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EXCELLENCE IN MOUSE PHENOGENOMICS

MODEL GENERATION TECHNICAL REPORT

Rabbit β - globin intron-RFP657-Cre-F3-ER^{T2}-F3 Knock-In into Mcpt8

Project code: IR6279 / Ros6279b

Report updated: 31/03/2022

cnrs
Université de Strasbourg
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CERBM centre européen de recherche en biologie et en médecine gie
Aix*Marseille université

MODEL GENERATION TECHNICAL REPORT

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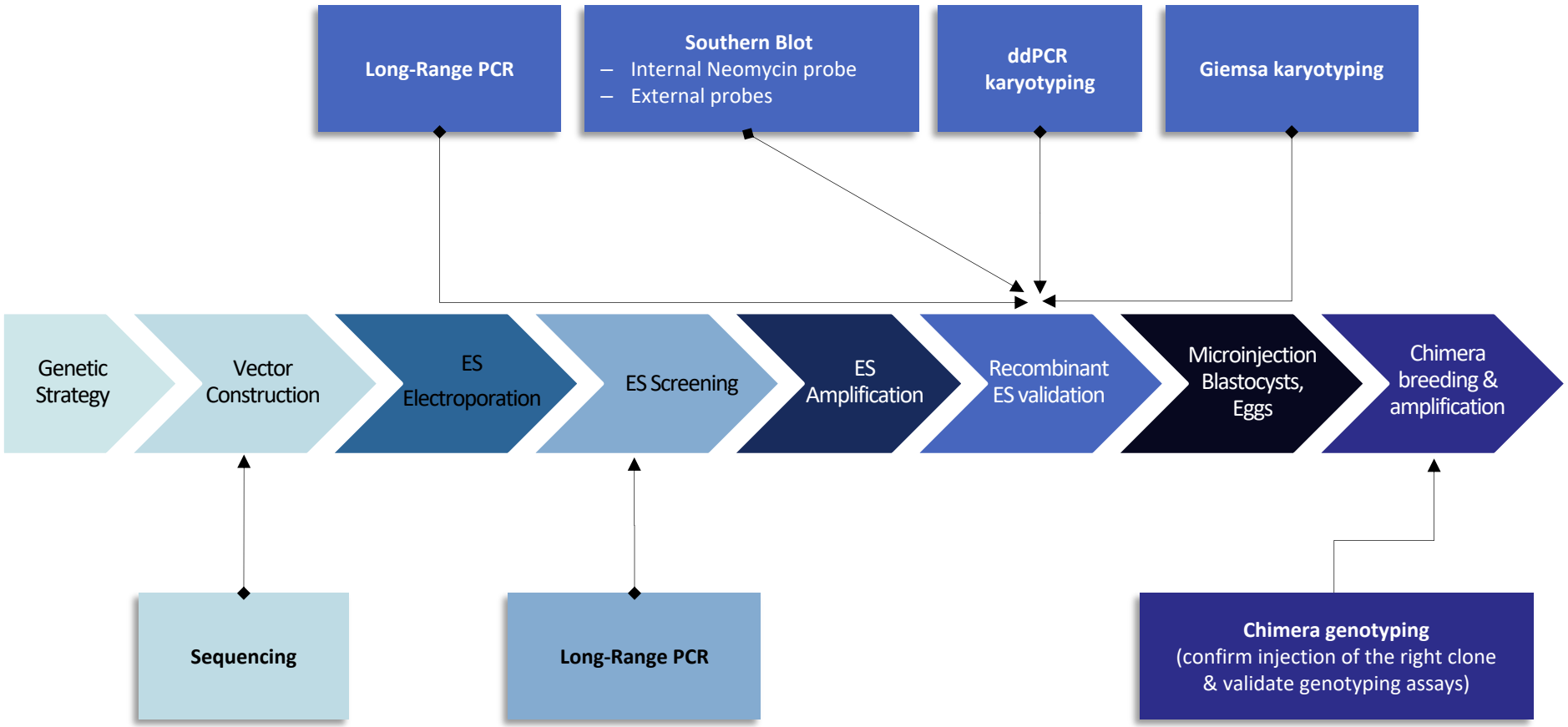
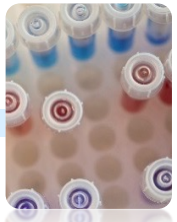
5 MICROINJECTION & BREEDING

