCTCCTCCTTTTCTGCCA
CCTCACGCACCAGGTGCTCGACCTCTTCGCCCCGAGCCCAGTGAGCAGACGGCTAGGATCCCGGCCACGGGCAACCTCTGAC
CCTGTGGGGGACTCCTGACCCCTAGGGTTAGCCTGTAAATTCCAGGGTGACCTCTGACACTACCTCCCCATTGTAAGCTCAG
GTTGCTTTGTGGGTGACCCCTGACCCCAGGTGACAACACGGGCGACCCCGTGTGTGACCCCAGTTTGACCCCTTACACCAGG
TGTCGTCACGGGTGACCCCAGC

Aneuploidy screening in ES recombinant clones



Selected recombinant ES cells clones were karyotyped by ddPCR as described in Codner *et al.* [1] and by Giemsa metaphase staining. Results of aneuploidy analysis are presented in the table below.

Clone ID	ddPCR	Giemsa
#49	Not Done	Pass
#88	Not Done	Failed
#161	**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**

MICROINJECTION & BREEDING

- Microinjection
- Breeding to F1 generation

Microinjection

- The ES cells used in the injection experiment were originally derived from a C57BL/6NCrl 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 #49 and #161 validated in previous project phase were 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
#49	0	0	0	0
#161	**3**	**1**	**13**	**17**

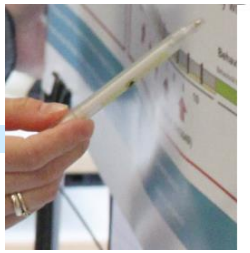
Breeding to F1 generation

- Three highly chimeric males generated in the previous phase by blastocyst injection of ES clone #161 were mated with C57BL/6N Flp deleter 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 : 23/09/2015

- Allele nomenclature (following MGI guidelines) : **Crlf2$^{em1.1Ics}$**

6 SEQUENCE OF THE DELIVERED ALLELE



CTTTTCCTTTTCTCTGTTTGTGAATATGCAAATGAGGTGTCGCCCACCGCTACAGTTCTTGGGCCTCTCGTTTACATATTCATTCCCTATATTTGCGTACGAGCCAGAATGTCTTCCT
CTGTCTTCCCCTGCCTAGTGCATATGCAAATGAGGCCCGGCCTGCTCCCAGCGCCCTAGCCAAGGGTCTGAGTCAGGGAAATGGCTGCGATGGCTCTTCTGGAACGCGCTGACGTCAC
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TAGATGCATGGCCTTTGACCCCACCCATGACATTTGAATCCTTCCCGCCTCAGCCCGACCTCTCTCGCCTCTGGCCCCAAACTGTGACCT

LoxP FRT Exon 3

phenomin
EXCELLENCE IN MOUSE PHENOGENOMICS

REPORT REDACTION & VALIDATION
Protocol finalized on 2024/10/25
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Genotyping protocol

TSLPR

/ K515b

(ICS internal reference)

