phenomin
EXCELLENCE IN MOUSE PHENOGENOMICS

MODEL GENERATION TECHNICAL REPORT

Generation of a Dre deleter C57BL/6N line

Project code: Ros6728 / IR6728

Report finalized: 1/03/2023

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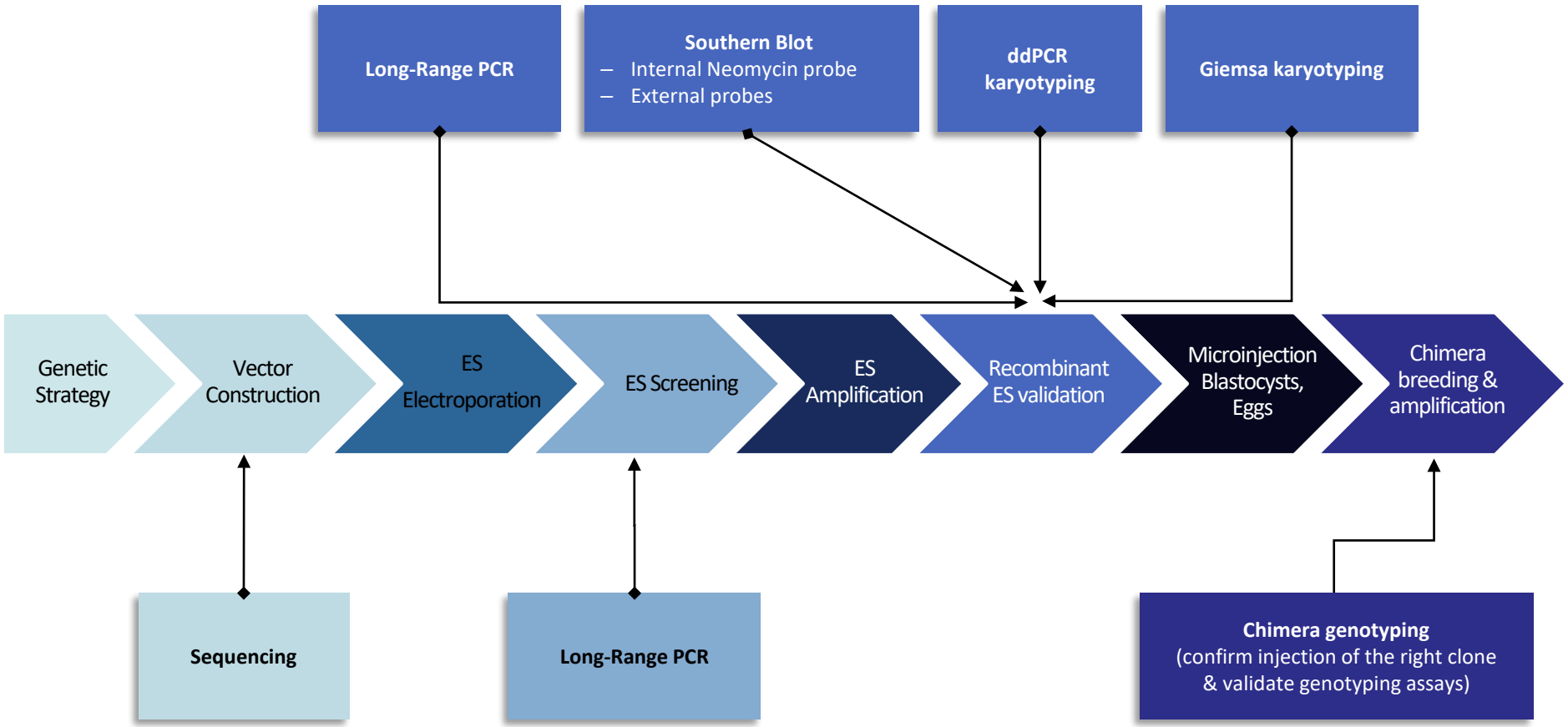
MODEL GENERATION TECHNICAL REPORT

