phenomin
EXCELLENCE IN MOUSE PHENOGENOMICS

MODEL GENERATION TECHNICAL REPORT

Generation of a RFP657-LF2A-Cre-F3-ERT2-F3 into Prg2 Knock-In mouse line

Project code: Ros6280 / IR6280

Report updated: 16/05/2023

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

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1 PROJECT PROCESS & QUALITY CONTROL

2 GENETIC STRATEGY

3 HOMOLOGOUS RECOMBINATION VECTOR CONSTRUCTION

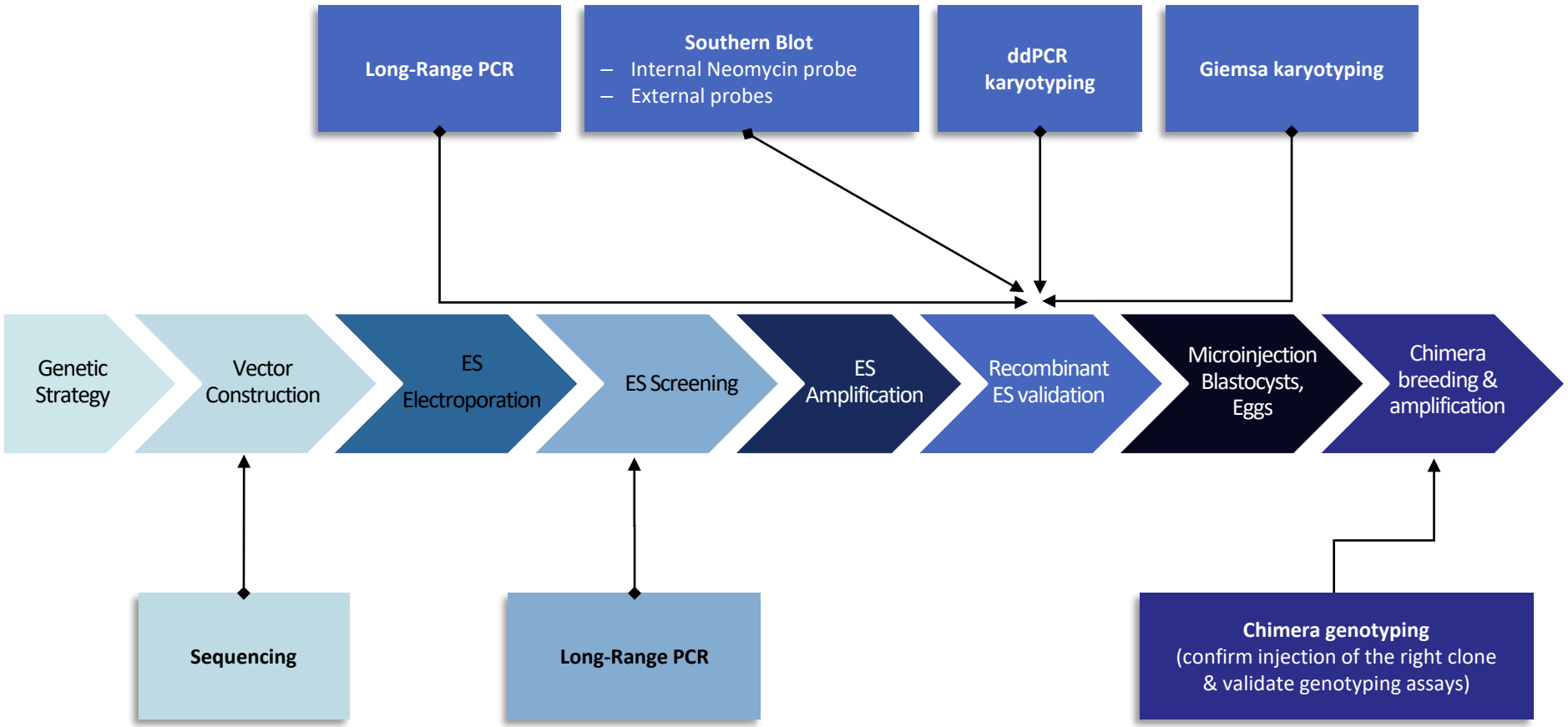
4 ES ELECTROPORATION & SCREENING OF RECOMBINANT CLONES

5 MICROINJECTION & BREEDING

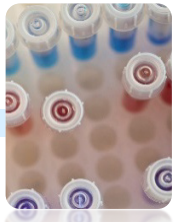
6 SEQUENCE OF THE DELIVERED ALLELE

1 PROJECT PROCESS & QUALITY CONTROL

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

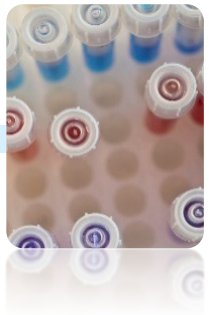


2 GENETIC STRATEGY

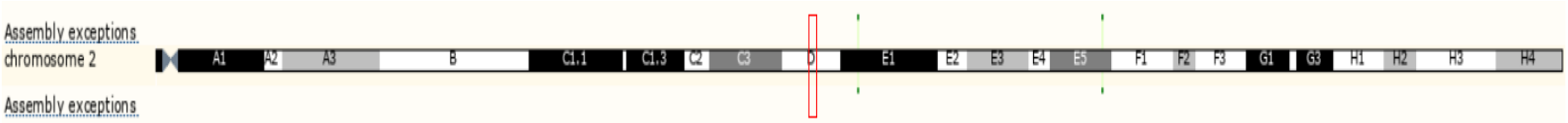


- Target locus structure
- mRNA and protein
- Genetic strategy
- PRO & CONS evaluation of the strategy

