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MODEL GENERATION TECHNICAL REPORT

Generation of mouse model :

Cdkl5 conditionnal Knock-out

Project code: G20 / IR00003734

Report finalized: 08/09/2023

cnrs
Université de Strasbourg
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CERBM centre européen de recherche en biologie et en médecine gie
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MODEL GENERATION TECHNICAL REPORT

1 PROJECT PROCESS & QUALITY CONTROL

2 GENETIC STRATEGY

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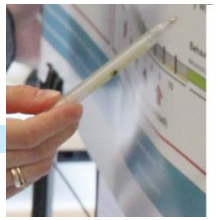
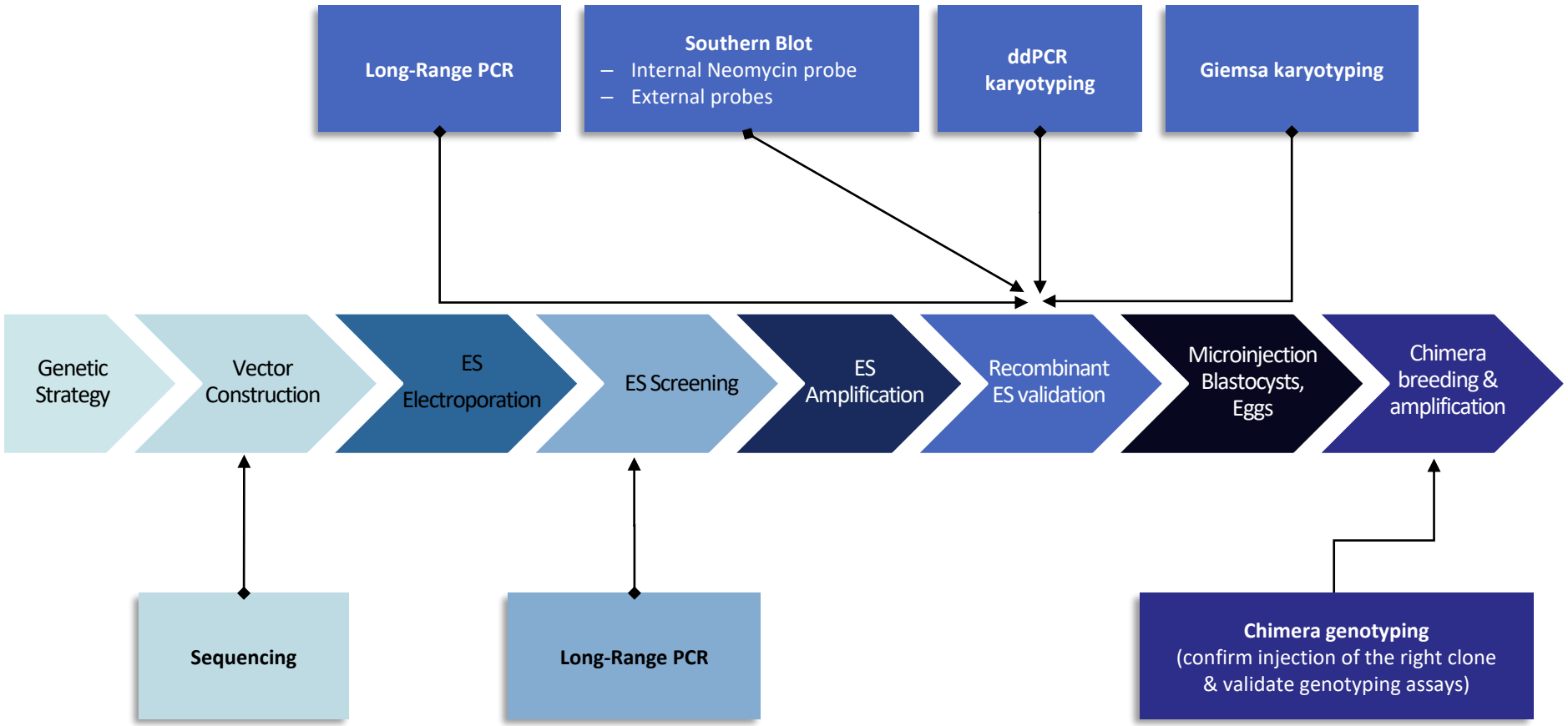
4 ES ELECTROPORATION & SCREENING OF RECOMBINANT CLONES

5 MICROINJECTION & BREEDING

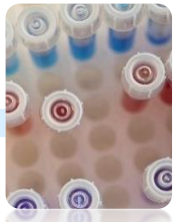
6 SEQUENCE OF THE DELIVERED ALLELE

PROJECT PROCESS & QUALITY CONTROL



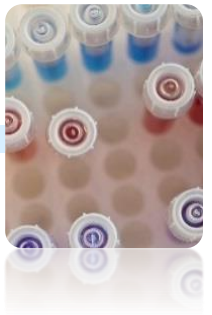


2 GENETIC STRATEGY

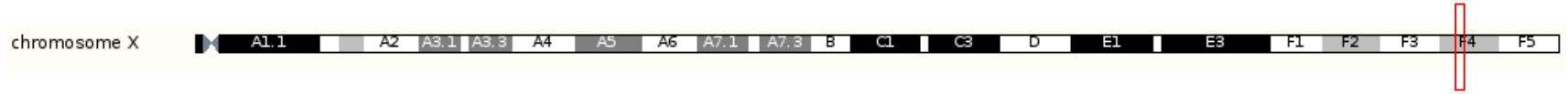


- Target locus structure
- mRNA(s) and protein(s)
- Genetic strategy
- PRO & CONS evaluation of the strategy