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TABLE OF CONTENTS

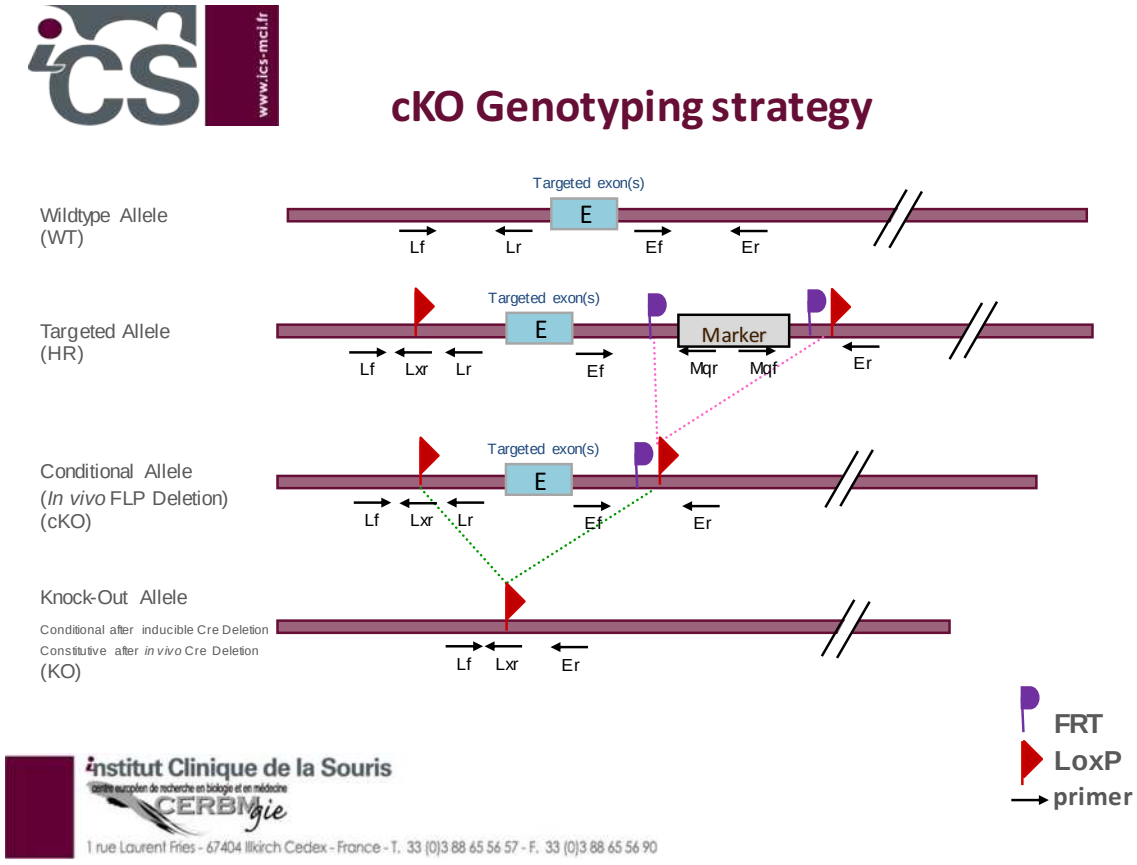
Table of contents **2**
1. Genotyping protocol and data **2**
1.1. Genotyping strategy 2
1.2. PCR protocol 4
2. Cre and Flp genotyping method **5**

1. Genotyping protocol and data

This section describes the condition used at the Mouse Clinical Institute (ICS) to genotype your **TSLPR** Conditional Knockout (cKO) project.

1.1. Genotyping strategy

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



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1 rue Laurent Fries - 67404 Illkirch Cedex - France - T. 33 (0)3 88 65 56 57 - F. 33 (0)3 88 65 56 90

Sequence of primers used for genotyping:

Position	Primers	Sequence
Ef	8700	CGGCGCTTAGACGGGAAGTTG
Er	8701	GTGGCGGTGACATCACTTTCTGTCC
Er[2]	8702	GGCTTAATCCACACCCACCGAG
Lf	8698	CGGGATTTCCTCTCATTCCTCTTCAC
Lf[2]	8697	CAACTTGAGCCTGGAGTTCCGGTG
Lr	8699	GAAAGTGATGGGCAGAAAGGAAGTACC
Lxr	4724	CGAAGTTATCTGCAGGTCGACCTTAAG
Mqf	6	GAAGAACGAGATCAGCAGCCTCTGTTCC
Mqr	238	TGACTAGGGGAGGAGTAGAAGGTG

[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)	cKO allele	KO allele	WildType allele
Presence of the distal loxP	8698-8699	Lf / Lr	256	256	---	176
Excision of the selection marker	8700-8701	Ef / Er	2220*	367	---	241
5' part of the selection marker	8700-238	Ef / Mqr	389	---	---	---
3' part of the selection marker	6-8702	Mqf / Er[2]	336	---	---	---
LoxP specific PCR	8697-4724	Lf[2] / Lxr	200	200	200	---
Excision of the floxed exon(s), i.e. knock out	8697-8701	Lf[2] / Er	2954*	1101*	356**	895*
Excision of the floxed exon(s), i.e. knock out 2	8698-8702	Lf / Er[2]	2890	1037	292**	831

*: this PCR product will not be observed using our PCR genotyping 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.

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.