phenomin
EXCELLENCE IN MOUSE PHENOGENOMICS

MODEL GENERATION TECHNICAL REPORT

Generation of mouse model : Tubb3 conditional point mutation

Project code: G5 / IR00003727

Report finalized: 05/09/2023

cnrs
Université de Strasbourg
Instituts thématiques Inserm institut national de la santé et de la recherche médicale
CERBM centre européen de recherche en biologie et en médecine gie
Aix*Marseille université

MODEL GENERATION TECHNICAL REPORT

1 PROJECT PROCESS & QUALITY CONTROLS

2 GENETIC STRATEGY

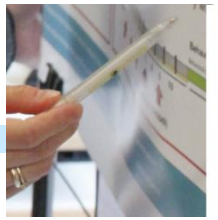
3 HOMOLOGOUS RECOMBINATION VECTOR CONSTRUCTION

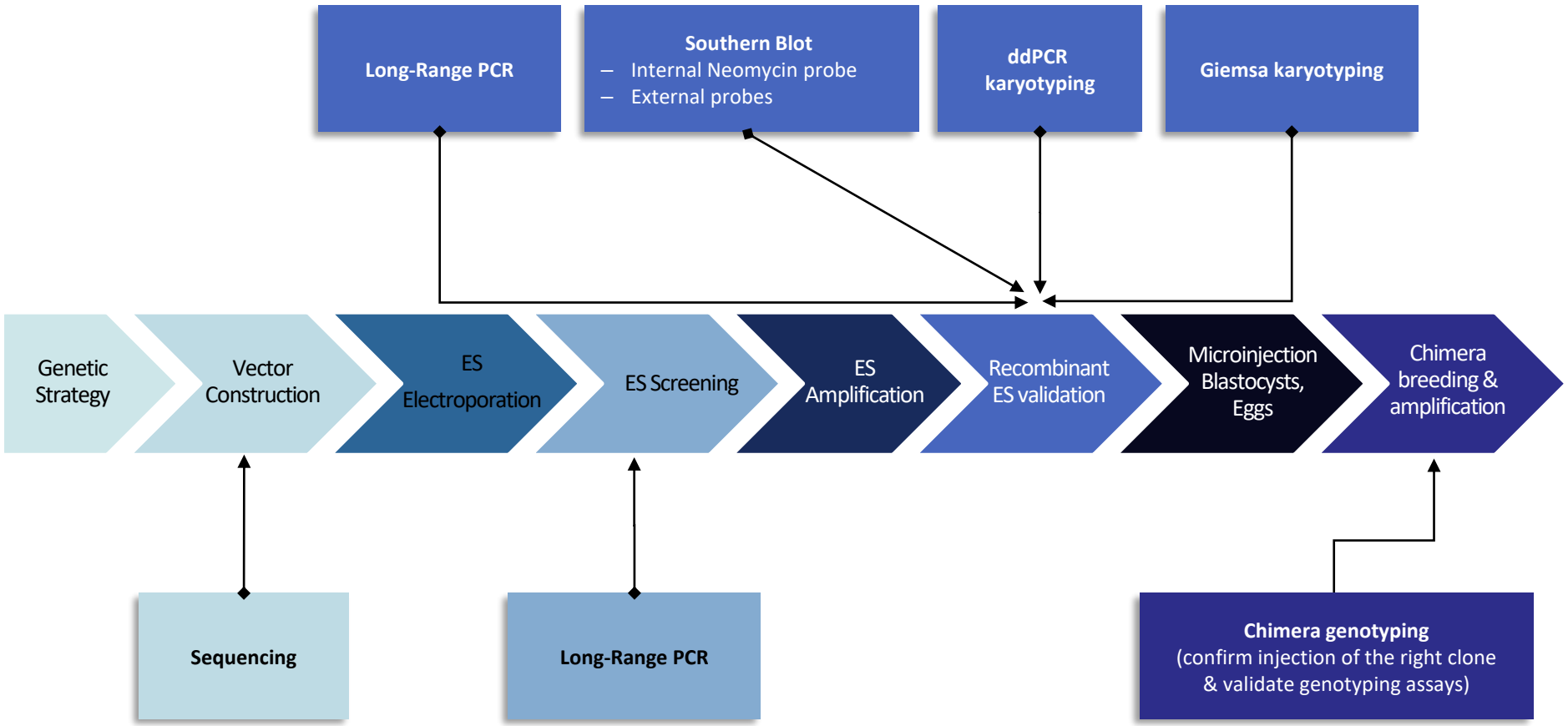
4 ES ELECTROPORATION & SCREENING OF RECOMBINANT CLONES

5 MICROINJECTION & BREEDING

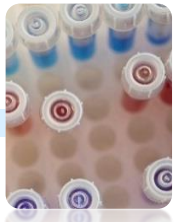
6 SEQUENCE OF THE DELIVERED ALLELE

PROJECT PROCESS & QUALITY CONTROLS



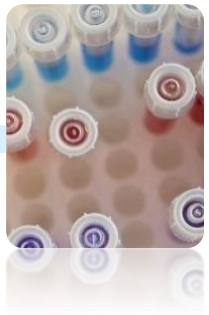


2 GENETIC STRATEGY

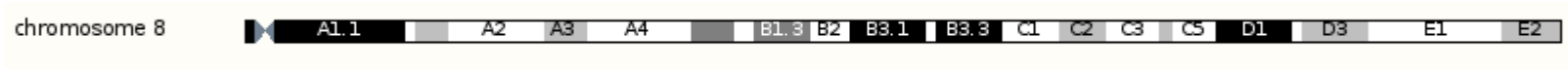


- Target locus structure
- mRNA(s) and protein(s)
- Genetic strategy
- PRO & CONS evaluation of the strategy