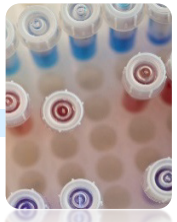
1 PROJECT DESCRIPTION

- Project process & quality controls

Project process & quality controls



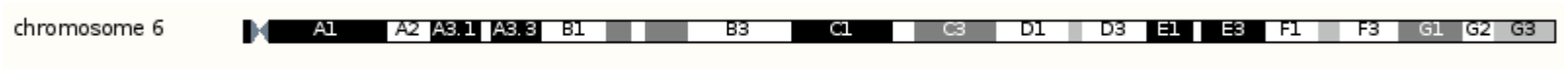
2 GENETIC STRATEGY



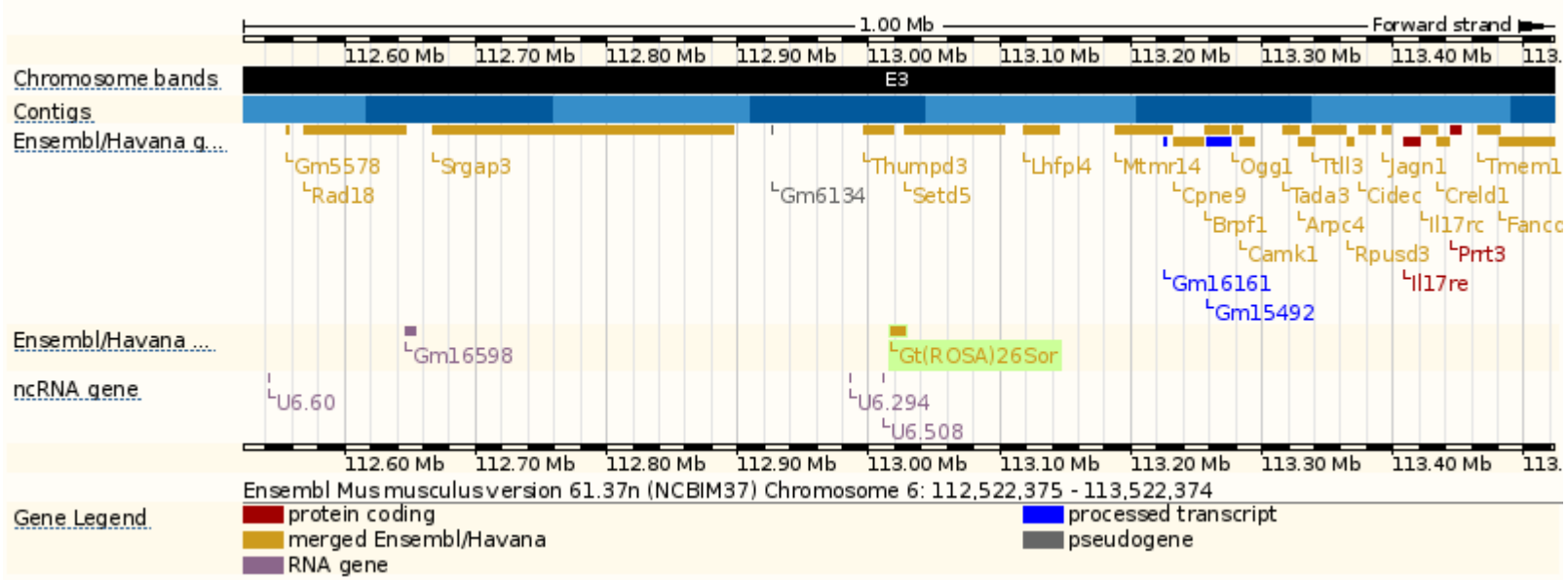
- Target locus structure
- Genetic strategy
- Sequence of the Dre recombinase

Rosa26 mouse genomic locus – structure

Location:

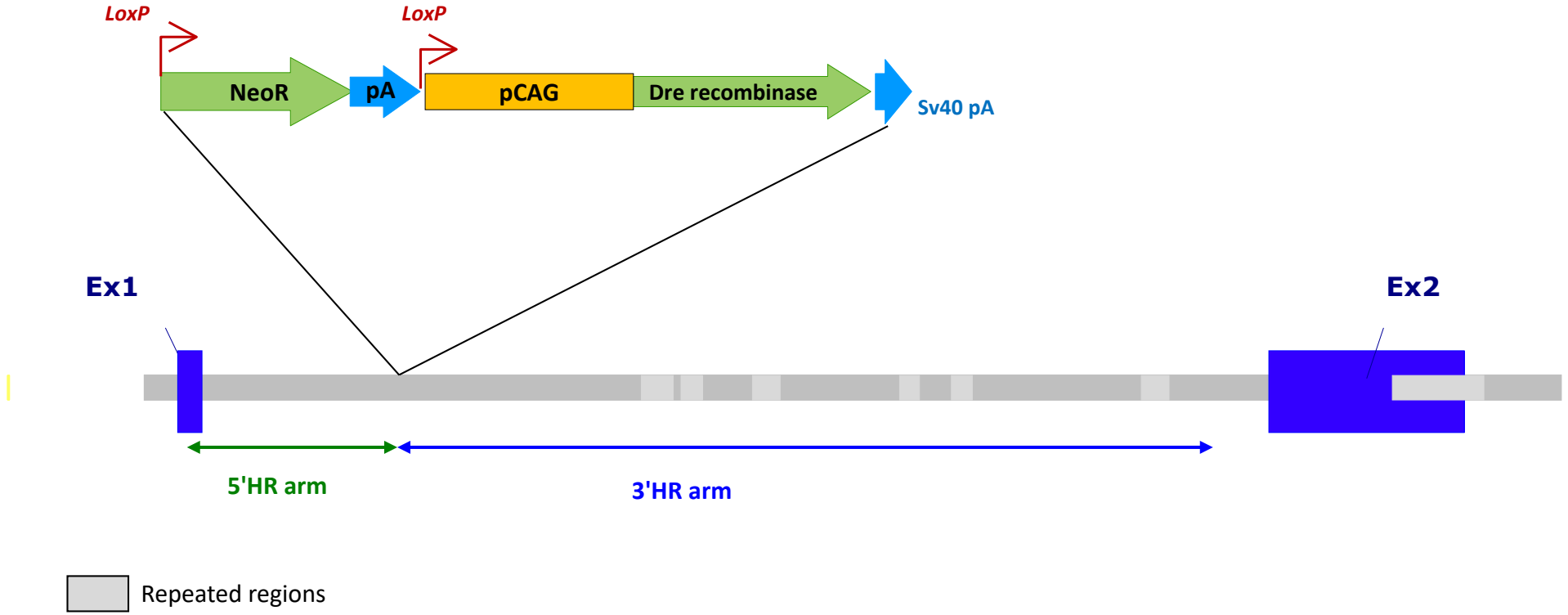


Gt(ROSA)26Sor (ENSMUSG00000086429)