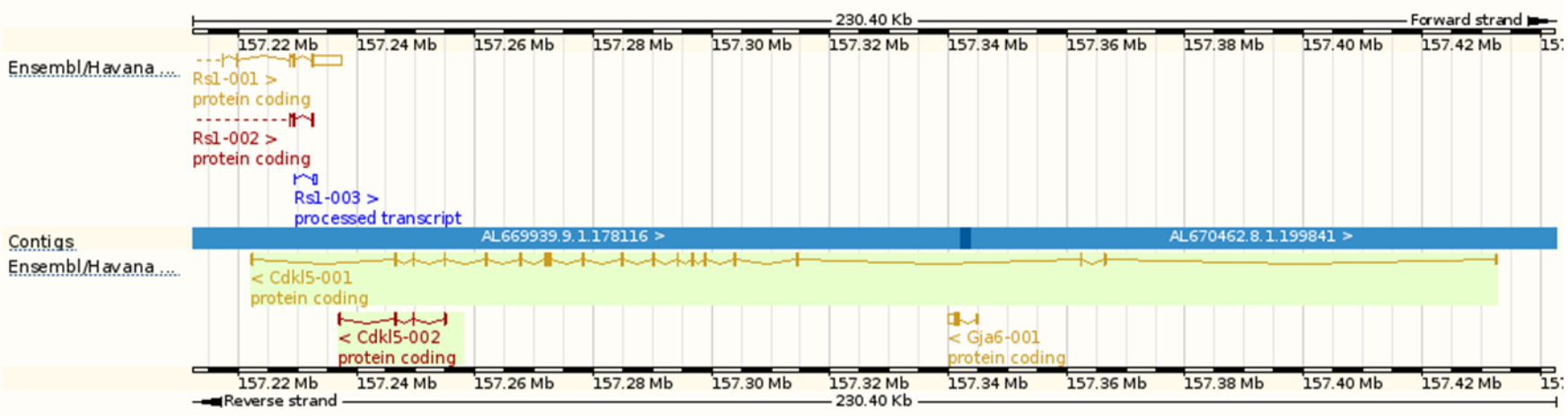
Cdkl5 mouse genomic locus – structure



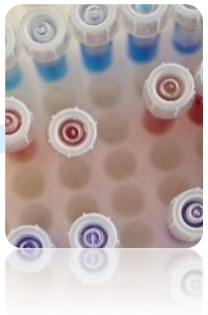
Location :



Ensembl Gene ID: Cdkl5 **ENSMUSG00000031292**

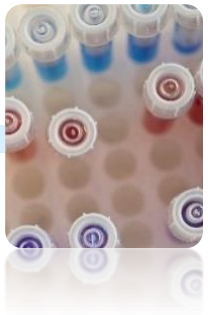


Cdkl5 mRNA(s) and protein(s)

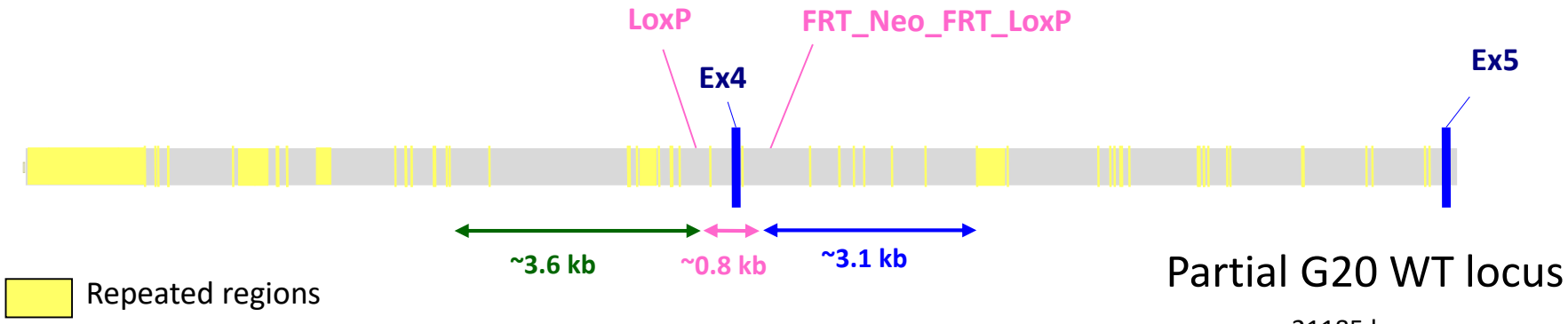


Name	Transcript ID	Length (bp)	Protein ID	Length (aa)
Cdkl5-001	ENSMUST00000087104	3267	ENSMUSP00000084342	938
Cdkl5-002	ENSMUST00000112355	654	ENSMUSP00000107974	149

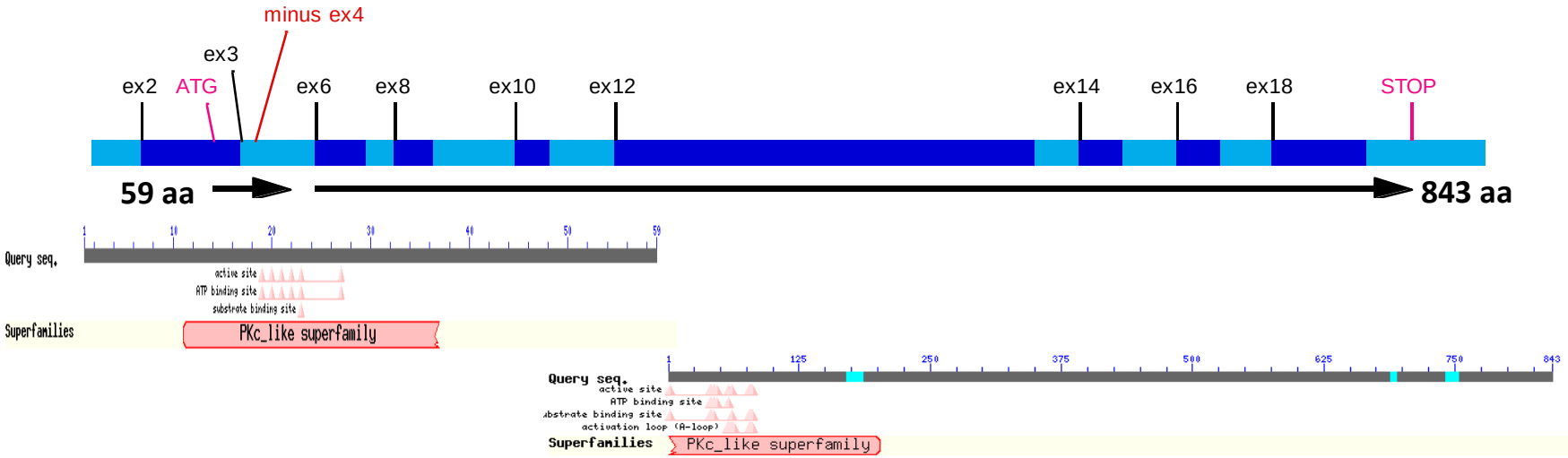
Approach chosen: flox exon 4



Targeted locus



mRNA and protein expected after Cre mediated recombination



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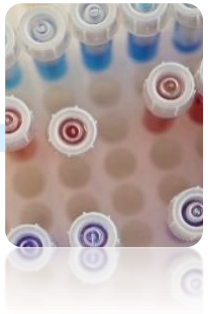
PROs& CONs evaluation of the strategy