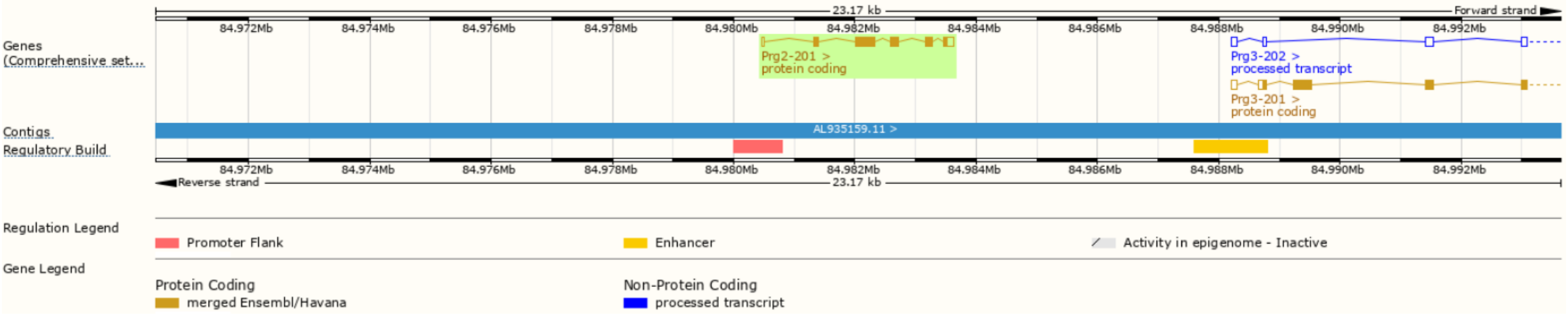
mouse genomic locus – structure



Chromosome 2: 84,980,461-84,983,632



Ensembl Gene ID : ENSMUSG00000027073



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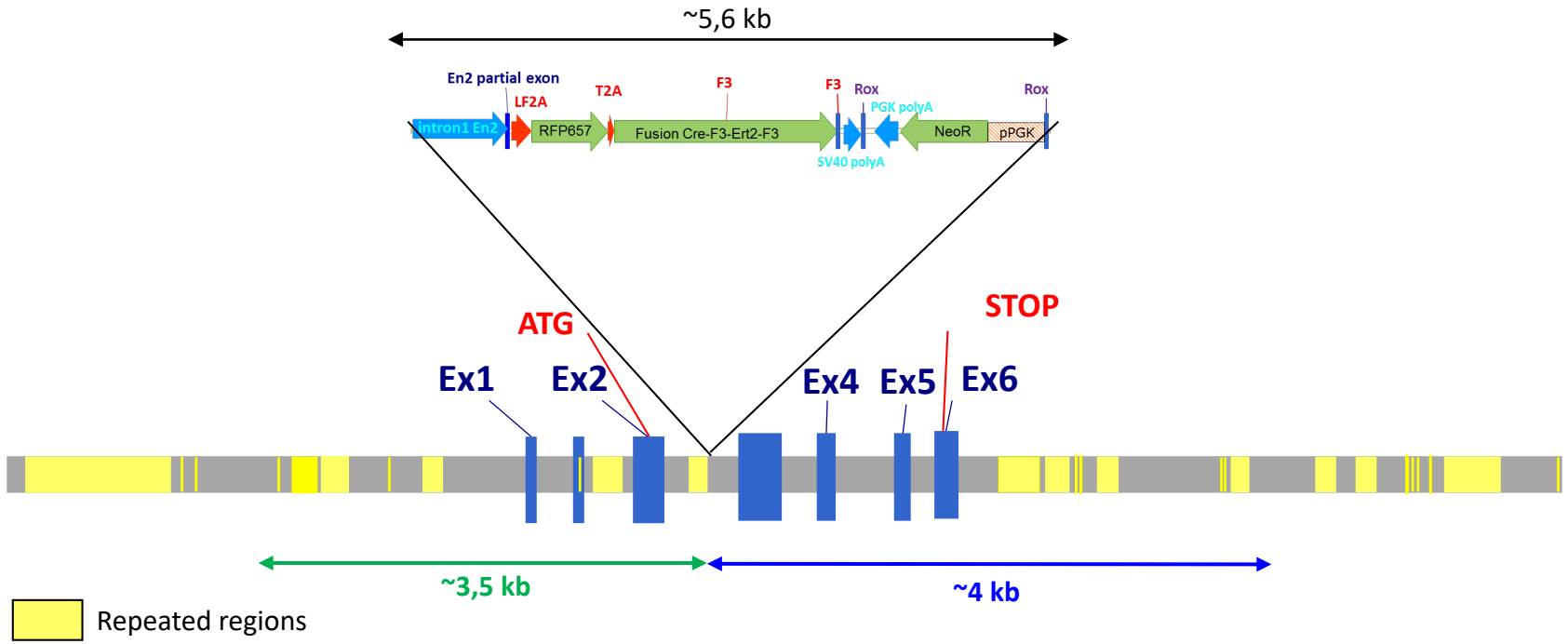
mRNA and protein