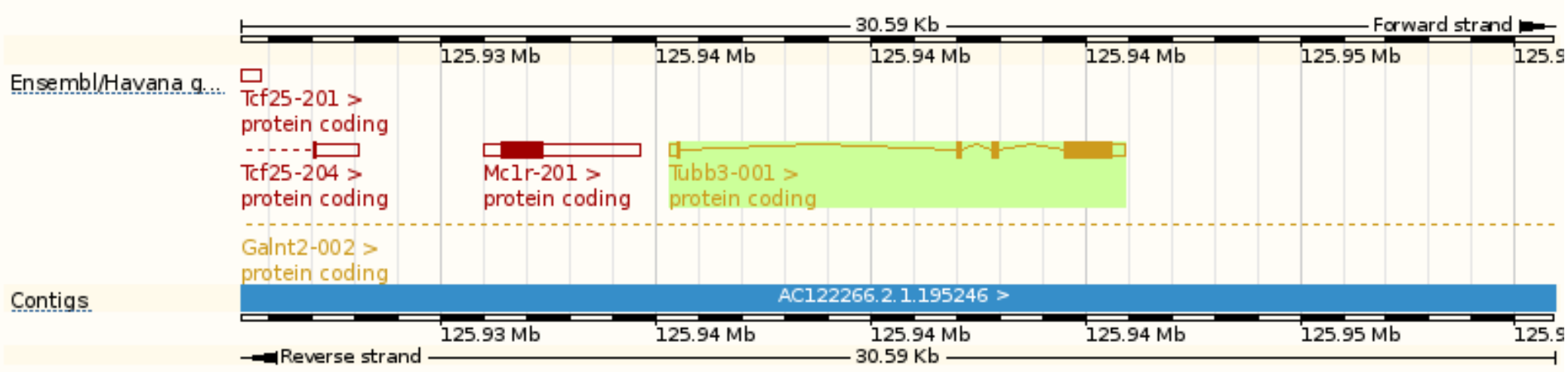
Tubb3mouse genomic locus – structure



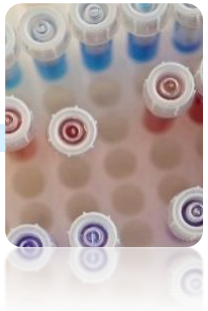
LOCATION: Chr8



Gene: Tubb3 ENSMUSG00000062380

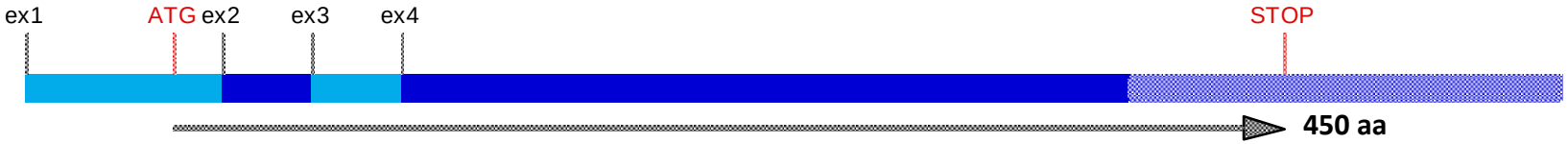


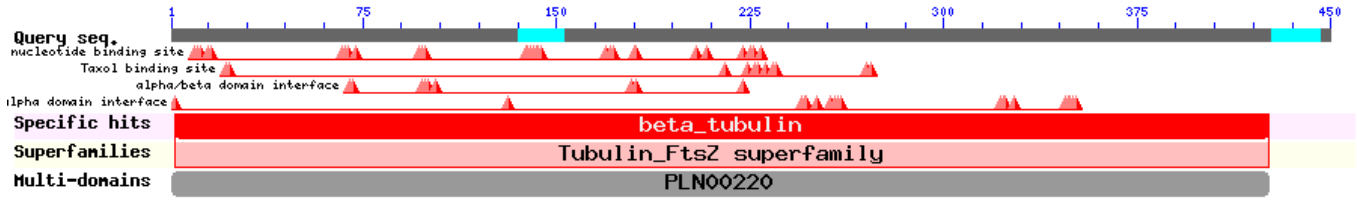
Tubb3 mRNA(s) and protein(s)



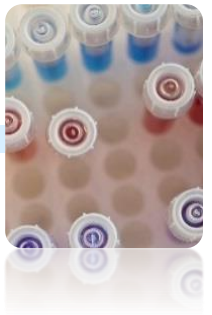
Name	Transcript ID	Length (bp)	Protein ID	Length (aa)
Tubb3-001	ENSMUST00000071134	1868	ENSMUSP00000071134	450

Tubb3-001 (ENSMUST00000071134)

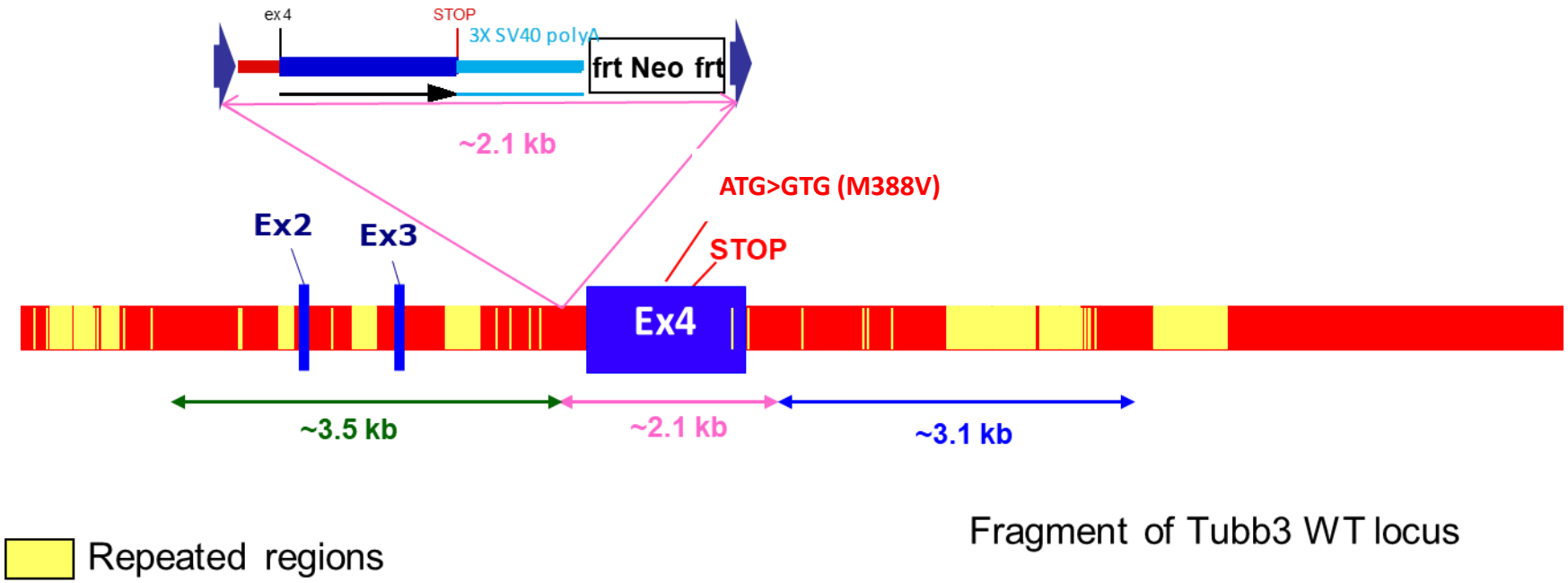




Approach chosen: conditional M388V point mutation (equivalent to human p.M388V)



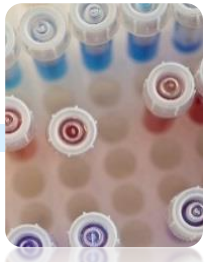
Targeted locus



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.