6 SEQUENCE OF THE DELIVERED ALLELE

PROJECT PROCESS & QUALITY CONTROLS

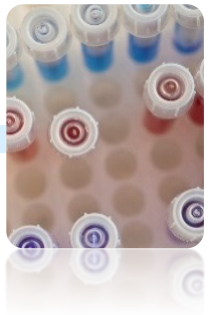


2 GENETIC STRATEGY



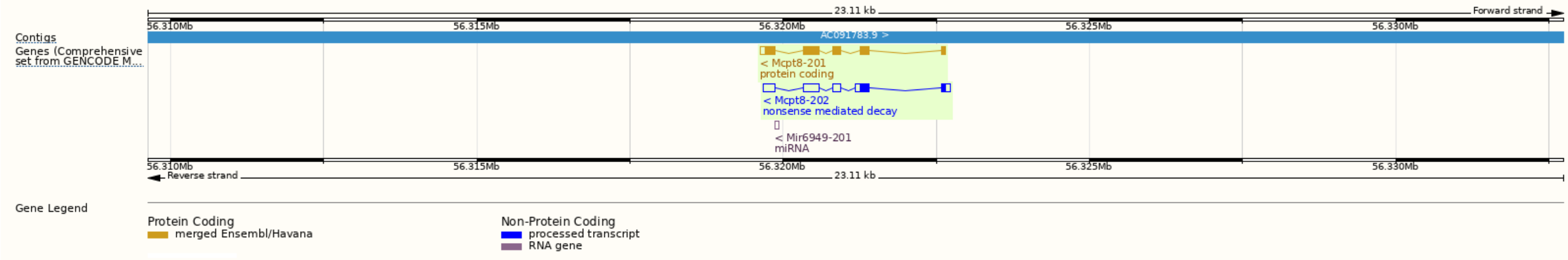
- Target locus structure
- mRNAs and protein
- Genetic strategy
- PRO & CONS evaluation of the strategy