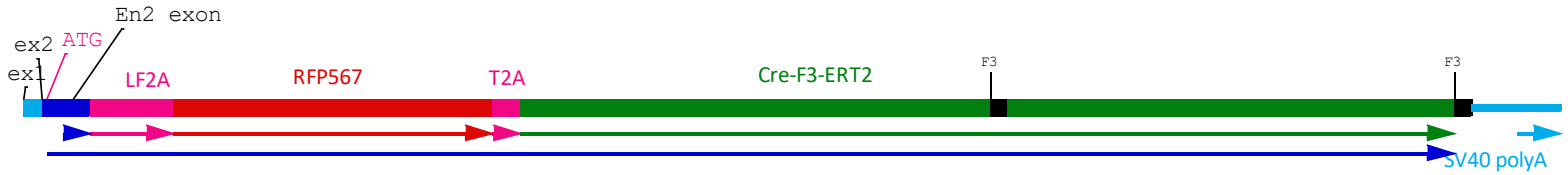
Name	Transcript ID	bp	Protein	Biotype	CCDS	UniProt	RefSeq	Flags
Prg2-201	ENSMUST00000028467.5	838	223aa	Protein coding	CCDS16198	Q545D8 Q61878	NM_008920 NP_032946	TSL:1GENCODE basicAPPRIS P1

Strategy

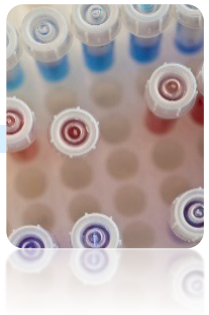
Targeted allele



mRNA and protein expressed after Cre mediated excision (sequence detail see next page)



PROs& CONs evaluation of the strategy



Pros

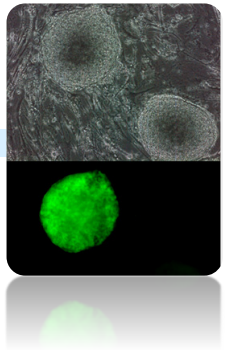
- Use of the TagRFP657 and dual cassette (Cre-F3-ER^{T2}-F3

Cons

- Presence of repeated sequences in both homology arms might render PCR amplification or LR-screen difficult

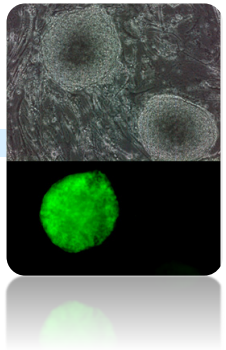
3 HOMOLOGOUS RECOMBINATION - VECTOR CONSTRUCTION

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

The targeting vector was co-electroporated with a CRISPR vector (expressing the WT Cas9 and a guide RNA –atagcacccccatgaagtac- 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. Six 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 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**.

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1 PROJECT PROCESS & QUALITY CONTROL