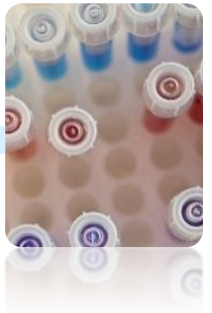


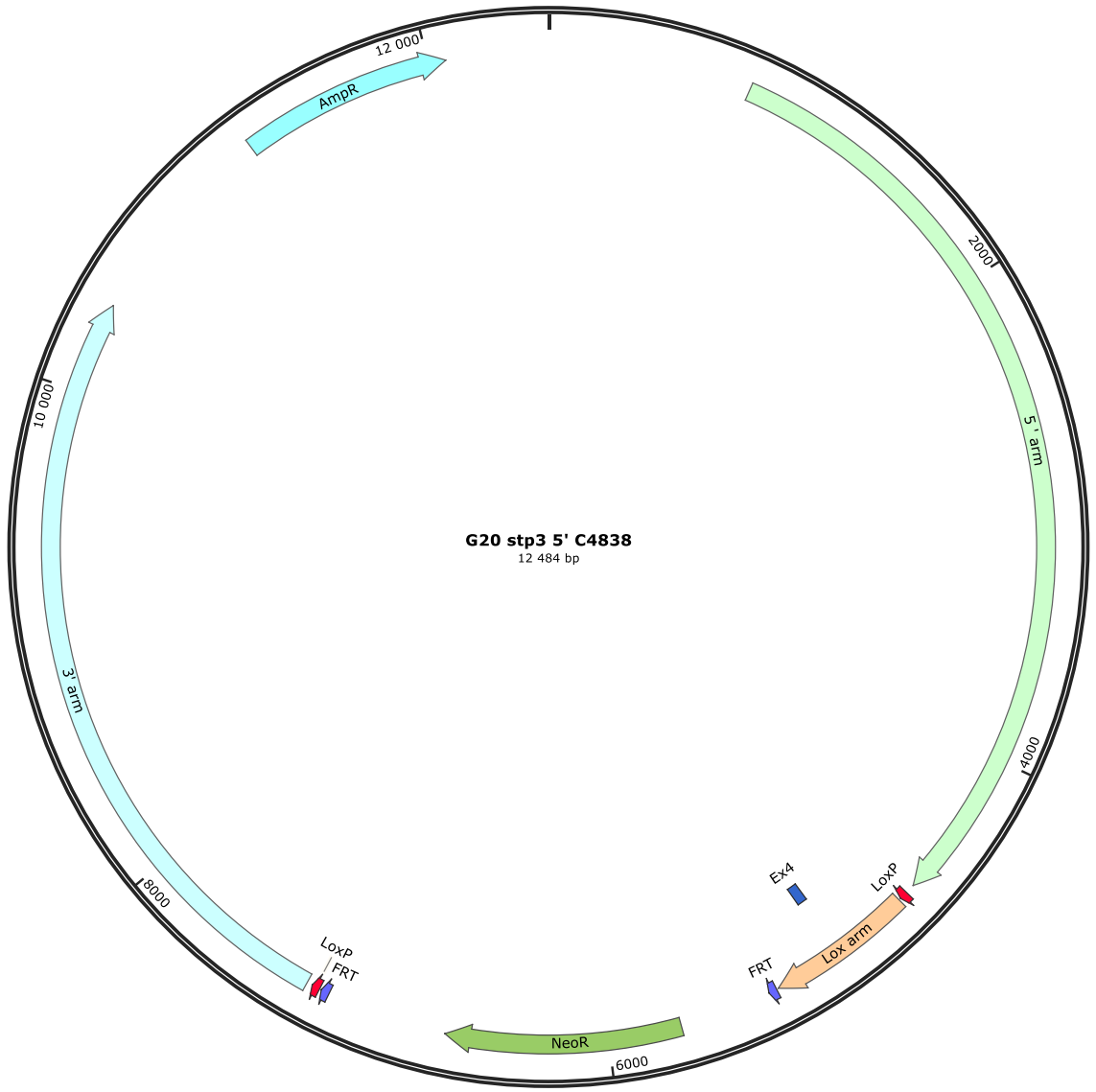
- **Pros**
  - Same strategy than the one proposed by EUCOMM
- **Cons**
  - Presence of repeated regions might render PCR amplification and screening difficult.

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)

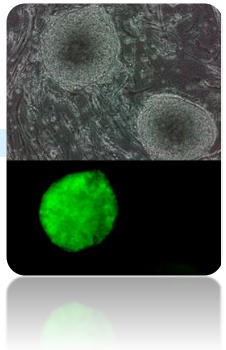
Highly-efficient, fluorescent, locus directed cre and FlpO deleter mice on a pure C57BL/6N genetic background.
Birling MC, Dierich A, Jacquot S, Hérault Y, Pavlovic G. Genesis. 2012 Jun;50(6):482-9. doi: 10.1002/dvg.20826.

3 HOMOLOGOUS RECOMBINATION - VECTOR CONSTRUCTION



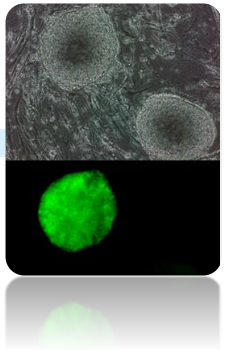


4 ES cell electroporation & Screening of recombinant clones



- 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



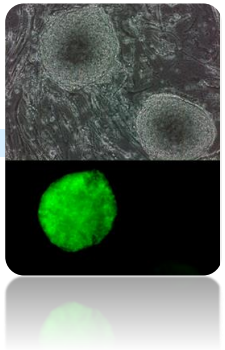
The targeting vector was electroporated in the proprietary C57BL/6N BD10 cell line.

Transfected ES clones were submitted to neomycin selection (G418) and 93 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.

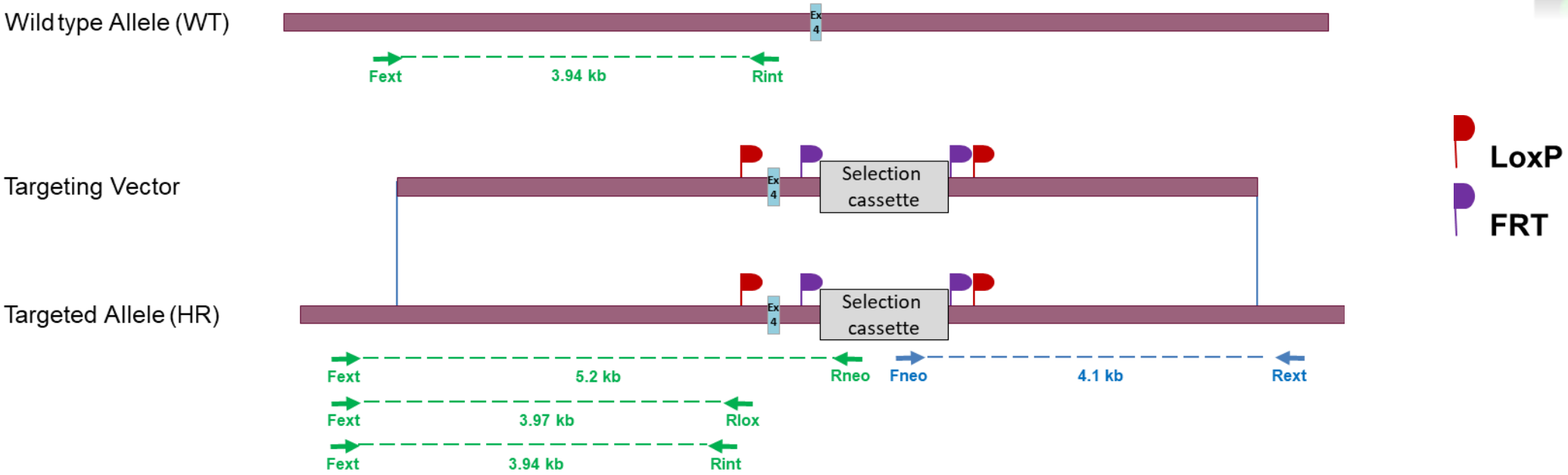
Screening process steps:

1. Identification of candidate recombinant clones by initial 5’ Long-Range PCR
2. Nine of 5’ PCR positive clones are confirmed for 3’ 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

Long range PCR screening – strategy

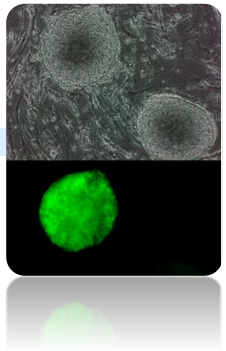


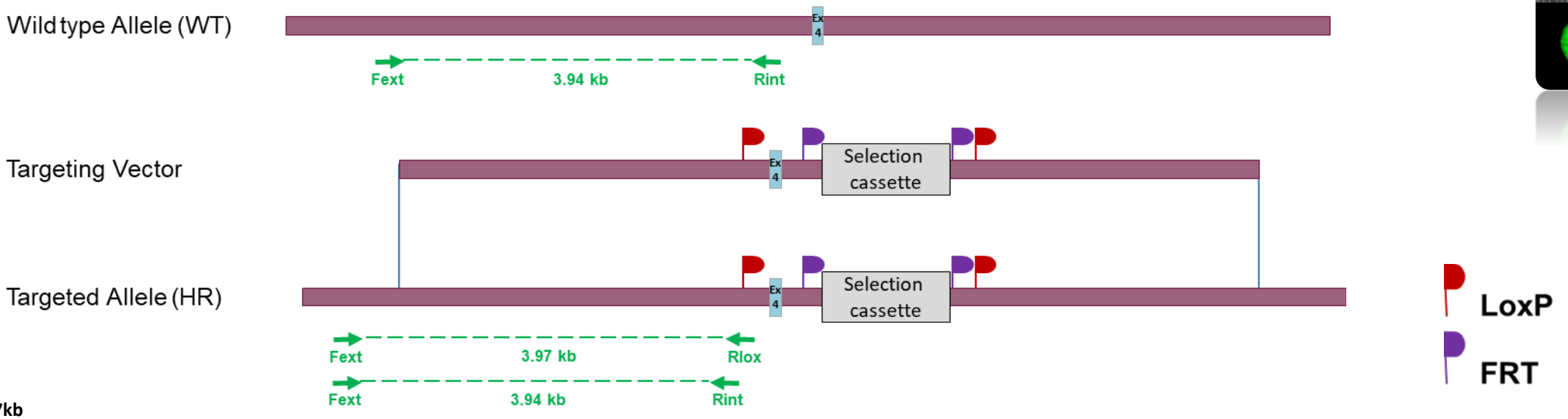
Schematic 5’ and 3’ PCR screening strategy



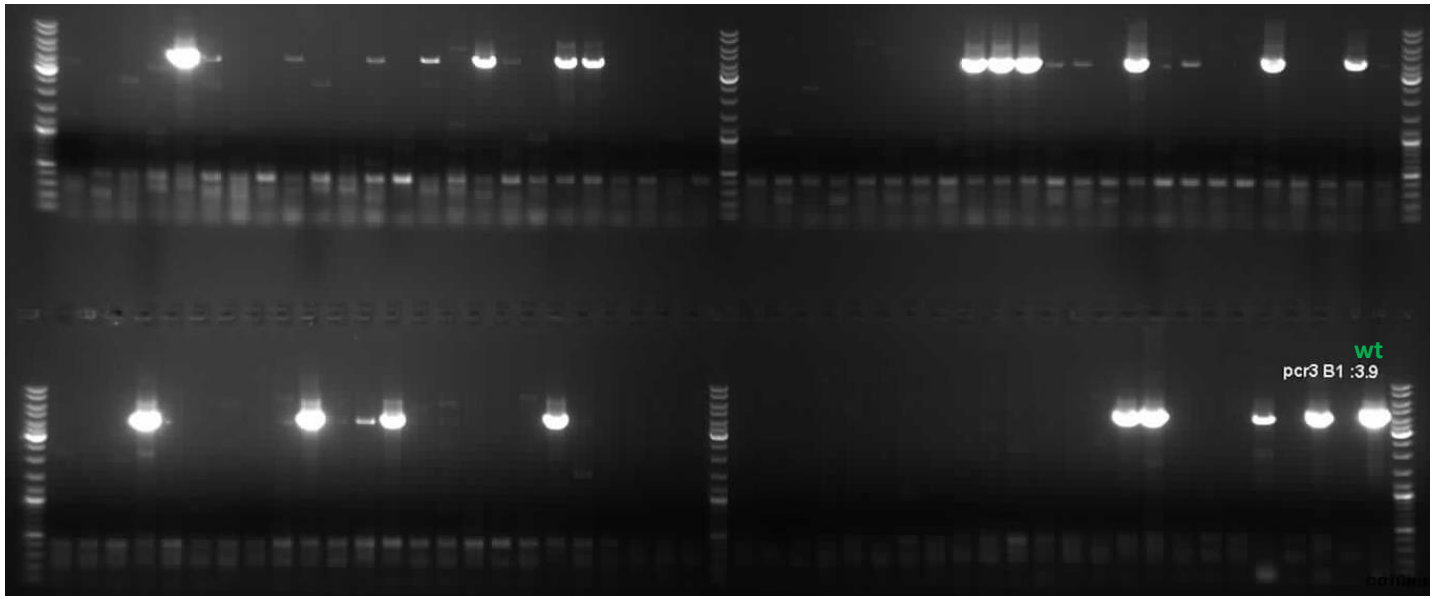
PCR	Primer Name	Primer sequences	PCR product size
5' PCR	Fext	GCCTTTTTGAATCCCATACTGGCCA	3.97 kb
	Rlox	GTTATCTGCAGGTCGACCTTAAGCT	
5’ PCR	Fext	GCCTTTTTGAATCCCATACTGGCCA	3.94 kb
	Rint	AATATGGCCGGCCTCTTACCAGTTTGGTATATTTCTTC	
5’ PCR	Fext	GCCTTTTTGAATCCCATACTGGCCA	5.2 kb
	Rneo	GCGGCCGGAGAACCTGCGTGCAATC	
3’ PCR	Fneo	AGGGGCTCGCGCCAGCCGAACTGTT	4.1 kb
	Rext	TAATGGCCTCAGTGGCCAATCTCAAAACTTCTTTTAACCAA	

Long-Range 5’ PCR screening – results

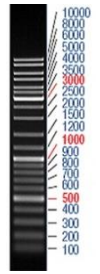




PCR Fext – Rlox : 3.97kb



wt : Control DNA



Ladder pattern

PCR Fext – Rint : 3.94 kb

Ten candidate clones out of the 10 positive clones were selected for 3’ Long-Range PCR and Southern blot validation.