mouse genomic locus – structure

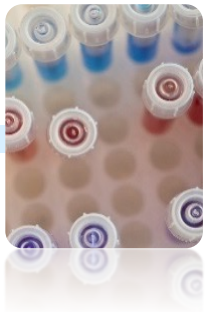


Chromosome 14: 56,319,623-56,322,730

Gene: Mcpt8 ENSMUSG00000022157

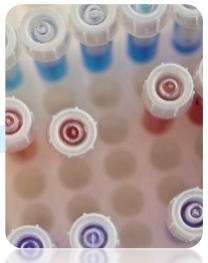


mRNAs and protein

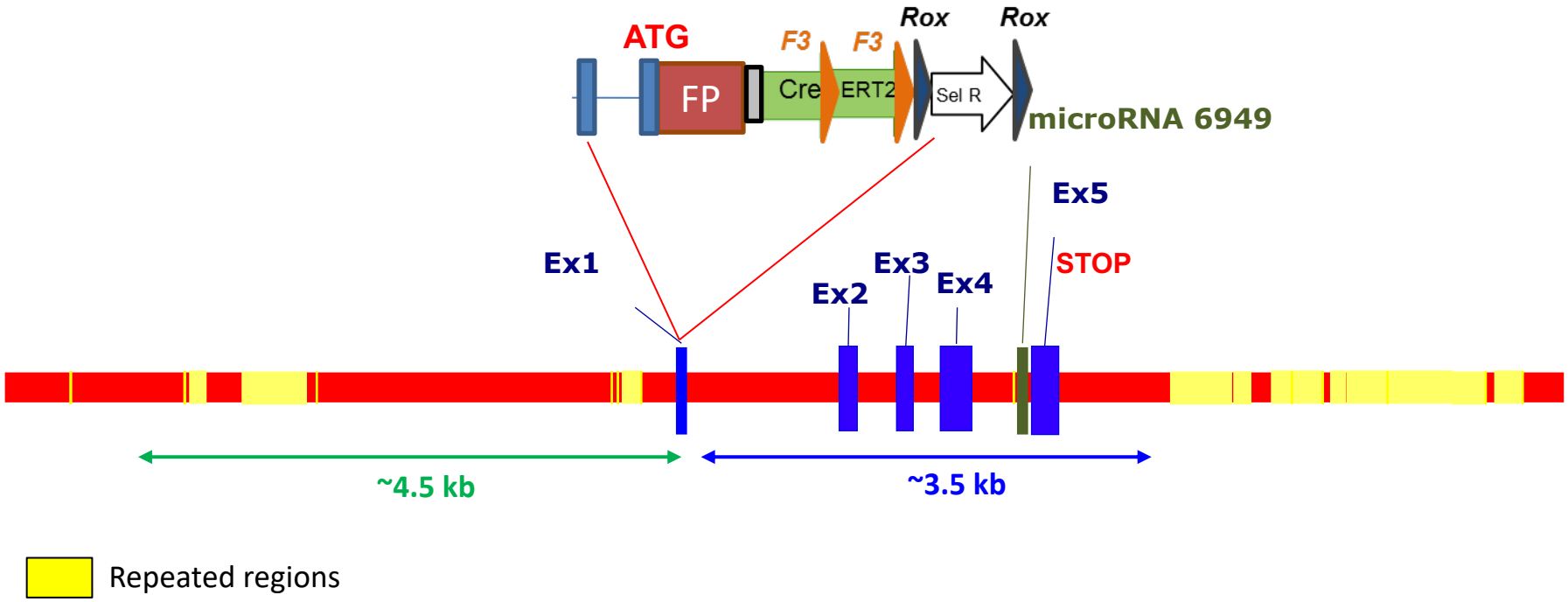


Transcript ID	Name	bp	Protein	Biotype	CCDS	UniProt Match	Flags
ENSMUST00000015594.9	Mcpt8-201	836	247aa	Protein coding	CCDS27141	P43430 Q3UWB6	GENCODE basic APPRIS P1 TSL:1
ENSMUST00000225107.2	Mcpt8-202	945	68aa	Nonsense mediated decay	-	A0A286YDN6	-

Strategy



Strategy : FP-LF2A-CreER^{T2} KI at the start codon (accepted the 28/05/2016)

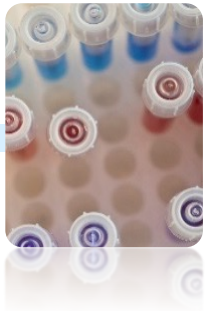


Repeated regions

FP for fluorescent protein; ie TagRFP657 a far - red monomeric fluorescent protein with an emission peak at 657 nm

In project Ros6279b, an intron is added in the first exon (in order to stabilize the transgenic cDNA)

PROs& CONs evaluation of the strategy



PROs

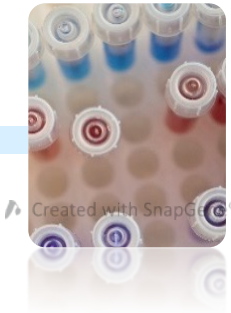
- A reporter (TagRFP657 followed by a LF2A sequence will be added at the ATG)
- Expression of CreER^{T2} is expected to follow the same pattern than the gene itself
- The ER^{T2} sequence (F3-ER^{T2}-F3) can be deleted in vivo by breeding males from the established line with Flp deleter females (see *Birling et al. Genesis. 2012 Jun;50(6):482-9*). So we will be able to obtain the non - inductible Cre line also.