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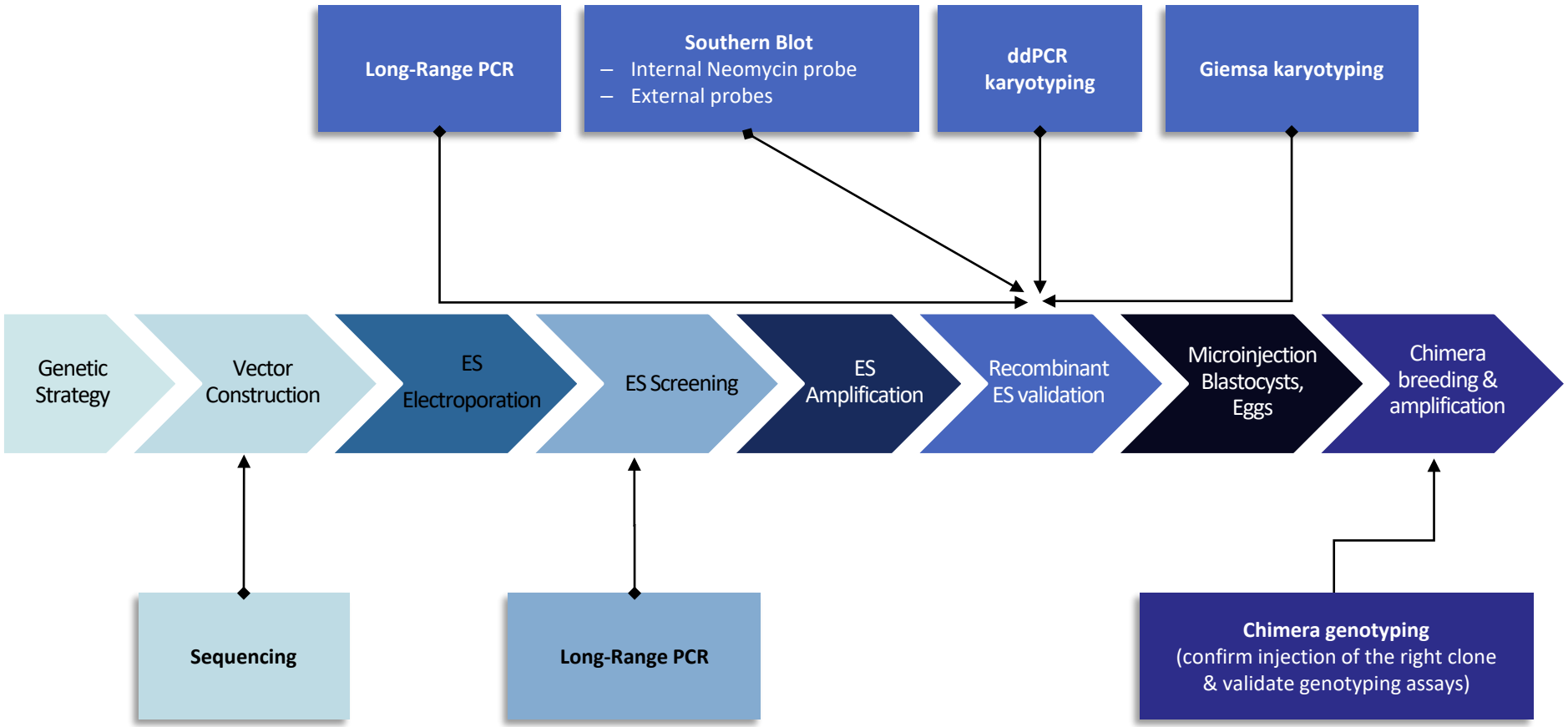
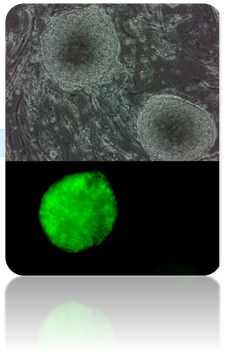
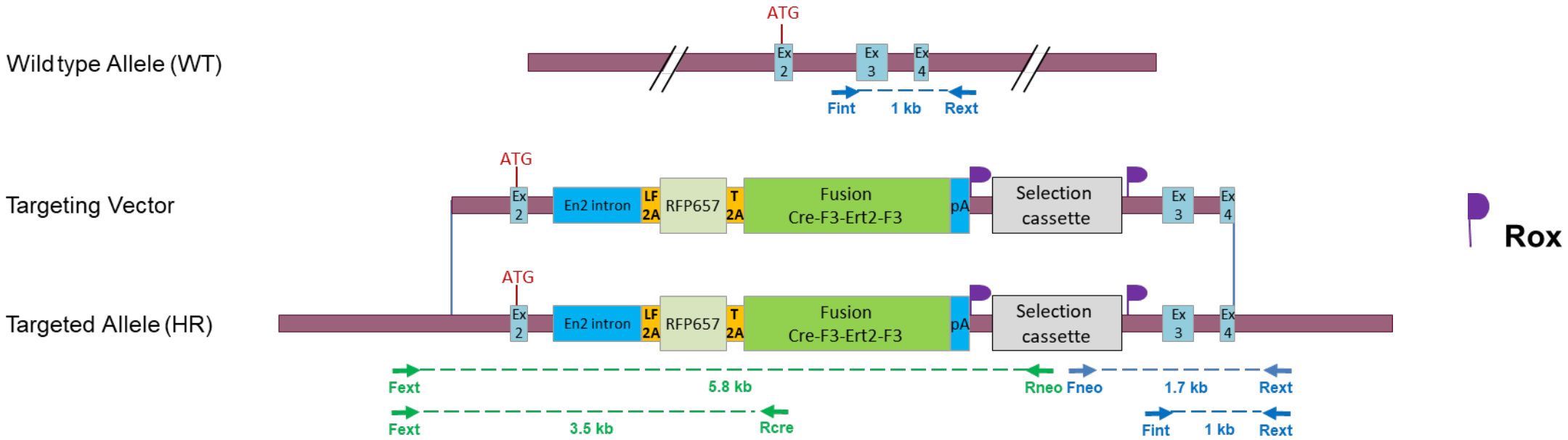