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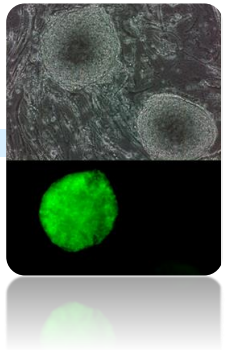
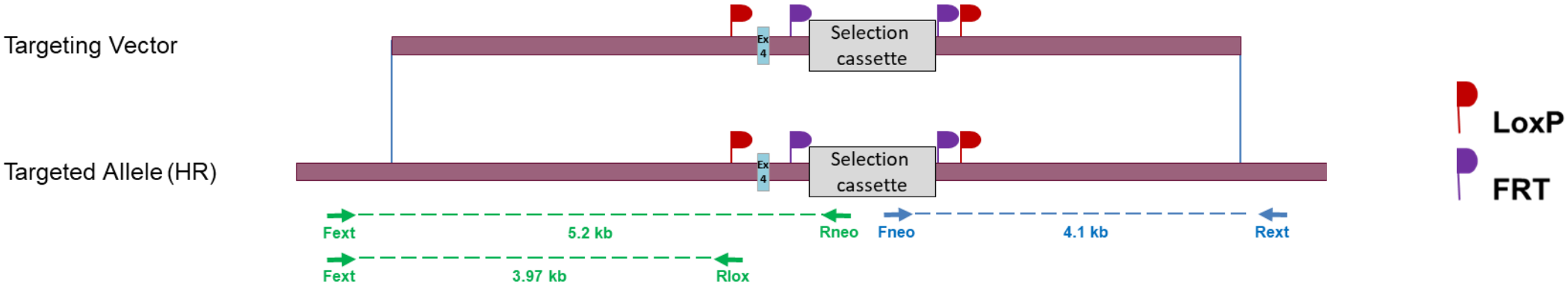
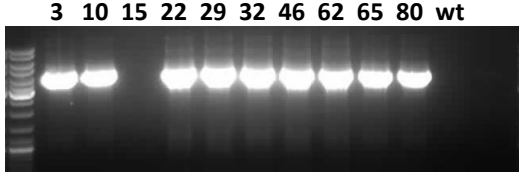
Recombinant ES validation by Long Range PCR

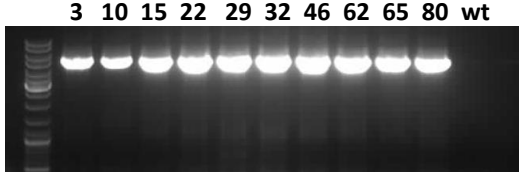


Confirmation and Validation of candidate recombinant ES clones by 5’ and 3’ PCRs

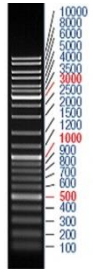




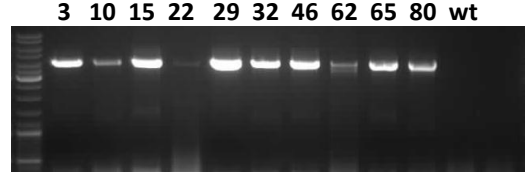
PCR Fext – Rlox : 3.97 kb



PCR Fext – Rneo : 5.2 kb



Ladder pattern



PCR Fneo – Rext : 4.1 kb

Ten candidate clones identified by 5’ PCR screening were further analysed by 3’ PCR screening.
Nine clones (clones #3, #10, #22, #29, #32, #46, #62, #65 and #80) were confirmed.

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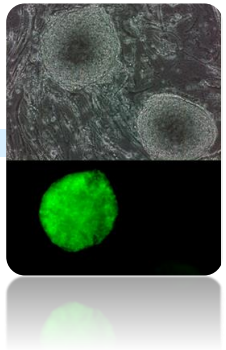
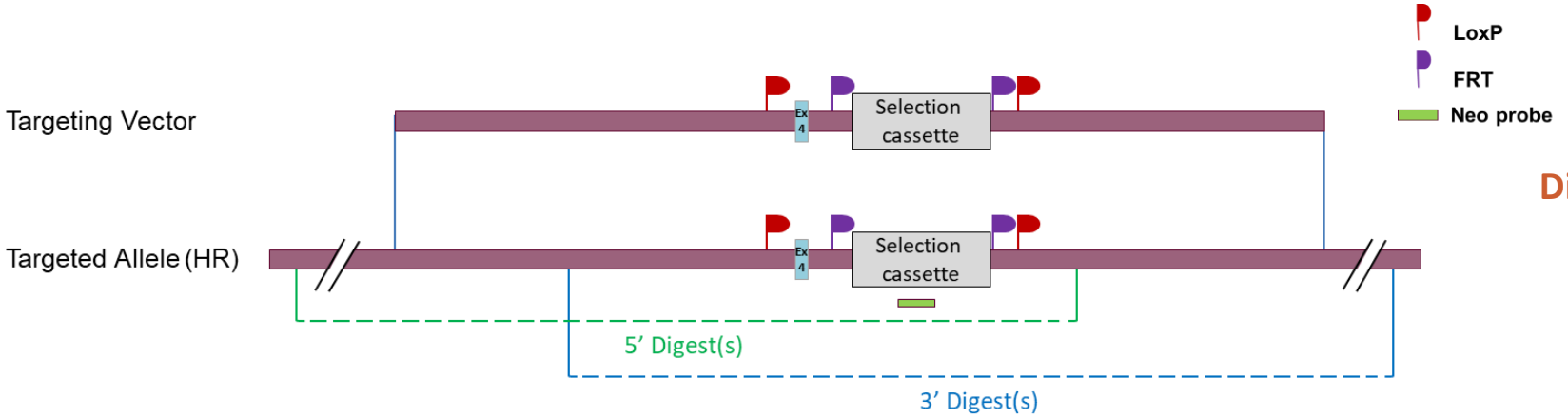
Recombinant ES clones validation by Southern Blot – Internal probe



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.