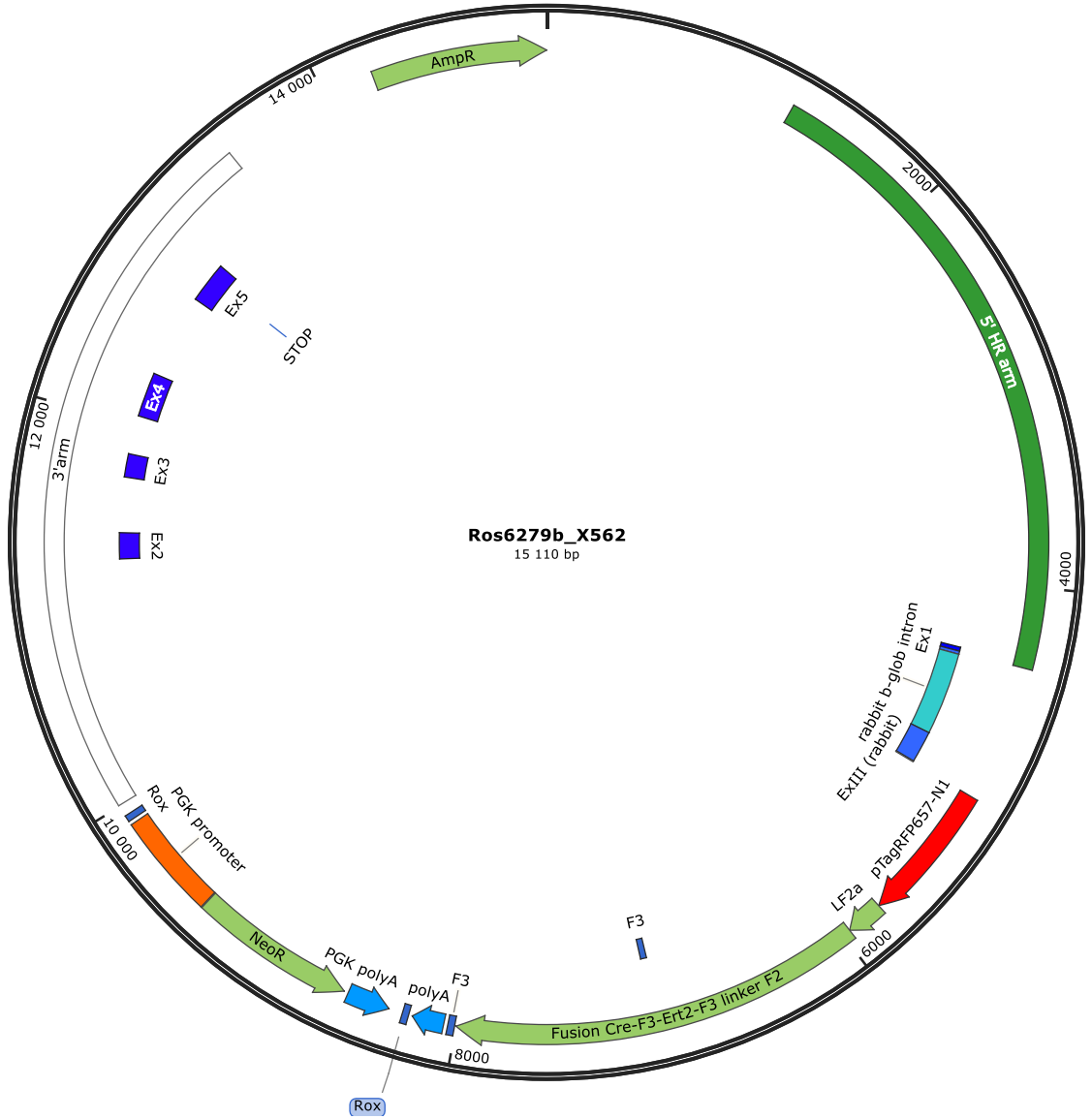
CONs

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

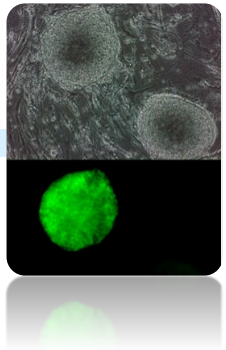
The selection cassette (FRT- Neo -FRT) will be removed by breeding male chimera with a flp deleter line which shows maternal contribution (Birling et al., 2012)

3 HOMOLOGOUS RECOMBINATION - VECTOR CONSTRUCTION



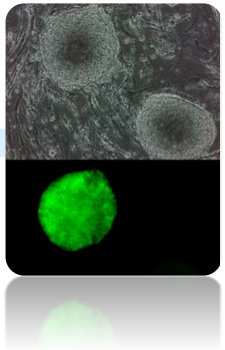


4 ES cell electroporation & Screening of recombinant clones



- 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 electroporated in the proprietary C57BL/6NCrl S3 cell line.