PROs& CONs evaluation of the strategy



Pros

- This strategy allows the introduction of a conditional point mutation after Cre mediated excision
- Higher chance of expression of the wildtype protein before Cre mediated excisio
- Expression of the mutated protein after Cre mediated excision (less chance to obtain mosaic animals)

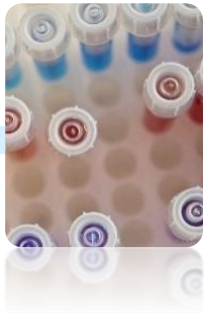
Cons

- A 3X polyA will replaced the endogenous 3'UTR in the WT RNA, specificity of expression or post transcriptional modification might be lost (or changed)
- Presence of repeated regions in both homology arms could render homologous recombination and/or PCR amplification difficult

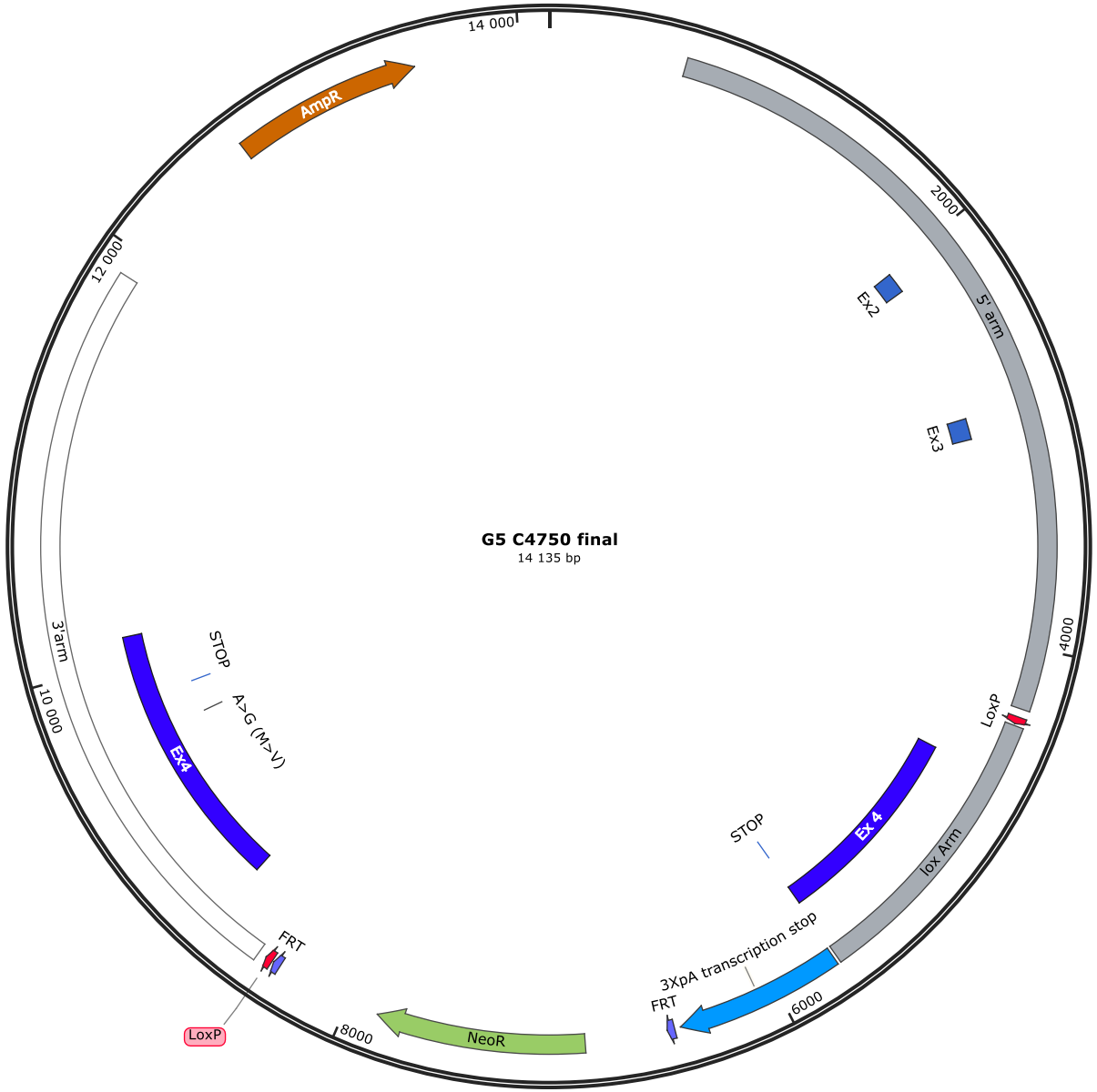
The selection cassette (FRT-Neo-FRT) will be removed by breeding male chimera with a flp deleter line which shows maternal contribution (*Birling et al.*, 2012)

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

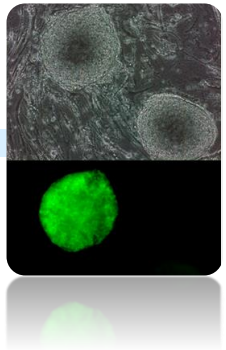


Created by SnapGene



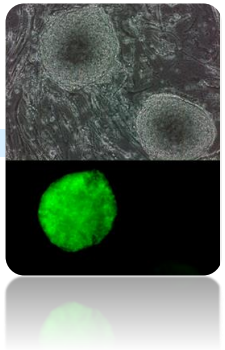
phenomin
EXCELLENCE IN MOUSE PHENOGENOMICS

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
- Validation of the introduced PM in Recombinant ES clones
- Aneuploidy screening in ES recombinant clones

Electroporation and screening process



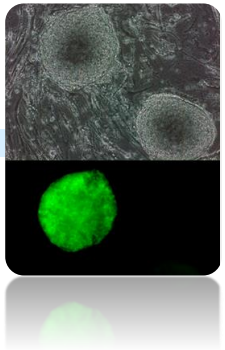
The targeting vector was electroporated in the proprietary C57BL/6NCrl BD10 cell line.

Transfected ES clones were submitted to neomycin selection (G418) and 186 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.