Strategy

Targeted locus

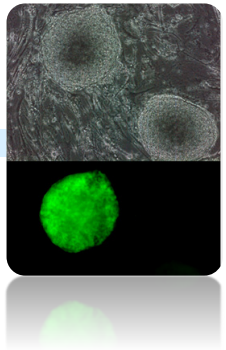


Sequence of the Dre recombinase (cf. Stewart's paper)

```
ATGGGTGCTAGCGAGCTGATCATCTCTGGCTCCTCTGGAGGATTCCTGAGGAACATCGGCAAGGAGTACCAGGAGGCTGCTGAGAACTTCATGAGATTCATGAATGACCAGGGA
 M  G  A  S  E  L  I  I  S  G  S  S  G  G  F  L  R  N  I  G  K  E  Y  Q  E  A  A  E  N  F  M  R  F  M  N  D  Q  G
GCCTACGCCCCTAACACCCTGAGAGACCTGAGGCTGGTGTTCCACTCCTGGGCTAGATGGTGCCACGCTAGACAGCTGGCCTGGTTCCCTATCTCTCCTGAGATGGCTAGGGAG
 A  Y  A  P  N  T  L  R  D  L  R  L  V  F  H  S  W  A  R  W  C  H  A  R  Q  L  A  W  F  P  I  S  P  E  M  A  R  E
TACTTCCTTCAGCTGCACGATGCTGACCTGGCCTCTACCACCATCGACAAGCACTACGCCATGCTGAACATGCTGCTGTCCCACTGTGGCCTGCCTCCTCTGTCTGATGACAAG
 Y  F  L  Q  L  H  D  A  D  L  A  S  T  T  I  D  K  H  Y  A  M  L  N  M  L  L  S  H  C  G  L  P  P  L  S  D  D  K
TCTGTGAGCCTGGCCATGAGGAGAATCCGGAGAGAGGCTGCCACCGAGAAGGGAGAGA GAACCGGCCAGGCCATCCCTCTGAGATGGGATGACCTGAAGCTGCTGGATGTGCTG
 S  V  S  L  A  M  R  R  I  R  R  E  A  A  T  E  K  G  E  R  T  G  Q  A  I  P  L  R  W  D  D  L  K  L  L  D  V  L
CTGTCTAGATCTGAGAGACTGGTGGACCTGAGGAATAGGGCCTTCCTGTTTGTGGCCTACAACACCCTGATGAGGATGTCTGAGATCTCTAGGATCAGAGTGGGAGACCTGGAC
 L  S  R  S  E  R  L  V  D  L  R  N  R  A  F  L  F  V  A  Y  N  T  L  M  R  M  S  E  I  S  R  I  R  V  G  D  L  D
CAGACCGGAGACACCGTGACCCTGCACATCTCCCACACCAAGACCATCACCACCGCTGCTGGCCTGGACAAAGTGCTGTCTAGGAGGACCACCGCTGTGCTGAATGACTGGCTG
 Q  T  G  D  T  V  T  L  H  I  S  H  T  K  T  I  T  T  A  A  G  L  D  K  V  L  S  R  R  T  T  A  V  L  N  D  W  L
GATGTGTCTGGCCTGAGAGAGCACCCTGACGCTGTGCTGTTCCCTCCTATCCACCGGAGCAACAAGGCTAGGATCACCACCACCCCTCTGACCGCCCCTGCCATGGAGAAGATT
 D  V  S  G  L  R  E  H  P  D  A  V  L  F  P  P  I  H  R  S  N  K  A  R  I  T  T  T  P  L  T  A  P  A  M  E  K  I
TT TAGCGATGCCTGGGTGCTGCTGAACAAGAGGGATGCCACCCCTAACAAGGGCCGCTACCGGACCTGGACCGGCCACTCTGCTAGAGTGGGAGCTGCCATCGACATGGCTGAG
 F  S  D  A  W  V  L  L  N  K  R  D  A  T  P  N  K  G  R  Y  R  T  W  T  G  H  S  A  R  V  G  A  A  I  D  M  A  E
AAGCAAGTGTCCATGGTGGAGATCATGCAGGAGGGCACCTGGAAAAAGCCTGAGACACTGATGAGATACCTGAGGAGGGGAGGAGTGTCTGTGGGAGCCAACTCTAGGCTGATG
 K  Q  V  S  M  V  E  I  M  Q  E  G  T  W  K  K  P  E  T  L  M  R  Y  L  R  R  G  G  V  S  V  G  A  N  S  R  L  M
GACTCCGCTAGCGGCGCCGGTCCTAAGAAGAAGAGGAAAGTGTGA
 D  S  A  S  G  A  G  P  K  K  K  R  K  V
```