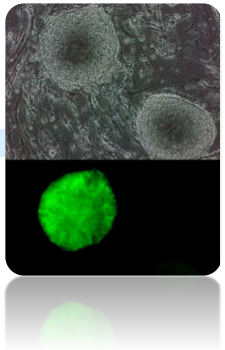
Transfected ES clones were submitted to neomycin selection (G418) and 93 resistant ES clones were isolated. The clones were then submitted to the screening process allowing secured identification of those harbouring the expected recombination events at both ends of targeting vector.

Screening process steps:

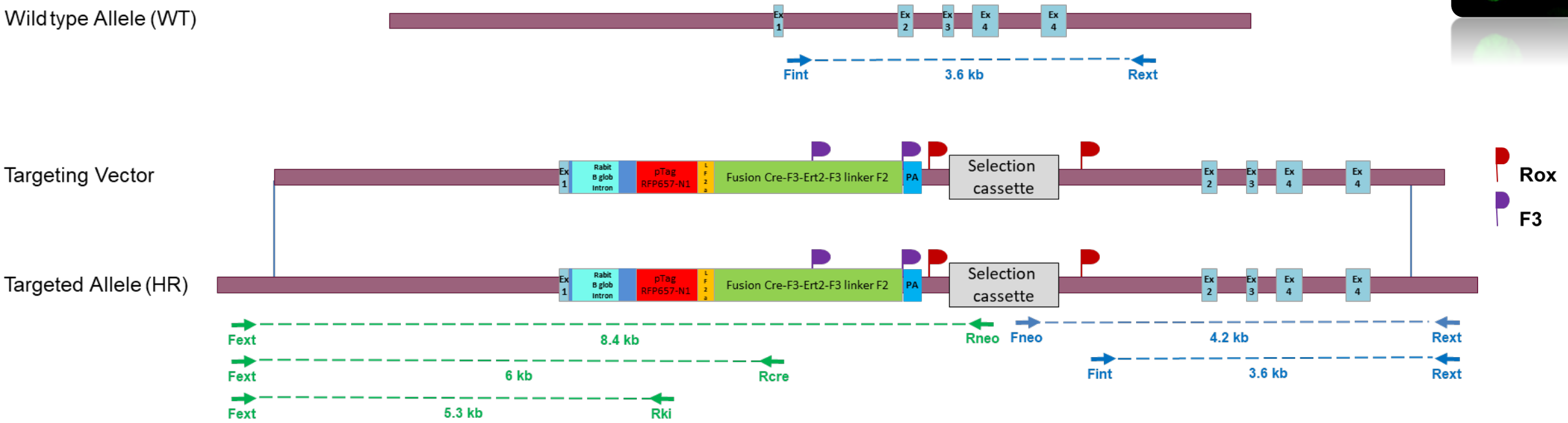
1. Identification of candidate recombinant clones by initial 3' Long-Range PCR
2. Six of 3' PCR positive clones are confirmed for 5' recombination event by Long-Range PCR
3. Positive clones in step2 are further validated by Southern blot analysis using internal and external probes
4. The karyotype of 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**