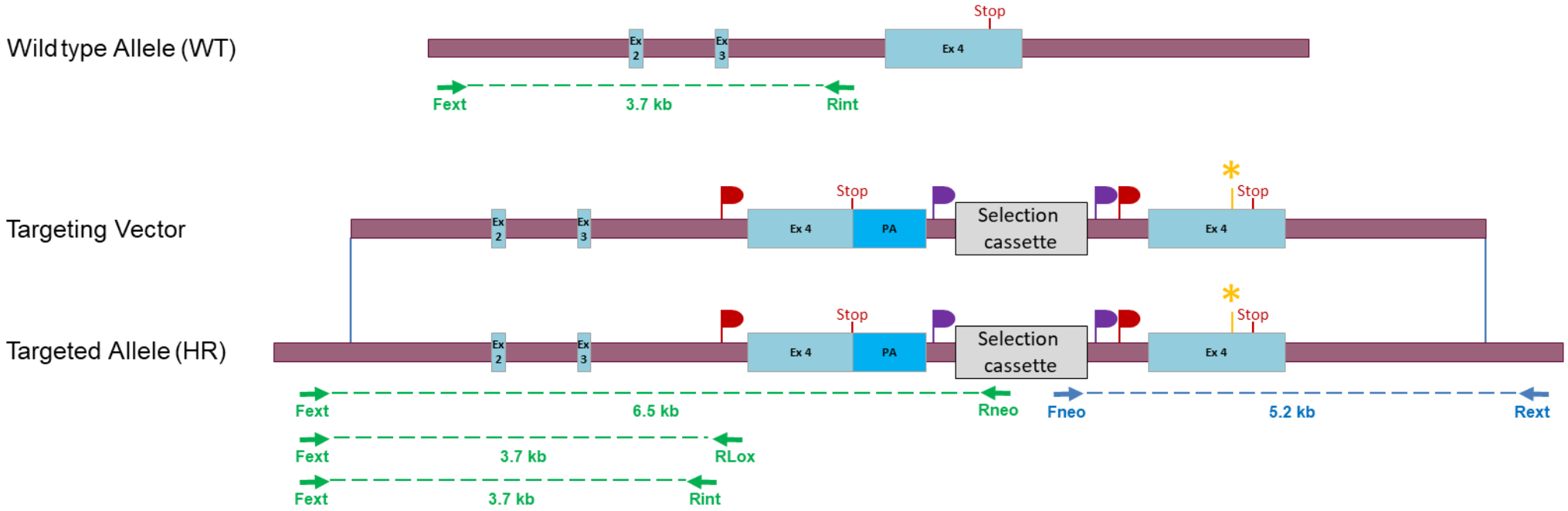
Screening process steps:

1. Identification of candidate recombinant clones by initial 5’ Long-Range PCR
2. Eight 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. Validation of the introduced PM in Recombinant ES clones by Sanger sequencing
5. The karyotype of at least 2 validated clones is verified using Giemsa staining

Long range PCR screening – strategy

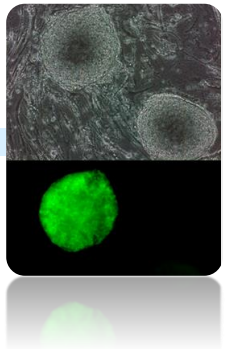


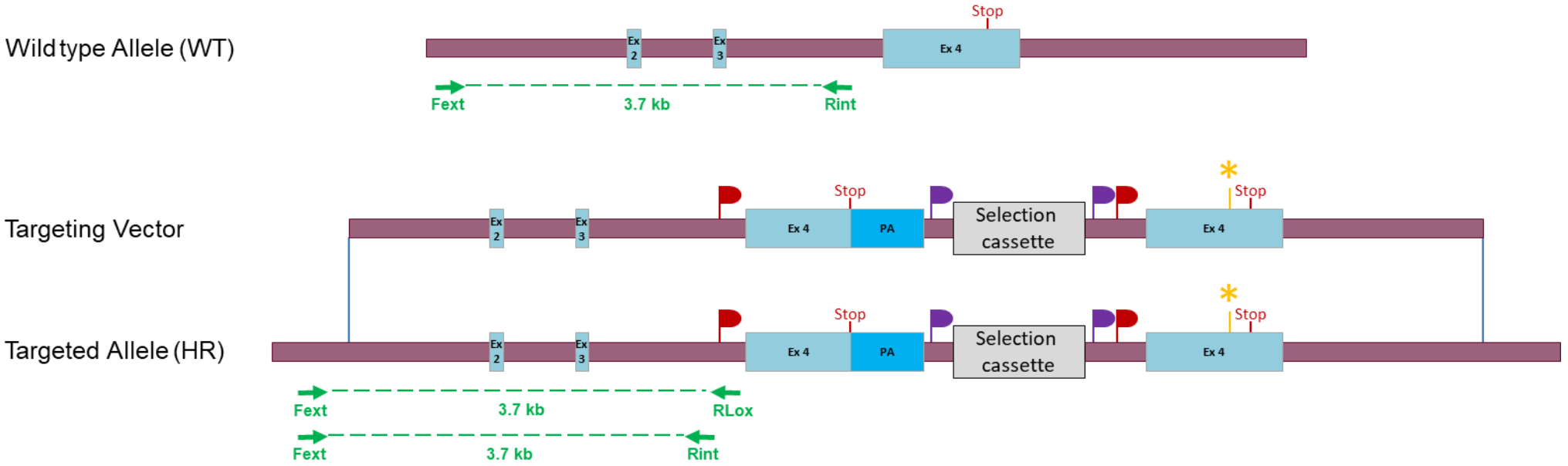
Schematic 5’ and 3’ PCR screening strategy



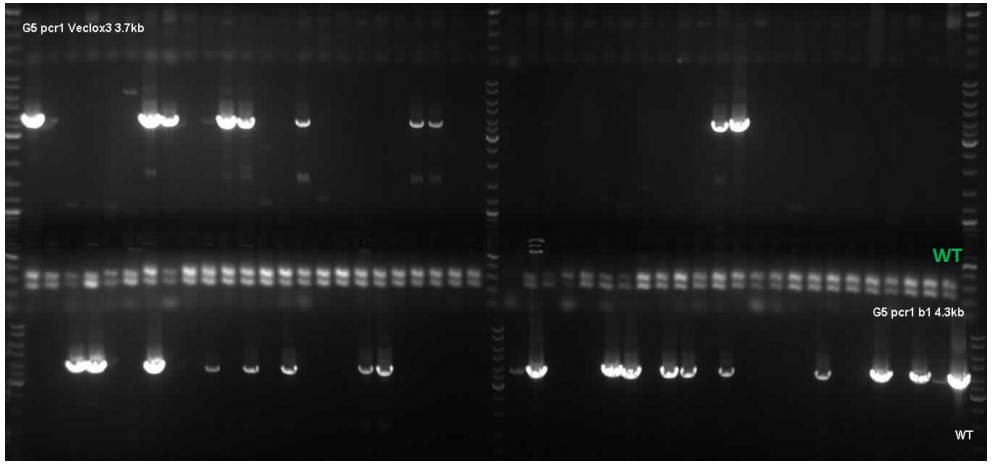
PCR	Primer Name	Primer sequences	PCR product size
5' PCR	Fext	CCTCTGAGCTGTCTTCTCCACAAGA	3.7 kb
	Rlox	GTTATCTGCAGGTCGACCTTAAGCT	
5’ PCR	Fext	CCTCTGAGCTGTCTTCTCCACAAGA	3.7 kb
	Rint	AATATGGCCGGCCTGGATAAATAGGTCAGCCTTCCC	
5’ PCR	Fext	CCTCTGAGCTGTCTTCTCCACAAGA	6.5 kb
	Rneo	GCGGCCGGAGAACCTGCGTGCAATC	
3’ PCR	Fneo	AGGGGCTCGCGCCAGCCGAACTGTT	5.2 kb
	Rext	TAATTGGCCTCAGTGGCCTCATGGGCCACTATCTATGTTGTTG	