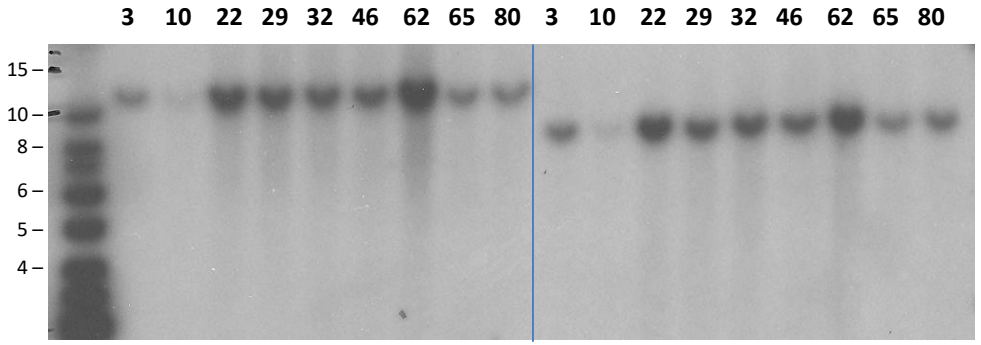
Digestions used to validate the 5' and 3' insertion

Probe		Genomic DNA digest	Targeted Allele (kb)
Neo	5' digest	NheI	12.1
		SbfI	9
	3' digest	PacI	6.5
		AflII	12.1

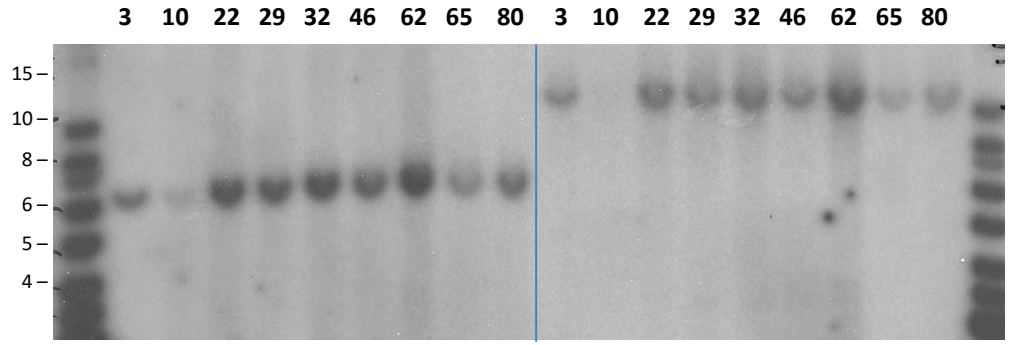
Neo probe sequence

CTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTC
CTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGT
ACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCAT
GGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAG
CTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGGGGATCCGCTGTAAGTCT

Southern blot - Neo 5'



Southern blot - Neo 3'



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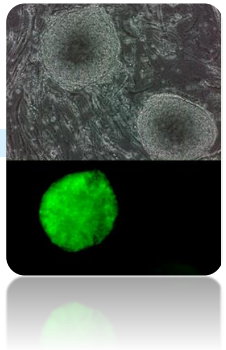
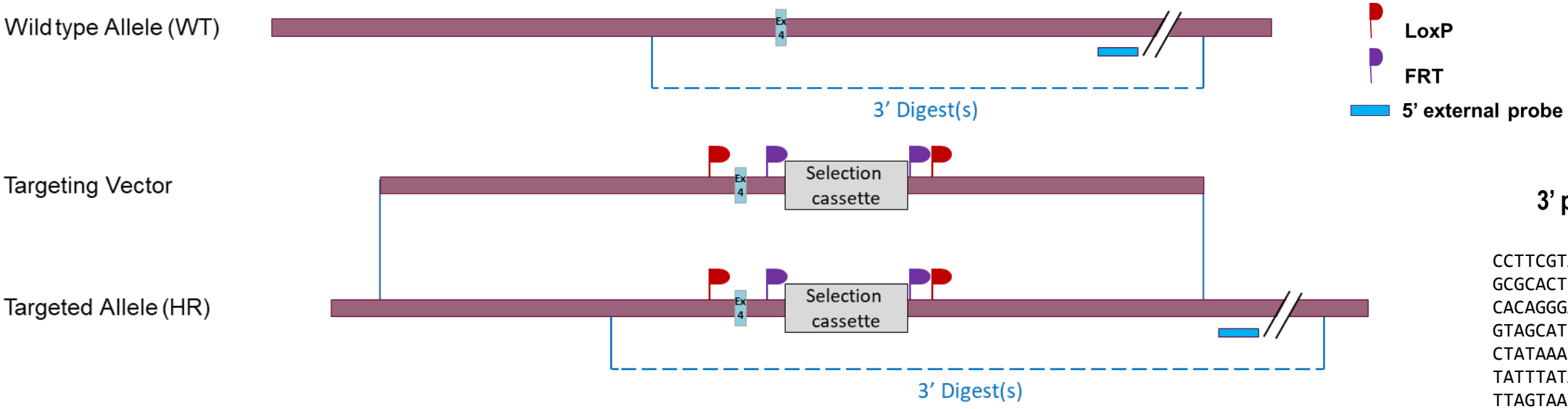
Recombinant ES clones validation by Southern Blot – External probe



Schematic Southern Blot validation strategy

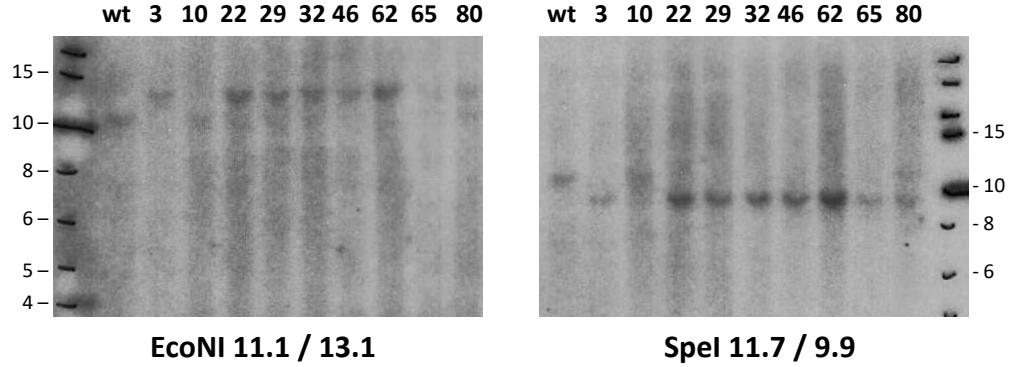
Digests on the scheme illustrate the position of the chosen restriction sites relative to the probe. They don't show the exact position of the restriction sites.