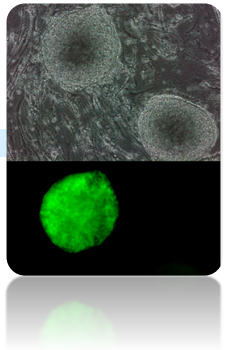
Sequence (codon optimized) from Anastassiadis et al, 2009 Disease Models &Mechanisms 2,508-515

4 ES cell electroporation & Screening of recombinant clones



- Electroporation and screening process
- Long range PCR screening – strategy
- Long-Range 3’ 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 vector was co-electroporated with a pX330 derived CRISPR/Cas9 vector expressing spCas9 and the RNA (CGCCCATCTTCTAGAAAGAC) in the proprietary C57BL/6NCrl S3 cell line.

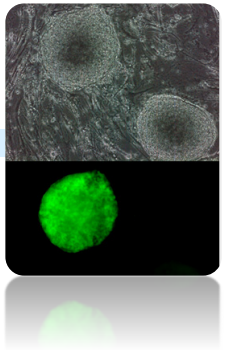
Transfected ES clones were submitted to neomycin selection (G418) and 93 resistant ES clones were isolated. The clones were then submitted to the screening process allowing secured identification of those harbouring the expected recombination events at both ends of targeting vector.

Screening process steps:

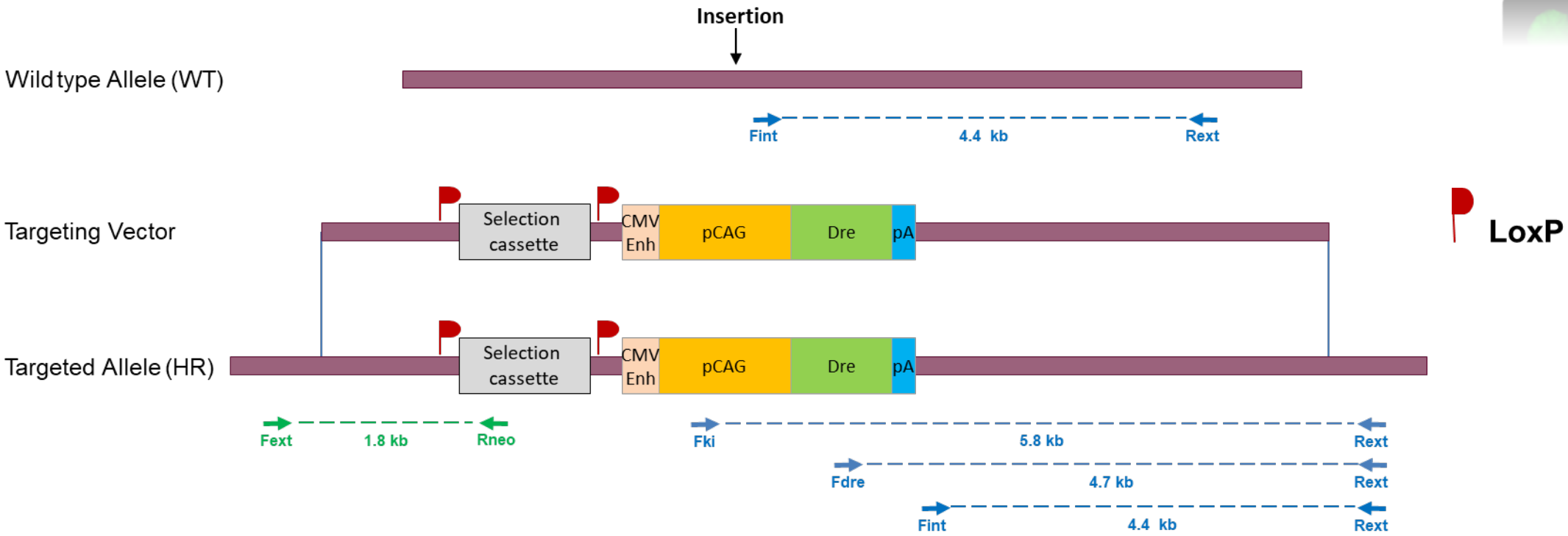
1. Identification of candidate recombinant clones by initial 3' Long-Range PCR
2. Four of 3' PCR positive clones are confirmed for 5' recombination event by Long-Range PCR. The absence of backbone was checked by PCR (Erbs et al., 2023*).
3. Positive clones in step2 are further validated by Southern blot 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. PMID: 36833328; PMCID: PMC9957269.