Long-Range 5’ PCR screening – results





PCR Fext – Rlox : 3.7 kb



WT : Controls DNAs

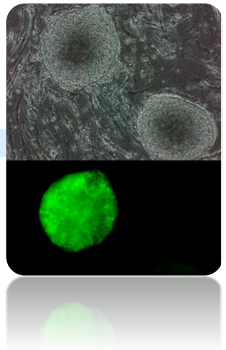
Ladder pattern

PCR Fext – Rint : 3.7 kb

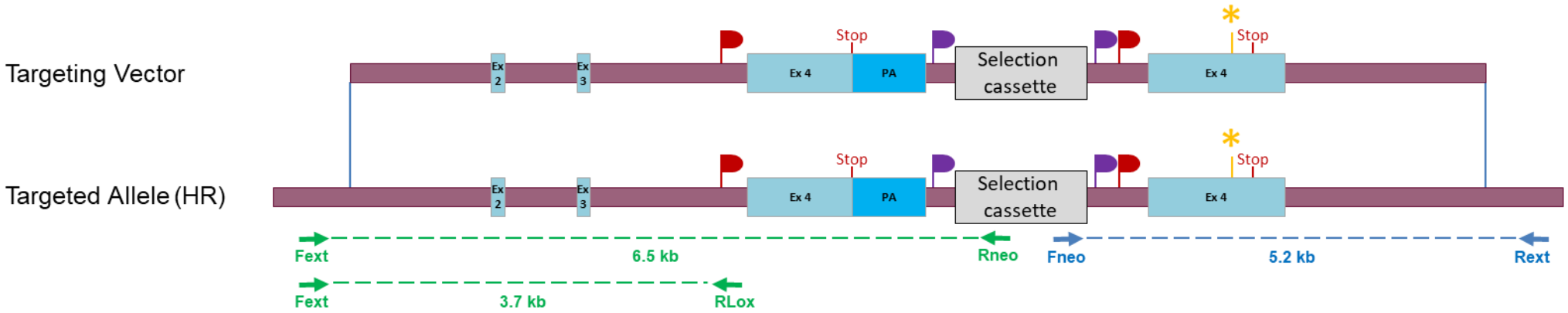
Eight candidate clones out of the 27 positive clones were selected for 3’ Long-Range PCR and Southern blot validation.

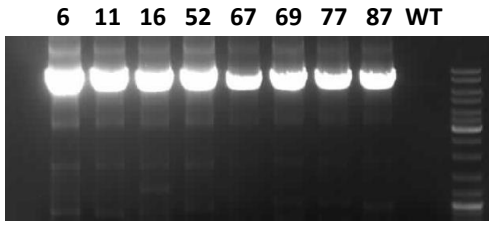
phenomin
EXCELLENCE IN MOUSE PHENOGENOMICS

Recombinant ES validation by Long Range PCR

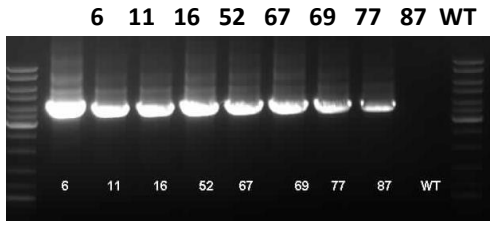


Confirmation and Validation of candidate recombinant ES clones by 5’ and 3’ PCRs

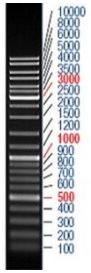




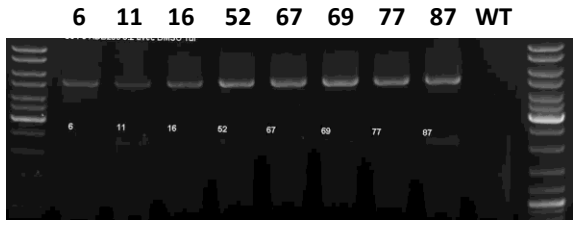
PCR Fext – Rneo : 6.5 kb



PCR Fext – Rlox : 3.7 kb



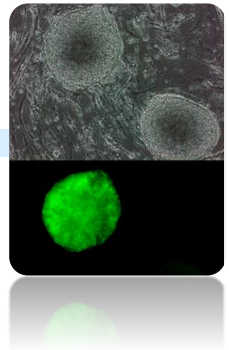
Ladder pattern



PCR Fneo – Rext : 5.2 kb

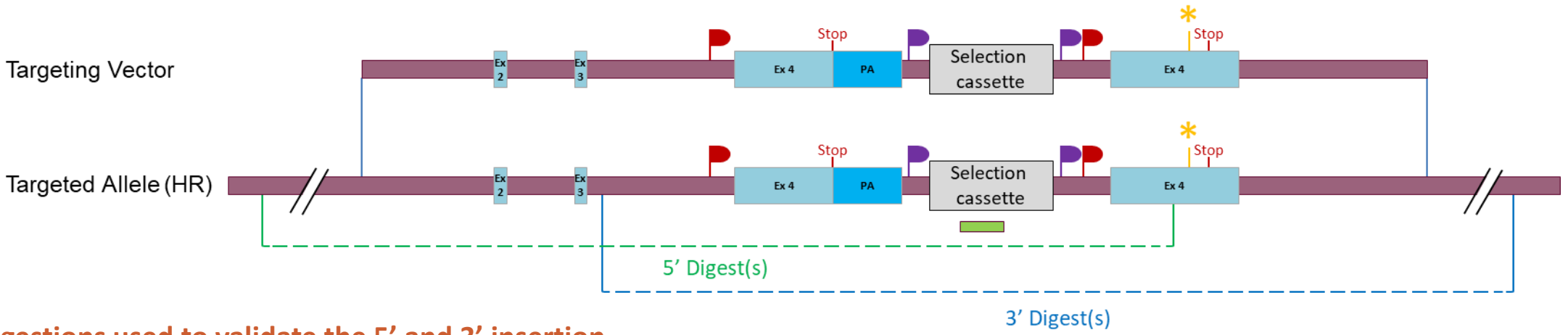
Eight candidate clones identified by 5’ PCR screening were further analysed by 3’ PCR screening.
Eight clones (clones #6, #11, #16, #52, #67, #69, #77 and #87) were confirmed.

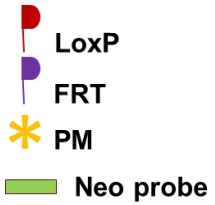
Recombinant ES clones validation by Southern Blot – Internal probe



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.