3' probe sequence

CCTTCGTACATGGCTACCTCCAGTGAGCT
GCGCACTCAGCTCTGTGTCACTGGGGTGA
CACAGGGATGCTTTTATATCAAAAAATGG
GTAGCATTTTATCTCCTGTTGTTTGTTTC
CTATAAATGTTTTTCTCTTGAAATGGACA
TATTTATATCTTTAATTCTTATGAAATGT
TTAGTAATTGTATAGTCTTTCCTACAGTT
GATTCCACTCTTATTTTTACCTAGAGACT
AGCAAACTCTGTACTGATCTCTTAGTTTG
CATGATGCTGTAAAACAAGAATTCGTGAA
TTGTAAACTTTAAGAGCCTAGATTCTGTA
TAAGAGAGGCCTTGAAACAAAATTTCCGG
CCTTGATTATCTAAAGAATCTGATTATCT
GAGCTGCAGCACCAGCTACTCC

Southern blot – 3' probe



EcoNI 11.1 / 13.1

SpeI 11.7 / 9.9

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	EcoNI	11.1	13.1
	3' second digest	SpeI	11.7	9.9

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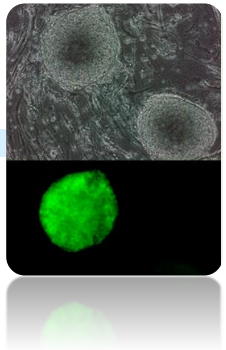
Aneuploidy screening in ES recombinant clones



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

Clone ID	Giemsa
#3	**Pass**
#22	Not done
#29	Failed
#32	Not done
#46	**Pass**
#62	Failed

5 MICROINJECTION & BREEDING

- Microinjection
- Breeding to F1 generation

Microinjection

- The ES cells used in the injection experiment were originally derived from a C57BL/6N 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 ESC clones #3 and #46 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
#3	4	4	9	17
#46	0	0	5	5

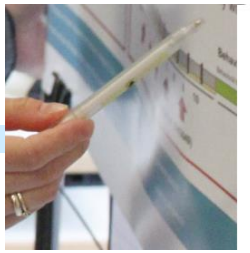
Breeding to F1 generation

- Eight 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 : 02/01/2013

- Allele nomenclature (following MGI guidelines) : **Cdkl5$^{tm1.1Ics}$**

6 SEQUENCE OF THE DELIVERED ALLELE



LLOYD'S REGISTER · LRQA
ISO 9001 · NFX 50-900

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CTCTGGGAATGAAGATGCTCTTGGAGTATGTTGATTGACAGACTATTTAAATAACCATCTATTACAAATGGAGCTTTAAGCAGTTTTTACCATCAGGTATAAATGTTACTTATATAATGGG
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TACCAAACTGGTAAGAGGCCGGCCAAGCTTCTCGAGCTTAAGGTCGACCTGCAG**ATAACTTCGTATAATGTATGCTATACGAAGTTAT**TTAATTAAGTTATATGAATATTTCTGGATGTCT
TCTTTATTTTGTTTCTTTTGTGTCCTGTACTTTTGAAGACATTGATAGTGAAGAATTATGTTTCATACATAGCGTGCCAGTGTCTGTTCTTTGCCCCCTTGCTACCCTCTTTCTTAACCTT
CCTTGGCAATGTCTCTAGGGCGGTAAGAGGATTCTGAAACAGAGTAGTGGGTGGATGGAGATGCTCTGTCTTAGCTCCTCTGCCCGCAGATAATTGTGATTCCCAGTTGGGACTAGAGAAT
GATTTTCCCTCTGCCTCTCTTTCCTTTTCAG**GAAACACATGAAATTGTGGCAATCAAGAAATTCAAGGACAGCGAAG**GTATATGTACATTTTTACTTCTGTACTTTTTTTTGTAAGTAAAA
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CTCATGCTATTAAAACATCTATTTTAATTTACAATCAAGGGTGGAATTTTTGCTCCAAAGGCCAGATAGAAGTTTTCTTAACTACATGTTTCACCGGTG**GAAGTTCCTATTCTCTAGAAAG**
TATAGGAACTTCGCGGCCGG**ATAACTTCGTATAATGTATGCTATACGAAGTTAT**GGATCCATCGACCCCCTGCAGGCACATAGATGACAATGGAAGAGATGATTAAAAGCAAATGCATGTG
TAGCTCCCATAAGTGTGAGTGCAGCATTTGGTCATGGAGTTCTCTAGCCCCTAGTCACAGCAAGGAATACAAGAAGTGGAAGGCAGGATGGAAAGGGGAATTTTTATATTTAGGACCATTT
TTTTGGAGCAAATAAGATTTTCAATAACAGATACCATATTTGGTGTGGGGTGGGGGGTTTGGTCACACTTTTAGAGCATTAAAATTCTCTGCTGGGCAAGAAAGTTTCAAACAGCCTTAGC
TGTTAACTCTTACCTAACCAAAGACAAAGAAAGACTGATAATGAAAGTCTACATGGCTTTTGAGATTTAGGCAGCAGTTCAGAGACCTCAGTAAAAAGACTTTCTAAATCCTAGCCCAGTT
TCCATATCGACACTATACTATAATGAAGCAAGTCCTACAAATTCGTGTGTTAATGAGGTACTCAATCTTCTACTTTCACTGCTTTATGTCAA

LoxP **FRT** **Exon 4**

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REPORT REDACTION & VALIDATION
Protocol finalized on 2023/09/08
Prepared by Romain LORENTZ, IE
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Genotyping protocol

Cdkl5

IR00003734 / G20

(ICS internal reference)