Long range PCR screening – strategy

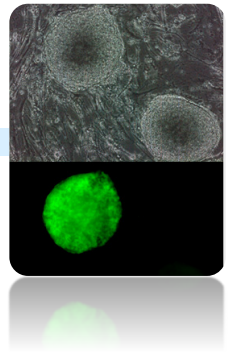


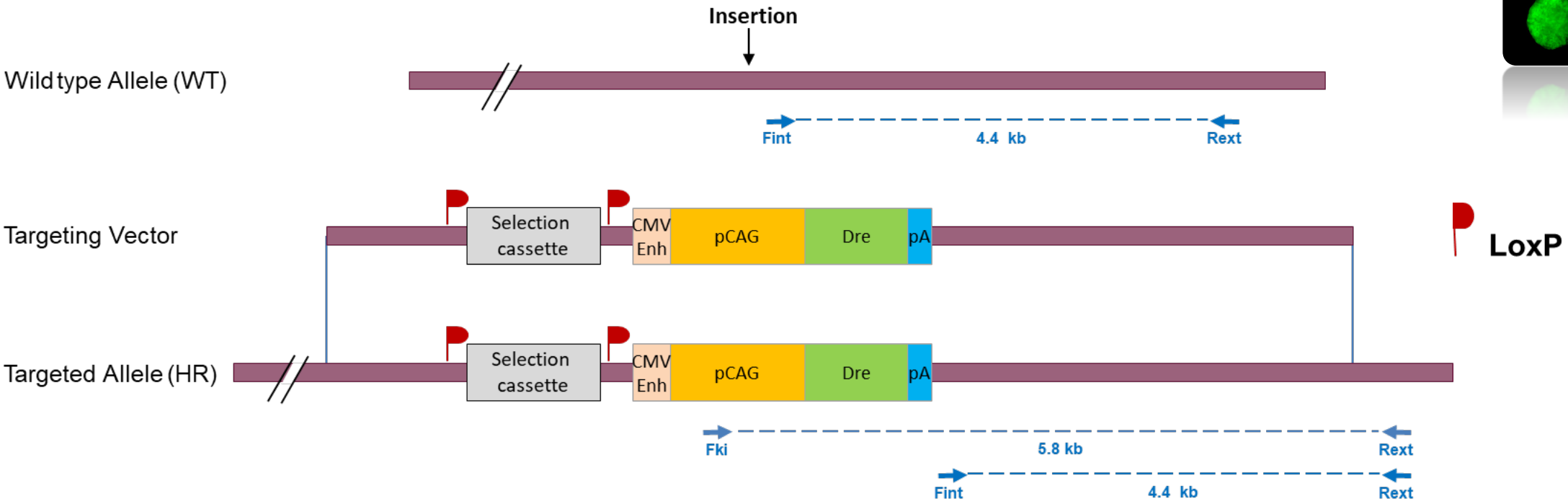
Schematic 5’ and 3’ PCR screening strategy



PCR	Primer Name	Primer sequences	PCR product size
5' PCR	Fext	CCTAAAGAAGAGGCTGTGCTTTGGG	1.8 kb
	Rneo	GCGGCCGGAGAACCTGCGTGCAATC	
3’ PCR	Fdre	ATGGCTGAGAAGCAAGTG	4.7 kb
	Rext	CTCAGTGGCTCAACAACACTTGGTC	
3’ PCR	Fki	GGCTCTAGAGCCTCTGCTAACCATG	5.8 kb
	Rext	CTCAGTGGCTCAACAACACTTGGTC	
3’ PCR	Fint	CTGGTGTGTGGGCGTTGTCCTGCAG	4.4 kb
	Rext	CTCAGTGGCTCAACAACACTTGGTC	

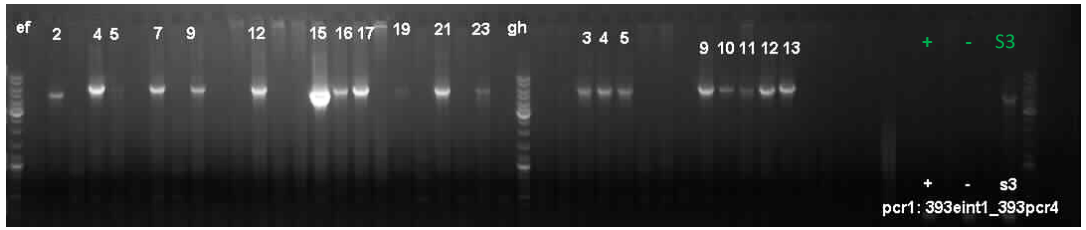
Long-Range 3’ PCR screening – results





Pcr Fki – Rext : 5.8 kb

+ / - / S3 : Controls DNAs



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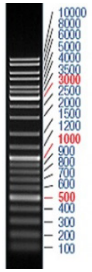

Ladder pattern

Four candidate clones out of the 18 positive clones were selected for 5’ Long-Range PCR and Southern blot validation.