Long range PCR screening – strategy

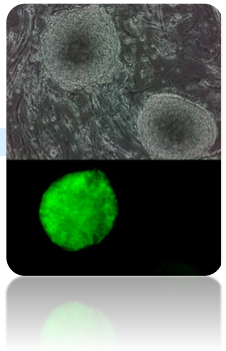


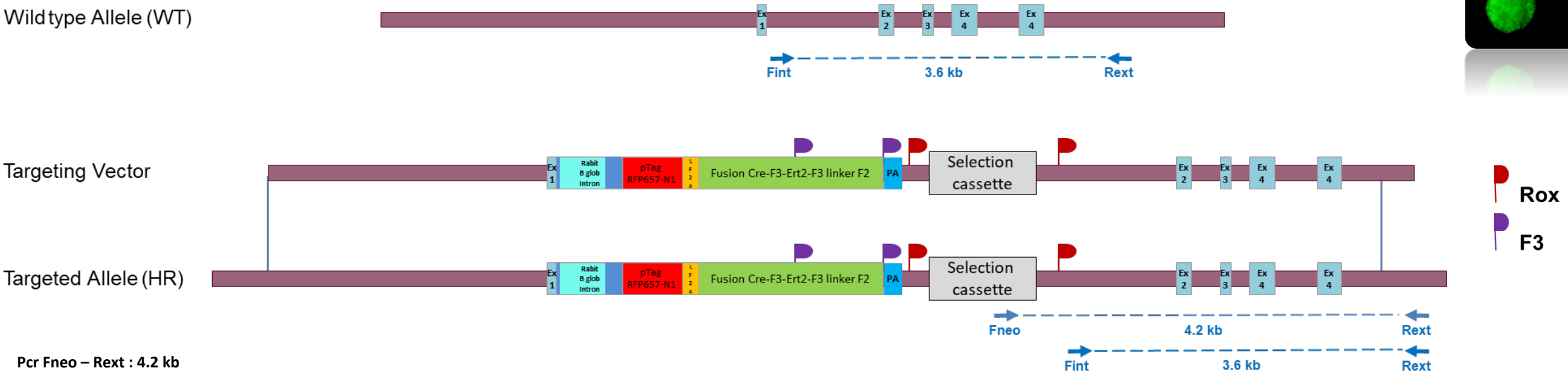
Schematic 5’ and 3’ PCR screening strategy



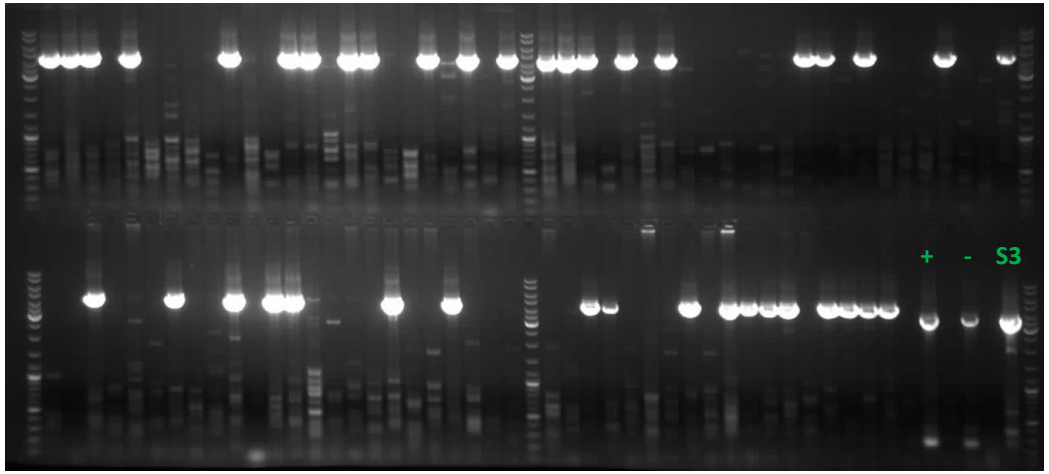
PCR	Primer Name	Primer sequences	PCR product size
5' PCR	Fext	GGTCATGCTCACCAGTAACTGACAG	8.4 kb
	Rneo	AGGGGCTCGCGCCAGCCGAACTGTT	
5' PCR	Fext	GGTCATGCTCACCAGTAACTGACAG	6 kb
	Rcre	CCAGATTACGTATATCCTGGCAGCG	
5’ PCR	Fext	GGTCATGCTCACCAGTAACTGACAG	5.3 kb
	Rki	GCTAGGGAGGTCGCAGTATCTGGCC	
3’ PCR	Fneo	GCGGCCGGAGAACCTGCGTGCAATC	4.2 kb
	Rext	GGAATTGAGTCTCTACTGGCTTGAA	
3’ PCR	Fint	TTCCTGCTCCTGGTCCTCCTGGTGG	3.6 kb
	Rext	GGAATTGAGTCTCTACTGGCTTGAA	