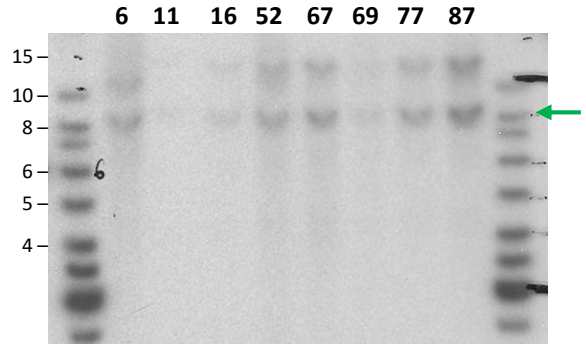




Digestions used to validate the 5' and 3' insertion

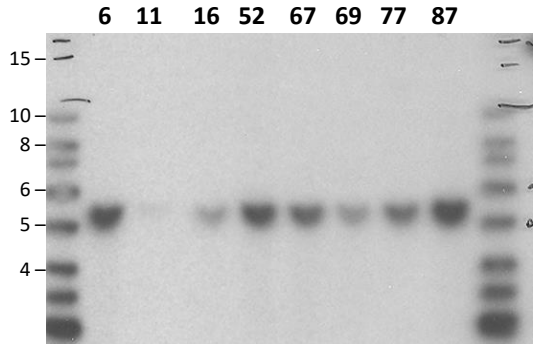
Probe		Genomic DNA digest	Targeted Allele (kb)
Neo	5' digest	AflIII	8.7
	3' digest	DrdI	5.6

Southern blot - Neo 5'



AflIII

Southern blot - Neo 3'



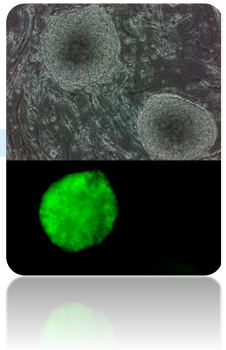
DrdI

Neo probe sequence

CTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTC
GACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTG
TCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACG
CTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGG
ATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAA
CTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCC
TGCTTGCCGAAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGT
GTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAA
TGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTAT
CGC

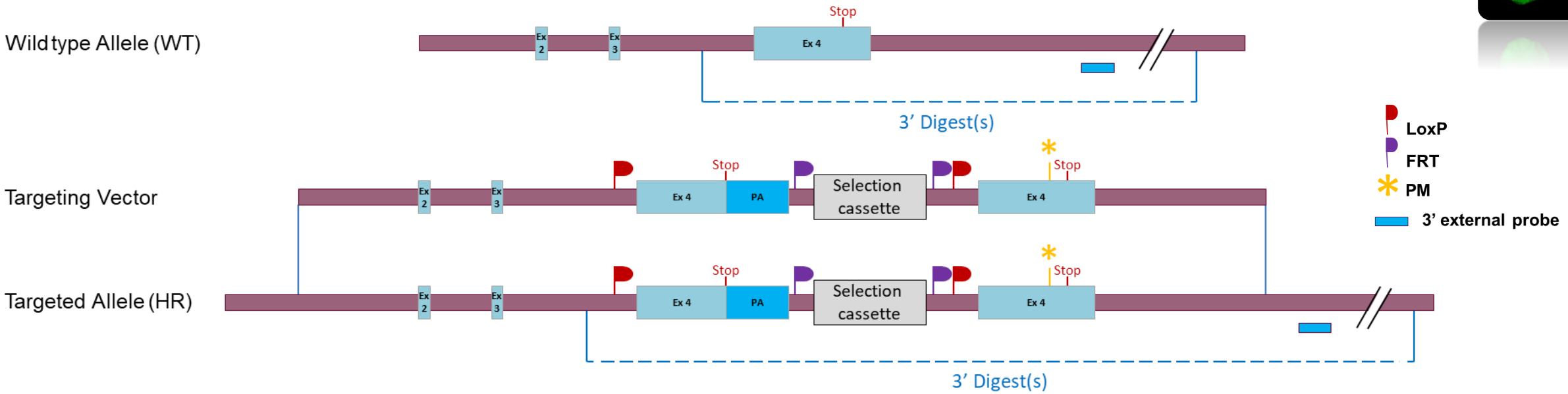
phenomin
EXCELLENCE IN MOUSE PHENOGENOMICS

Recombinant ES clones validation by Southern Blot – External probe

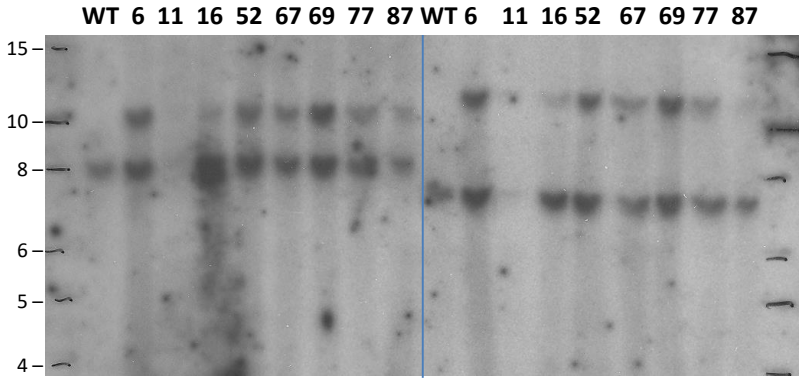


Schematic Southern Blot validation strategy

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 – 3' probe



AflII 8.6 / 10.2 EcoNI 7.2 / 11.4

3' probe sequence

CCATACTCAAGCTAGGCATAAAGGGGTGGGGTGTGC
AGCCCATTTTTCCCCCAGGCCTCCCACTGTGACCAT
GTGACTCCCCTTGACCATGGAGCAGGGAAACCGGAC
CCCACTCAAAGGCAACTTCACCAGCAGAGGGTGGTT
TCAGCTAGAGATGGGTGGGAGAGAGCAAGTGGACTC
CGGTGGAAGAAGAAAGAGATTTAAGAGGAACAGCGC
AGGAGTGGCGGATGGTGCTGGGGAGGAGAGGAGAGA
GATCAGAGCCGTGGCGTTGGAACCACCTGGCTCCTG
GAGAGAAAGGCTATCACTTTGCTCTGACATCTCTTC
CCCCACCCCCTTGTCCTCTTCCACCCTCCCCAACAT
CAGAGGGC

Digestions used to validate the 5' and 3' insertion

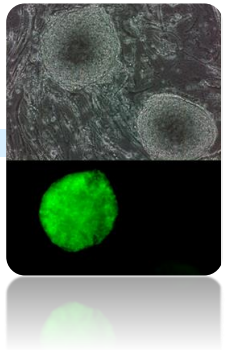
Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
3' external probe	3' first digest	AflII	8	10.2
	3' second digest	EcoNI	7.2	11.4

Validation of the introduced PM in Recombinant ES clones

Clone #87

Clone #69

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
#11	Failed
#16	Failed
#52	Failed
#67	Failed
#69	**Pass**
#87	**Pass**

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 ES clones #69 and #87 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
#69	2	3	17	22
#87	1	1	4	6

Breeding to F1 generation

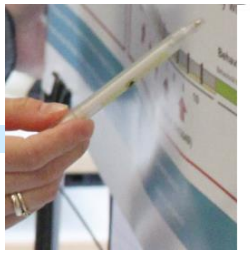
- Four highly chimeric males generated in the previous phase by blastocyst injection of the ES clones were mated with Flp deleter C57BL/6NCrl females showing maternal contribution* to investigate whether the recombined ES cells have contributed to the germ layer.