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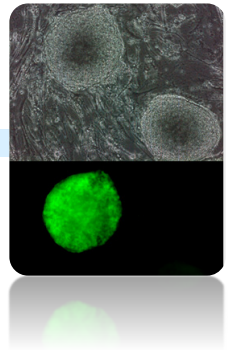
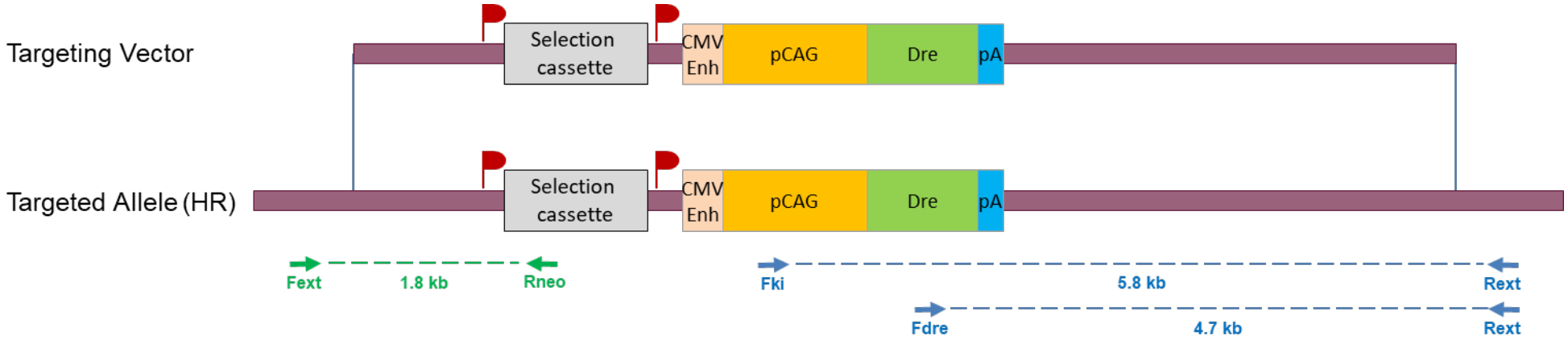
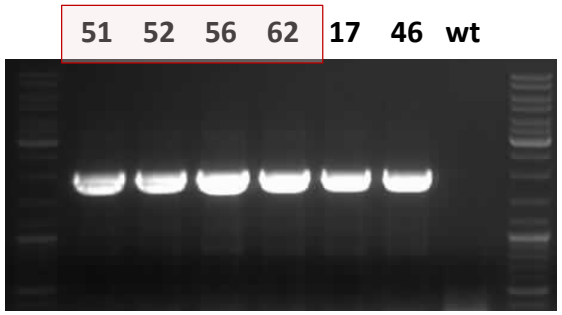
Recombinant ES validation by Long Range PCR

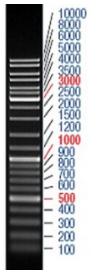


Confirmation and Validation of candidate recombinant ES clones by 5’ and 3’ PCRs

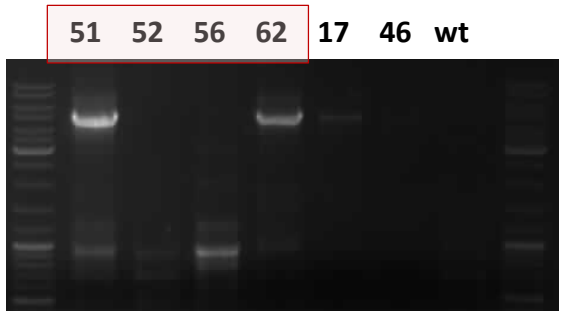




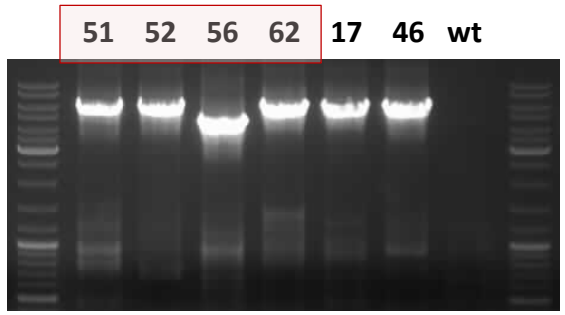
Pcr Fext – Rneo : 1.8 kb



Ladder pattern



Pcr Fdre – Rext : 4.7 kb



Pcr Fki – Rext : 5.8 kb

Clones of interest

Four candidate clones identified by 3’ PCR screening were further analysed by 5’ PCR screening.
Three clones (clones #51 #, #52 , #56 #) were confirmed. Although clone 56 was doubtful, it was included in the Southern Blot analyses.