Long range PCR screening – strategy

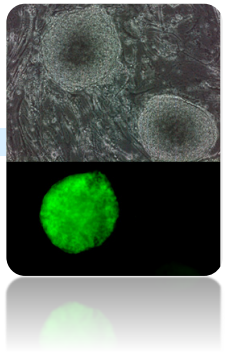


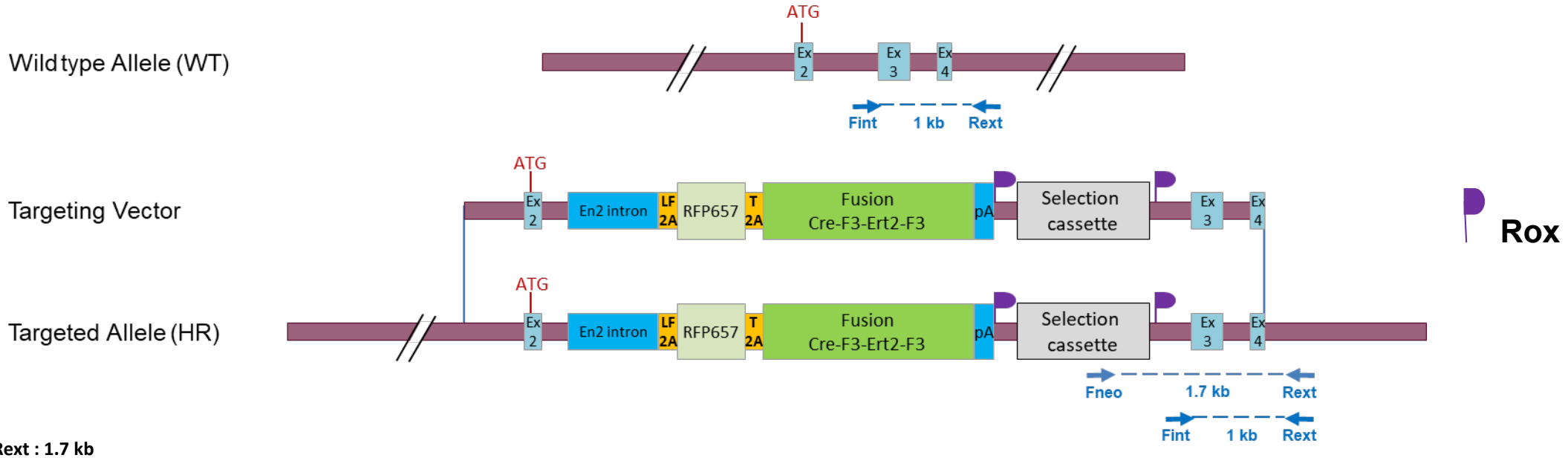
Schematic 5’ and 3’ PCR screening strategy



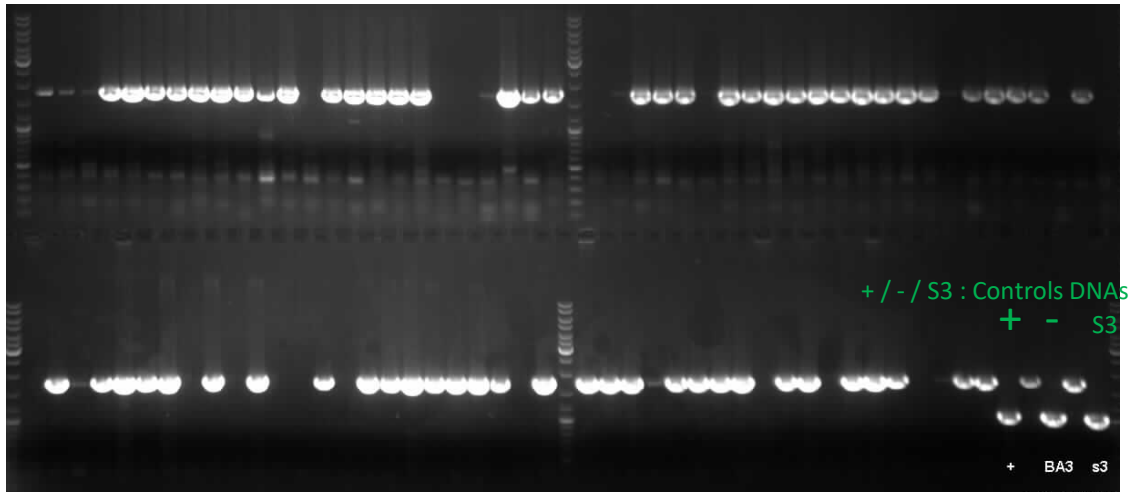
PCR	Primer Name	Primer sequences	PCR product size
5’ PCR	Fext	GCAGGTGTCATATGCTGCTTTGAGG	5.8 kb
	Rneo	AGGGGCTCGCGCCAGCCGAACTGTT	
5' PCR	Fext	GCAGGTGTCATATGCTGCTTTGAGG	3.5 kb
	Rcre	CTCTACACCTGCGGTGCTAACCAGC	
3’ PCR	Fint	GCTGGTAGTTGCTGGGTGCAGAACA	1 kb
	Rext	GGTAGATACTGGATGGGATCACAAG	
3’ PCR	Fneo	GCGGCCGGAGAACCTGCGTGCAATC	1.7 kb
	Rext	GGTAGATACTGGATGGGATCACAAG	