- Germ line transmission was obtained the : 26/09/2012

- Allele nomenclature (following MGI guidelines) : **Tubb3$^{tm1.1Ics}$**

***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.

6 SEQUENCE OF THE DELIVERED ALLELE

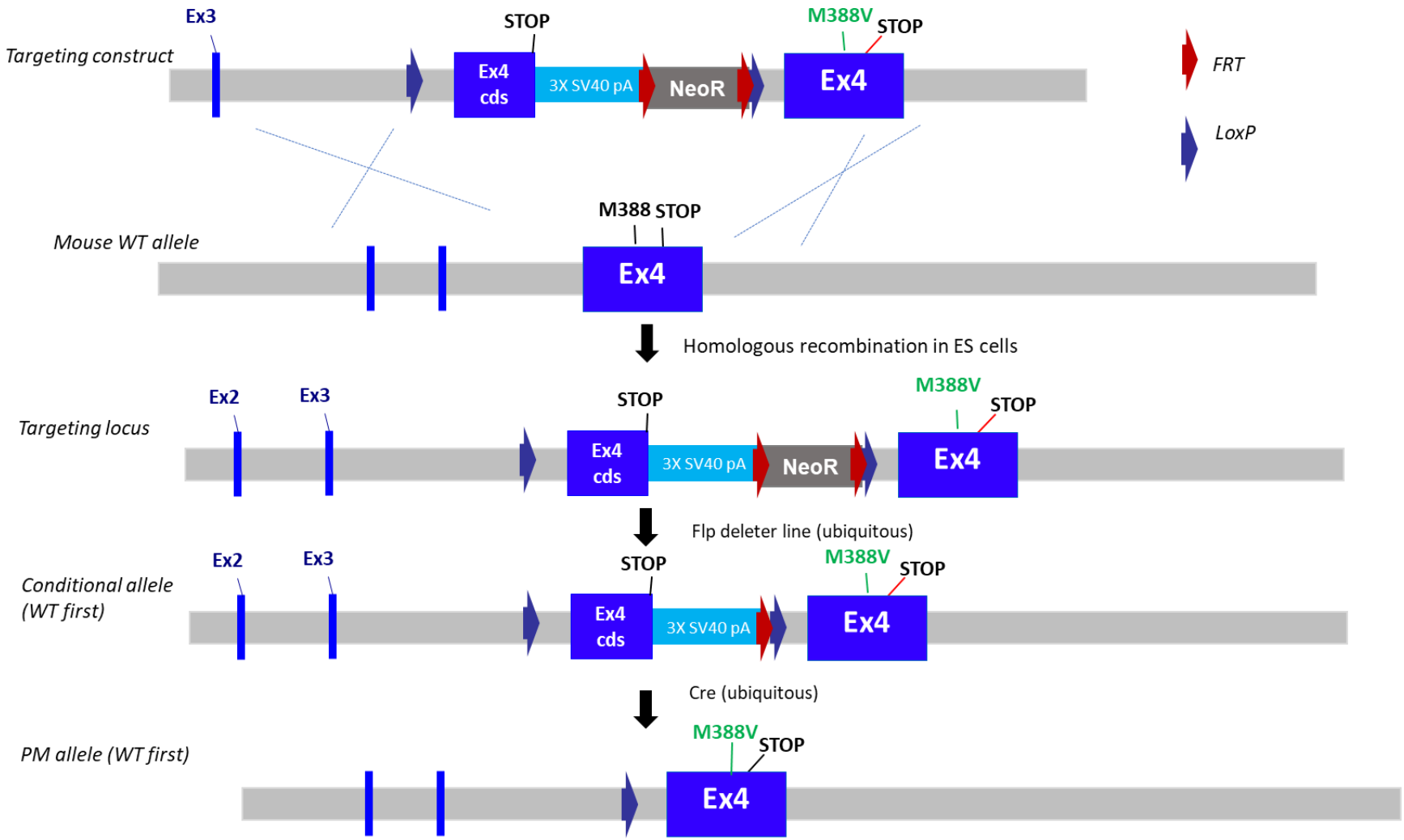


AGATAAAGGGCTTGCTTTCAAACCTGAAGATCTGAGTTCAATTCTTGGGACCCAAGTGGCAGAAATAGAGAACTAACTTCTTCTTCTTCTTTTTTTTTTTTTCCTCGAGACAGGGTTTCTCTGACCAGGCTGGCCTCGAACTCAGAAATCTGCCTGCC
TCTGCCTCCCAAGTGCTGGGAGAACCAACATGTGTGCCCTGGCATGTATCCACCTATACACATACACATAACCACCACACACATATTCTCTCTCTATAAACAAATGTGGTGTGTTGTTGTTGTTGTTGTTGTAAGATCTTAGACTAGACTAGCAGGT
GCCAGTTCTTAGTCAGTCCTCAGTTACAATAACTGCTCAGGGATTTTTTTTTTTCTGGAAAAGAGAAGTTGGCTTAGAGGGGTGAGGACAGCAGGCCAGGTGGCTGAATAAGGGACAGCCCTCATCTGACATCACAGTCTCAGCAGACCAGTGGGAG
GGAAGGGAAGGCTGACCTATTTATCCAGGGGCCGGCCAAGCTTCTCGAGCTTAAGGTCGACCTGCAG**ATAACTTCGTATAATGTATGCTATACGAAGTTAT**TTAATTAAATGCCCCTGAGAGATAGCAAGGCCCAGGTCTTATCCCAGATCCCCATT
CCCTGTTCAGAGCATCTGCAGCAGGGACCCCCTGCACTCAACAGTGATGCCCAGGGTGGAATGAGATGTTATGCAGTGCAGACATTTTATAGAATACAAGGGAACCAACTTTCTTCTAGAGGAGAGAGCGGTTGGCAGGTCCTAGAGGTCTCTGCAC
TGTAAACCCCCGACCTTACCTCTTACCTGCCTCTTCTCTCCTCATAGGTCAGAGTGGTGCTGGCAACAACTGGGCCAAAGGGCACTATACGGAGGGCGCGGAGCTGGTGGACTCAGTCCTAGATGTCGTGCGGAAAGAGTGTGAGAATTGTGACTGC
CTGCAGGGCTTCCAGCTGACACACTCACTGGGTGGGGGCACAGGCTCAGGCATGGGCACACTGCTCATCAGCAAGGTGCGTGAGGAGTACCCCGACCGCATCATGAACACCTTCAGCGTGGTGCCTTCACCCAAAGTGTCGGACACTGTGGTGGAGC
CCTACAACGCCACCCTGTCCATCCACCAGCTAGTGGAGAACACAGACGAGACCTACTGCATCGACAATGAAGCCCTCTACGACATCTGCTTCCGCACCCTCAAGCTGGCCACACCCACCTATGGGGACCTCAACCACCTTGTGTCTGCCACCATGAG
TGGAGTCACCACCTCCCTTCGATTCCCTGGTCAGCTCAATGCCGACCTCCGCAAGCTGGCTGTGAACATGGTGCCGTTCCCACGTCTCCACTTCTTCATGCCCGGCTTCGCCCCACTTACAGCCCGGGGCAGCCAGCAGTACCGTGCCCTGACGGTG
CCTGAGCTCACGCAGCAGATGTTCGATGCCAAGAACATGATGGCTGCCTGTGACCCGCGCCACGGTCGCTACCTGACCGTGGCCACTGTCTTCCGTGGGCGCATGTCTATGAAGGAGGTGGACGAGCAGATGCTGGCCATCCAGAGTAAGAACAGCA
GCTACTTCGTGGAGTGGATCCCCAACAACGTCAAGGTAGCCGTGTGTGACATCCCACCCCGTGGGCTCAAAATGTCATCCACCTTCATTGGCAACAGCACGGCCATCCAGGAGCTGTTCAAACGCATCTCGGAGCAGTTCACAGCCATGTTCCGGCG
CAAGGCCTTCCTGCACTGGTACACGGGCGAGGGCATGGATGAGATGGAGTTCACCGAGGCCGAGAGCAACATGAATGACCTGGTGTCCGAGTACCAGCAGTACCAGGACGCCACTGCGGAGGAGGAGGGGGAGATGTATGAAGATGATGACGAGGAA
TCGGAAGCCCAGGGGCCCAAGTGAAGT**TAATTAATCTAGAG**GATCTGCGACTCTAGAGGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGT
TGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCTGCGACTCTAGAGGA
TCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAA
TTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCTGCGACTCTAGAGGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACC
TCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAA
TGTATCTTATCATGTCTGGATCCCCATCAAGCTGATCCCACCGGTG**GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC**CGGCCGG**ATAACTTCGTATAATGTATGCTATACGAAGTTAT**GGATCCATCGACCCCCTGCAGGATTTAAATGGCCAC
TGAGGCCATGCCCCTGAGAGATAGCAAGGCCCAGGTCTTATCCCAGATCCCCATTCCCTGTTCAGAGCATCTGCAGCAGGGACCCCCTGCACTCAACAGTGATGCCCAGGGTGGAATGAGATGTTATGCAGTGCAGACATTTTATAGAATACAAGGG
AACCAACTTTCTTCTAGAGGAGAGAGCGGTTGGCAGGTCCTAGAGGTCTCTGCACTGTAAACCCCCGACCTTACCTCTTACCTGCCTCTTCTCTCCTCATAGGTCAGAGTGGTGCTGGCAACAACTGGGCCAAAGGGCACTATACGGAGGGCGCGGA
GCTGGTGGACTCAGTCCTAGATGTCGTGCGGAAAGAGTGTGAGAATTGTGACTGCCTGCAGGGCTTCCAGCTGACACACTCACTGGGTGGGGGCACAGGCTCAGGCATGGGCACACTGCTCATCAGCAAGGTGCGTGAGGAGTACCCCGACCGCATC
ATGAACACCTTCAGCGTGGTGCCTTCACCCAAAGTGTCGGACACTGTGGTGGAGCCCTACAACGCCACCCTGTCCATCCACCAGCTAGTGGAGAACACAGACGAGACCTACTGCATCGACAATGAAGCCCTCTACGACATCTGCTTCCGCACCCTCA
AGCTGGCCACACCCACCTATGGGGACCTCAACCACCTTGTGTCTGCCACCATGAGTGGAGTCACCACCTCCCTTCGATTCCCTGGTCAGCTCAATGCCGACCTCCGCAAGCTGGCTGTGAACATGGTGCCGTTCCCACGTCTCCACTTCTTCATGCC
CGGCTTCGCCCCACTTACAGCCCGGGGCAGCCAGCAGTACCGTGCCCTGACGGTGCCTGAGCTCACGCAGCAGATGTTCGATGCCAAGAACATGATGGCTGCCTGTGACCCGCGCCACGGTCGCTACCTGACCGTGGCCACTGTCTTCCGTGGGCGC
ATGTCTATGAAGGAGGTGGACGAGCAGATGCTGGCCATCCAGAGTAAGAACAGCAGCTACTTCGTGGAGTGGATCCCCAACAACGTCAAGGTAGCCGTGTGTGACATCCCACCCCGTGGGCTCAAAATGTCATCCACCTTCATTGGCAACAGCACGG
CCATCCAGGAGCTGTTCAAACGCATCTCGGAGCAGTTCACAGCC***G***TGTTCCGGCGCAAGGCCTTCCTGCACTGGTACACGGGCGAGGGCATGGATGAGATGGAGTTCACCGAGGCCGAGAGCAACATGAATGACCTGGTGTCCGAGTACCAGCAGTA
CCAGGACGCCACTGCGGAGGAGGAGGGGGAGATGTATGAAGATGATGACGAGGAATCGGAAGCCCAGGGGCCCAAGTGAAGTTGCTCGCAGCTGGGGTGTGGGGCCAAGTGGCAGCCAGGGCCAAGACAAGCAGCATCTGTCCCCCCCAGAGCCATC
TAGCTACTGACACTGCCCCCAGCTTTGCTTCTCACCAGCTCATTAGGGCTCCCAGGTTAAAGTCCTTCAGTATTTATGGCCACCCCCACTCCATGTGAGTCCACTTGGCTCTGTCCTCCCCATTTTAGCCACCTCTGTATTTATGTTGCTTATTCGT
CTGTTTTTATGGTTTGTTTTGTTTTTTTACTGGGTTGTGTTTATATTCGGGGGGAGGGGTATACTTAATAAAGTTACTGCTGTCTGTCAGATACCTCT

LoxP FRT Exon 4 3XpolyA Introduced PM : A>G (M>V)

phenomin EXCELLENCE IN MOUSE PHENOGENOMICS

Recap



Dominant PM embryonic lethale (as observed in an human embryo)

phenomin
EXCELLENCE IN MOUSE PHENOGENOMICS

REPORT REDACTION & VALIDATION
Protocol finalized on 2023/09/05
Prepared by Romain LORENTZ, IE
Finalized by Marie-Christine BIRLING, PhD

CONTACT US
By email at mutagenesis@igbmc.fr
By phone at +33 (0)3 88 65 56 57

www.phenomin.fr