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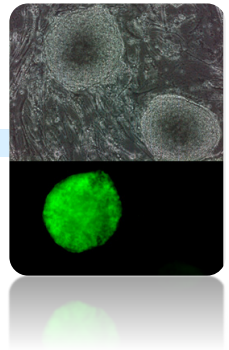
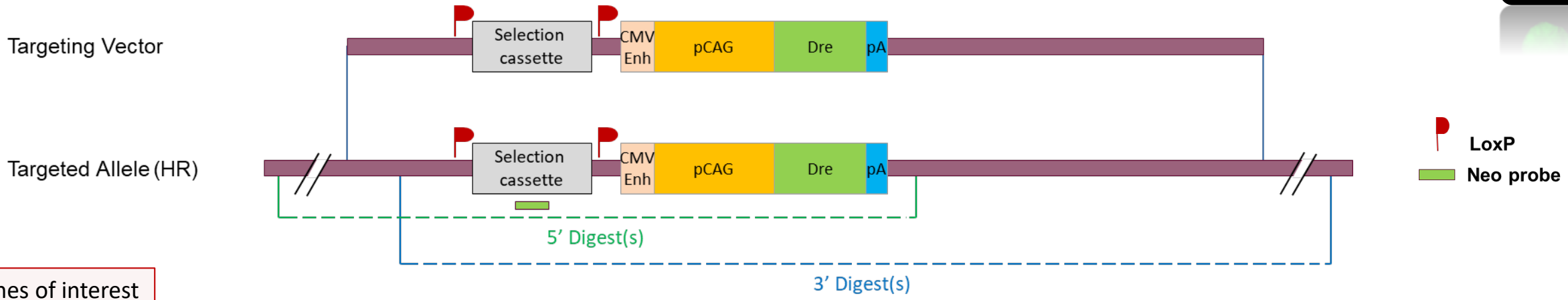
Recombinant ES clones validation by Southern Blot – Internal probe

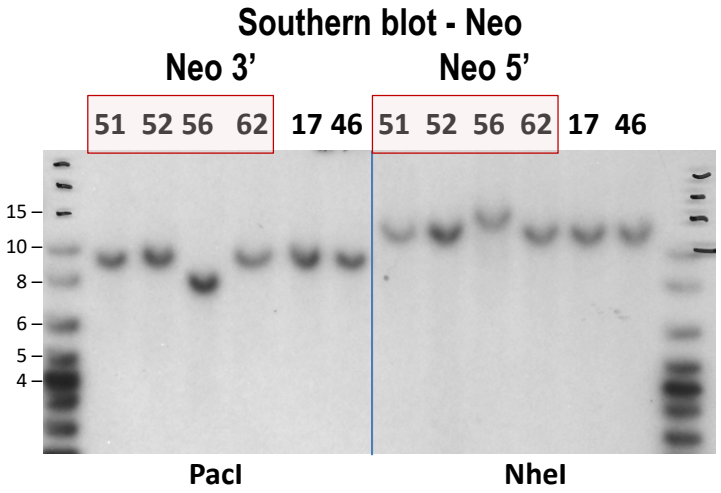


Schematic Southern Blot validation strategy

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.



Clones of interest



Neo probe sequence

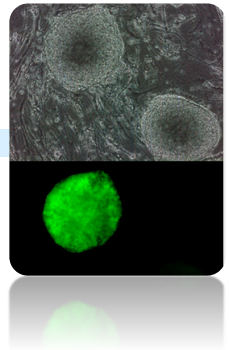
CTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTG
CGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGG
GCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAA
GTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTAC
CTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGA
TGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTC
GCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGA
TCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATG
GCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTAT
CAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATG
GGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCA
TCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGGGGATCCGCTGTAAGTC
T

Digestions used to validate the 5' and 3' insertion

Probe		Genomic DNA digest	Targeted Allele (kb)
Neo	5' digest	NheI	12.1
	3' digest	PacI	9.4

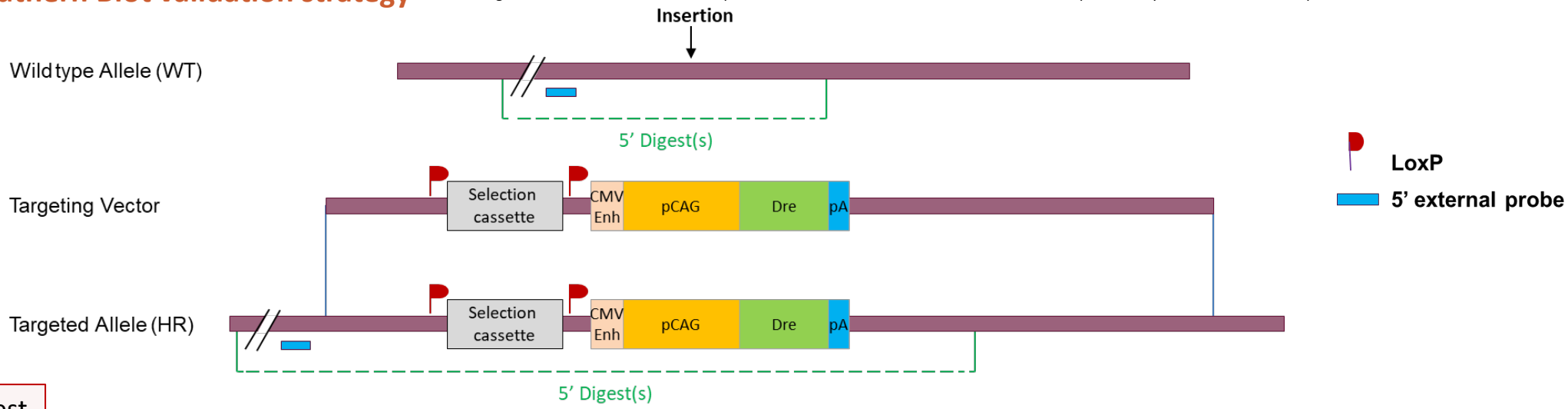
All clones (but #56) looked correct (only one band of the expected size observed with the 2 restriction digests)