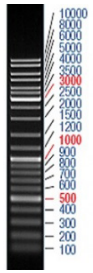
Long-Range 3’ PCR screening – results





Pcr Fneo – Rext : 1.7 kb



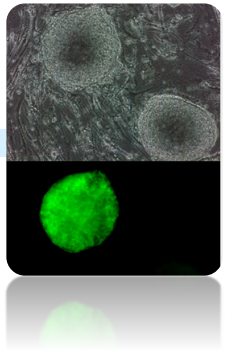


Ladder pattern

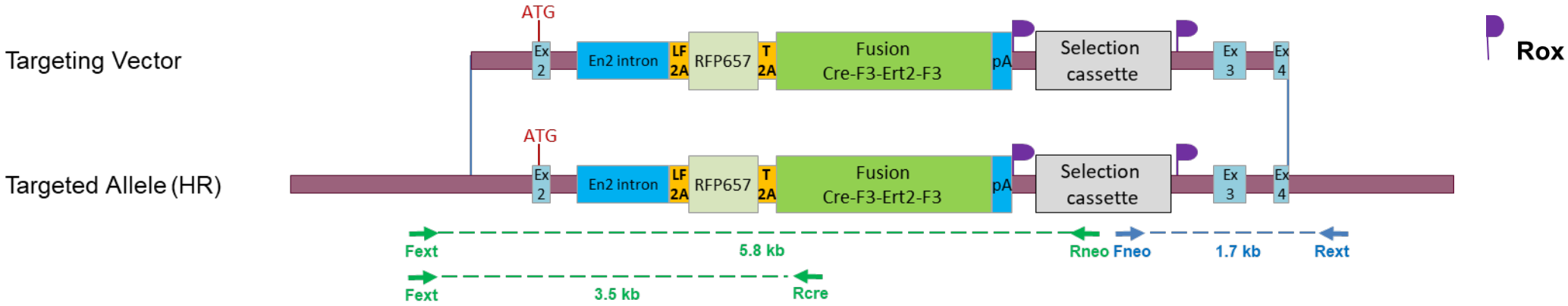
Pcr Fint – Rext : 1 kb

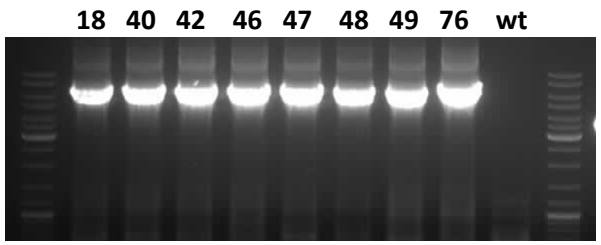
Six candidate clones out of the 74 positive clones were selected for 5’ Long-Range PCR and Southern blot validation.

Recombinant ES validation by Long Range PCR

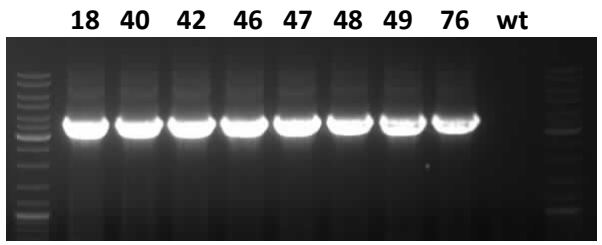


Confirmation and Validation of candidate recombinant ES clones by 5’ and 3’ PCRs

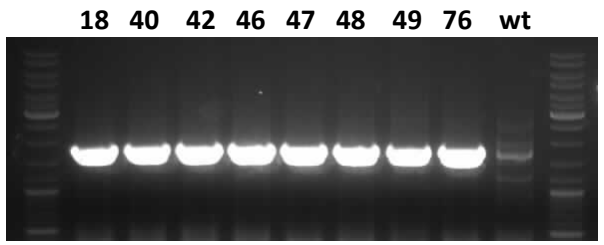




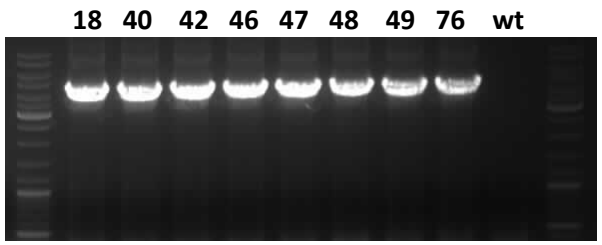
Pcr Fext – Rneo :5.8 kb (+DMSO)



Pcr Fext – Rcre :3.5 kb (+DMSO)



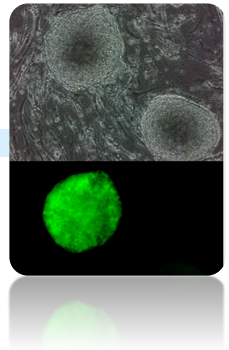
Pcr Fneo – Rext : 1.7 kb



Pcr Fcre – Rext : 4.4 kb

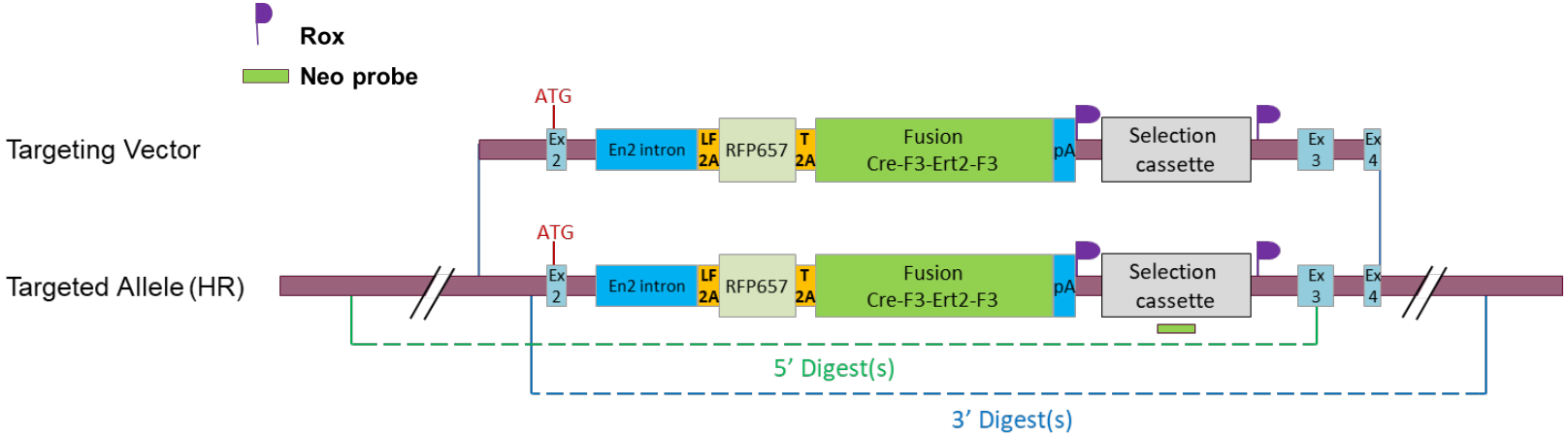
Eight candidate clones identified by 3’ PCR screening were further analysed by 5’ PCR screening.
Eight clones (clones #18, #40, #42, #46, #47, #48, #49 and #79) were confirmed.