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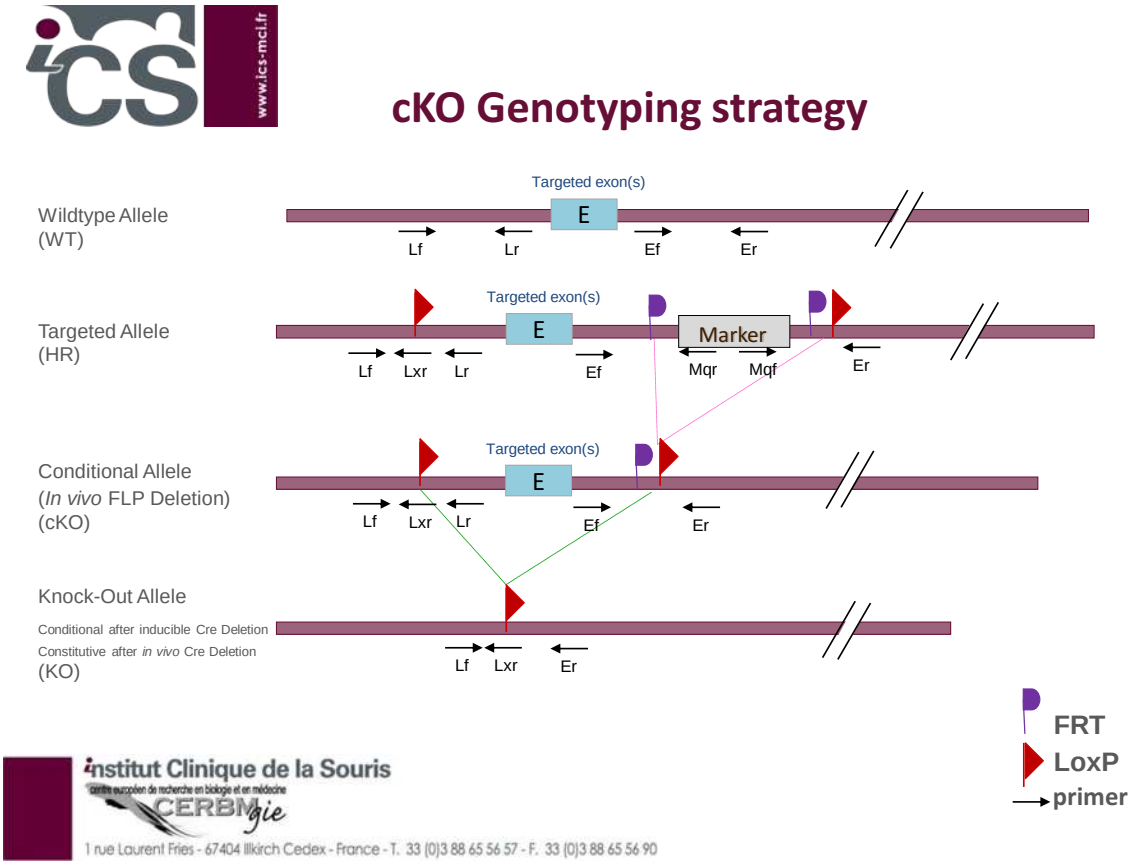
TABLE OF CONTENTS

1. Genotyping protocol and data

This section describes the condition used at the Mouse Clinical Institute (ICS) to genotype your **Cdkl5** 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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Sequence of primers used for genotyping:

Position	Primers	Sequence
Ef	6721	GGTGGAATTTTTGCTCCAAAGGCC
Ef[2]	6719	TGGGGAGGGAATGCAACTTGTTT
Er	6720	GAACTCCATGACCAAATGCTGCAC
Lf	6722	CTGAAGAAATATACCAAACTGGTAAGAG
Lr	6723	GGCACGCTATGTATGAAACATAATTC
Lxr	4724	CGAAGTTATCTGCAGGTCGACCTTAAG
Mq1f	1219	CAGCTCATTCCTCCCACTCATGATC
Mq1r	265	TGCTAAAGCGCATGCTCCAGACTGC

[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 (with DMSO)	6722-6723 (with 5% DMSO)	Lf / Lr	210	210	---	130
Excision of the selection marker (with DMSO)	6721-6720 (with 5% DMSO)	Ef / Er	2098*	244	---	140
5' part of the selection marker	6719-265	Ef[2] / Mq1r	258	---	---	---
3' part of the selection marker	1219-6720	Mq1f / Er	405	---	---	---
LoxP specific PCR (with DMSO)	6722-4724 (with 5% DMSO)	Lf / Lxr	74 !	74 !	74 !	---
Excision of the floxed exon(s), i.e. knock out	6722-6720	Lf / Er	2753*	899*	208**	715*

*: 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
! : GC rich region, taq Ozyme HS (réf: OZYA002-250) is used if no results is obtained with the Faststart PCR Master

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:

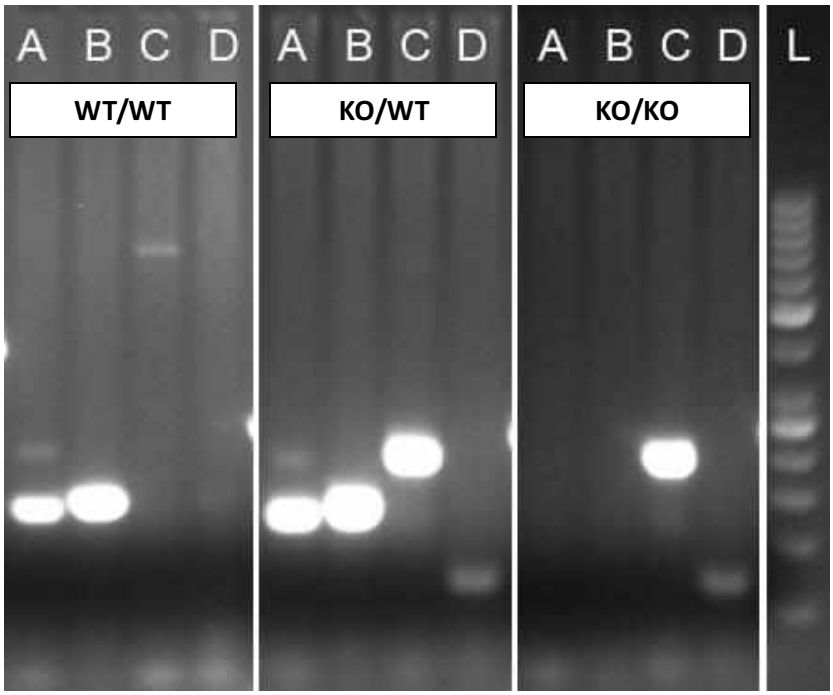
Temp	Time	#Cycles
95°C	4min	1
94°C	30s	
62°C	30s	34
72°C	1min	
72°C	7min	1
20°C	5min	1

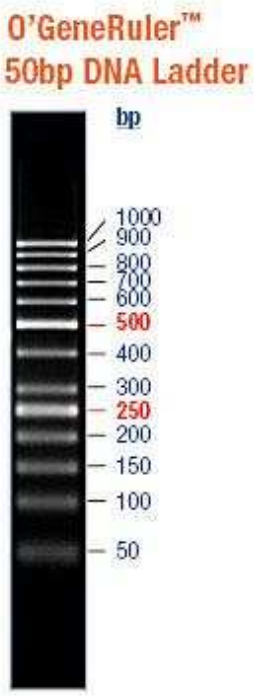
NB: These PCR conditions have been optimized for high-throughput genotyping. Adaptation to small-scale may be required.

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: Presence of the distal loxP
B: Excision of the selection marker
C: Excision of the floxed exon(s), i.e. knock out
D: 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.