Recombinant ES clones validation by Southern Blot – External probe



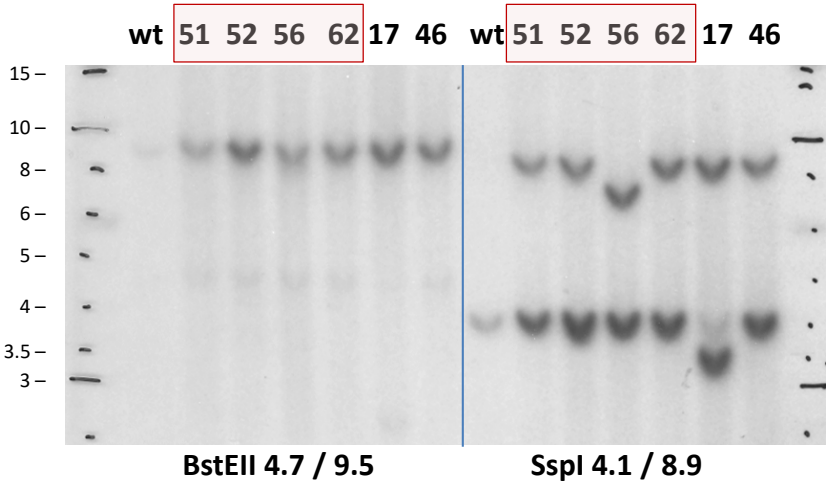
Schematic Southern Blot validation strategy

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.



Clones of interest

Southern blot – 5' probe



5' probe sequence

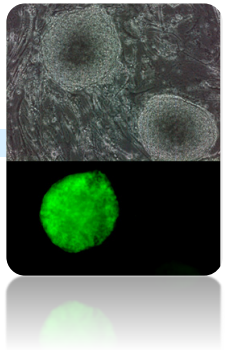
TATGTGTATTTTGAGAGCAGGGTTGGGAGGCC
TCTCCTGAAAAGGGTATAAACGTGGAGTAGGC
AATACCCAGGCAAAAAGGGGAGACCAGAGTAG
GGGGAGGGGAAGAGTCCTGACCCAGGGAAGAC
ATTAAAAAGGTAGTGGGGTCGACTAGATGAAG
GAGAGCCTTTCTCTCTGGGCAAGAGCGGTGCA
ATGGTGTGTAAAGGTAGCTGAGAAGACGAAAA
GGGCAAGCATCTTCCTGCTACCAGGCTGGGGA
GGCCCAGGCCCACGACCCCGAGGAGAGGGAAC
GCAGGGAGACTGAGGTGACCCTTCTTTCCCCC
GGGGCCCGGTCGTGTGGTTCGGTGTCTCTTTT
CTGTTGGACCCTTACCTTGACCCAGGC

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	BstEII	4.7	9.5
	5' second digest	SspI	4.1	8.9

Clone #51, #52 and #62 are correct.

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
#51	**Pass**	**Pass**
#52	Pass	Not done
#62	Pass	Not done

[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/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 #51 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
#51	4	1	13	18

Breeding to F1 generation

- Four highly chimeric males generated in the previous phase by blastocyst injection of the ES clones were mated with C57BL/6NCrl Cre deleter females that show maternal contribution (Birling et al 2012*; 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 06/06/2018

- Allele nomenclature as in MGI: **Gt(ROSA)26Sor$^{tm4.1(CAG-dre)Ics}$ (MGI:6467222)**

*Birling MC, Dierich A, Jacquot S, Hérault Y, Pavlovic G. Highly-efficient, fluorescent, locus directed cre and FlpO deleter mice on a pure C57BL/6N genetic background. Genesis. 2012 Jun;50(6):482-9. doi: 10.1002/dvg.20826. Epub 2012 Mar 20. PMID: 22121025

6 SEQUENCE OF THE Tm1.1 ALLELE (after Cre mediated excision of the floxed NeoR cassette)

GTCCATCCGCCGGCCAGCGGGGGCGGCGAGGAGGCGCTCCCAGGTTCCGGCCCTCCCCTCGGCCCCGCGCCGCAGAGTCTGGCCGCGCGCCCCTGCGCAACGTGGCAGGAAGCGCGCGCTGGGGGCGGGGACGGGCAGTAGGGCTGAGCGGCTG
CGGGGCGGGTGCAAGCACGTTTCCGACTTGAGTTGCCTCAAGAGGGGCGTGCTGAGCCAGACCTCCATCGCGCACTCCGGGGAGTGGAGGGAAGGAGCGAGGGCTCAGTTGGGCTGTTTTGGAGGCAGGAAGCACTTGCTCTCCCAAAGTCGCT
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CGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGGTCGAGGTGAGCCCCACGTTCTGCTTCACTCTCCCCATCTCCCCCCC
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LoxP CMV enh pCAG Dre SV40 pA

phenomin

EXCELLENCE IN MOUSE PHENOGENOMICS

REPORT REDACTION & VALIDATION

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