Recombinant ES clones validation by Southern Blot – Internal probe



Schematic Southern Blot validation strategy

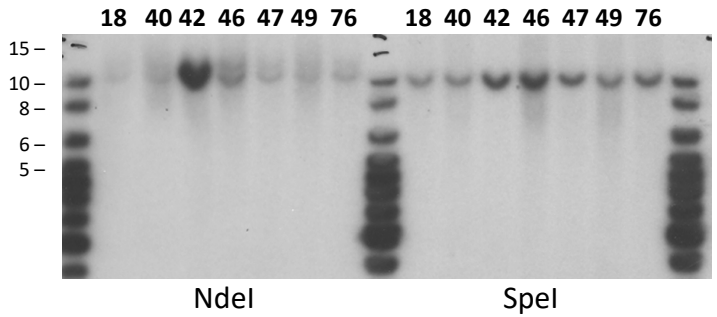
Digests on the scheme illustrate the position of the chosen restriction sites relative to the probe. They don't show the exact position of the restriction sites.



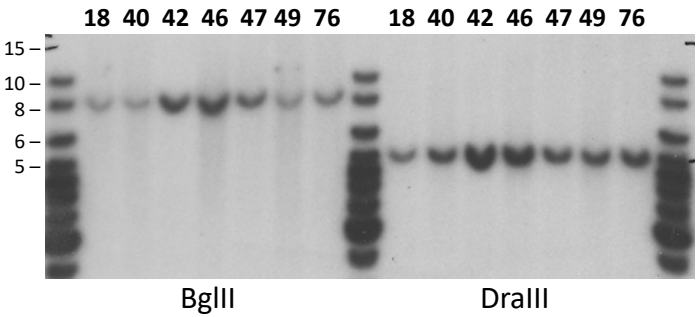
Neo probe sequence

AGACTTACAGCGGATCCCCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGC
GAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTC
TTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGC
CACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCG
CCATGGGTCACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTC
GGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCA
TCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGA
TCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAG
GTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTT
CAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGC
GCTGCCTCGTCCTGCAG

Southern blot - Neo 5'



Southern blot - Neo 3'

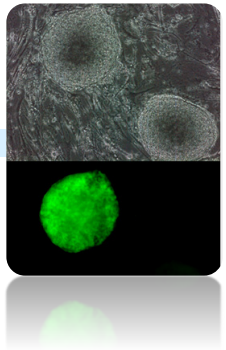


Digestions used to validate the 5' and 3' insertion

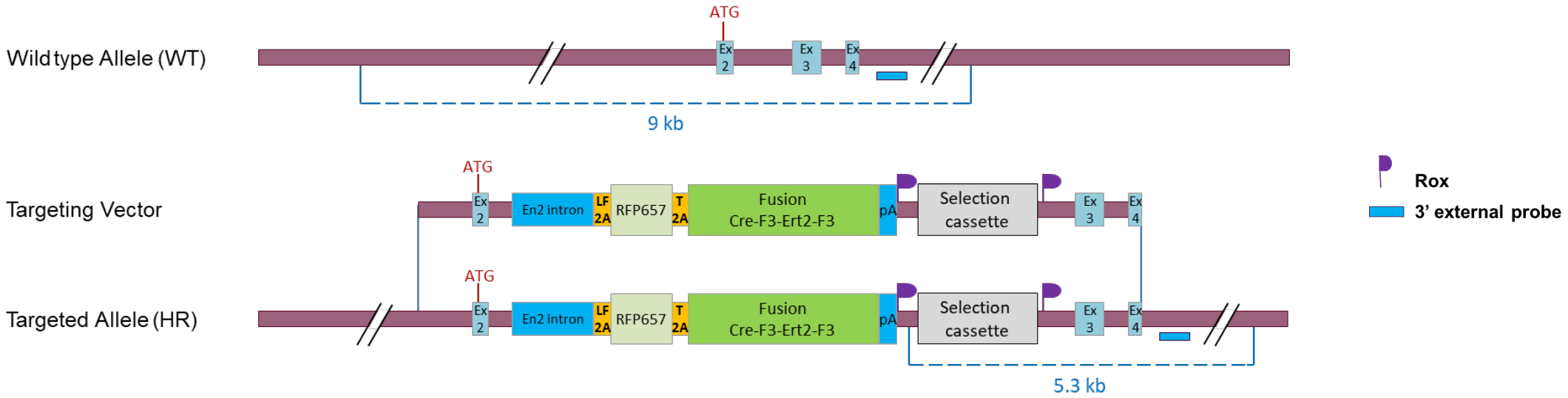
Probe		Genomic DNA digest	Targeted Allele (kb)
Neo	5' digest	NdeI	10.9
		SpeI	10.5
	3' digest	BglII	8.6
		DraIII	5.1

All 7 clones show only on band at the expected sizes with both restriction digests.

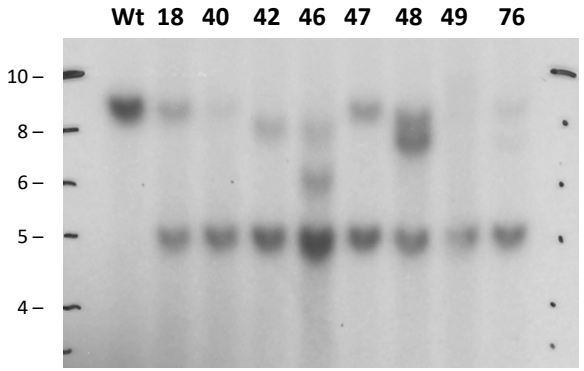
Recombinant ES clones validation by Southern Blot – External probe



Schematic Southern Blot validation strategy



Southern blot – 3' probe



BspHI 9 / 5.3

3' PROBE SEQUENCE

CCATCCAGTATCTACCTTGCTGCAGCTTTGTCCGTATTTCCC
CTTTACTATCCCCTAAAGAGCTGGGGACAGAAGGCCACCTAG
GCAAGACATCGAGATATCACAAAGGGTTTAGAATCAGGTTTG
AGTCCAAGTTCCTCCCAGTTTTTCTATCTTTGTGGCCTGGGG
ACTGTGTCCTTACCTGTAGGTTGCACATGGTTTCCCTTGCCC
CATTACTGGTTTGTGATATAAGGATGATCATGTCAGAGAATG
GGTTGGTGATGACCACTGTGGACCATTAGTTCATCTGCCCAG
TATCCATCATGGTCCTGTGGTCAAGGATGTCAGGAGGAGTTA
ACAGGGACAACGCTGGCTCTGCCTCTCTTCCTTCCCCCATGT
ATGCTTCTGCTTCCTACCTTCTCTAGGGCCGATGCAAACGCT
TTCGATGGGTTGATGGAAGTTCTTGG

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	BspHI	9	5.3

⇒ All clones were validated (targeted band at the expected size).

⇒ The additional band observed some clonesshown that the untargeted allele was edited by CRISPR.

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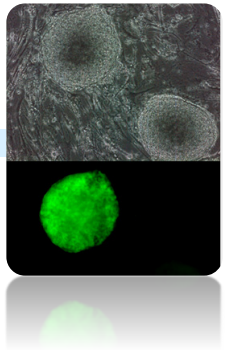
Aneuploidy screening in ES recombinant clones



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
#18	Limit	Not done
#40	Pass	Not done
#42	Pass	Not done
#46	Pass	Not done
#47	**Pass**	**Pass**
#48	Failed	Not done
#49	Pass	Not done
#76	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/6 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 #47 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
#47	1	2	3	6

Breeding to F1 generation

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

- Germ line transmission was obtained the 24/09/2018

- The Roxed NeoR cassette was excised by an additional breeding with a Dre deleter (Gt(ROSA)26Sor<tm4.1(CAG-dre)Ics>)

- Allele nomenclature (following MGI guidelines) : **Prg2$^{tm1.1Ics}$**

6 SEQUENCE OF THE DELIVERED ALLELE

ISO 9001·NFX 50-900

ATCTTCCTCGAACAGGTCCAAATGGTCTGGCTATGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGCAAAACTTCTGTGTTCTCTATTTCAGGACAAAGCCAAGATGAAATTCCCTCTACTTCTGGCTCTTCTAGTCGGGGGAGCGTCTGCTCTTCATCTGAGTGAGTTAGTTCGTT
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GGGACTAACAGAAGAACCCGTTGTGGGGAAGCTGTTGGGAGGGTCACTTTATGTTCTTGCCCAAGGTCAGTTGGGTGGCCTGCTTCTGATGAGGTGGTCCCAAGGTCTGGGGTAGAAGGTGAGAGGGACAGGCCACCAAGGTCAGCCCCCCCCCCCCTATCCCATAGGAGCCAGGTCCCTCT
CCTGGACAGGAAGACTGAAGGGGAGATGCCAGAGACTCAGTGAAGCCTGGGGTACCCTATTGGAGTCCTTCAAGGAAACAAACTTGGCCTCACCAGGCCTCAGCCTTGGCTCCTCCTGGGAACTCTACTGCCCTTGGGATCCCCTTGTAGTTGTGGGTTACATAGGAAGGGGGACGGGATT
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