Long-Range 3’ PCR screening – results



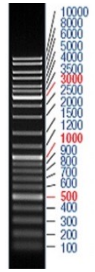


Pcr Fneo – Rext : 4.2 kb



+ / - / S3 : Controls DNAs

Pcr Fint – Rext : 3.6 kb



Ladder pattern

Six candidate clones out of the 40 positive clones were selected for 5’ Long-Range PCR and Southern blot validation.

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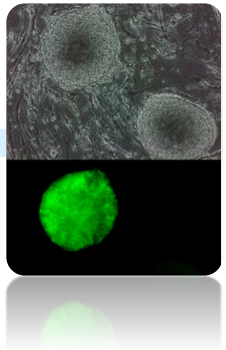
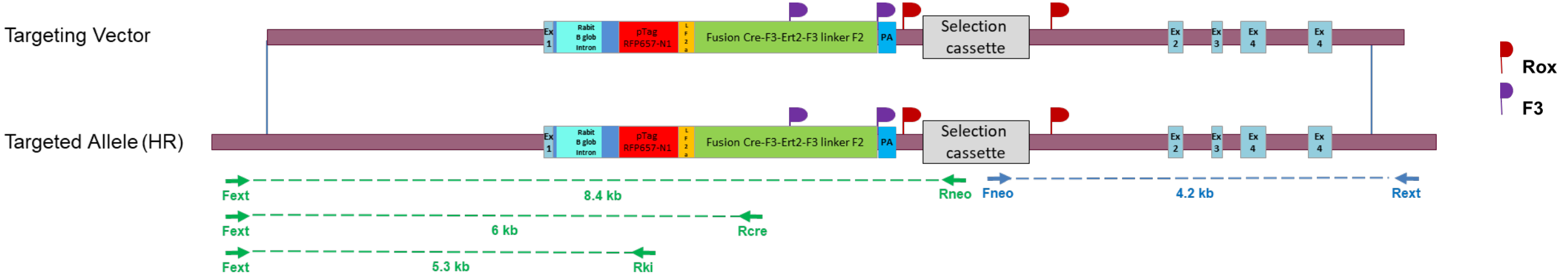
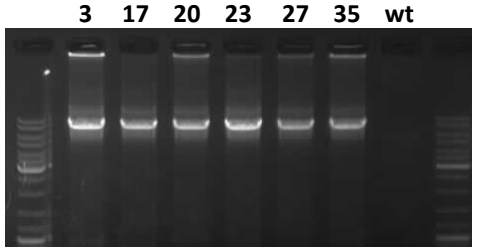
Recombinant ES validation by Long Range PCR

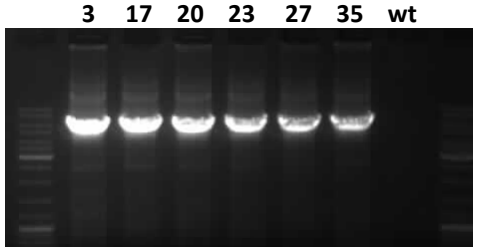


Confirmation and Validation of candidate recombinant ES clones by 5’ and 3’ PCRs

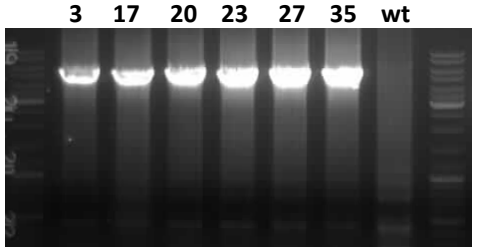




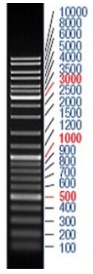
Pcr Fext – Rneo : 8.4 kb



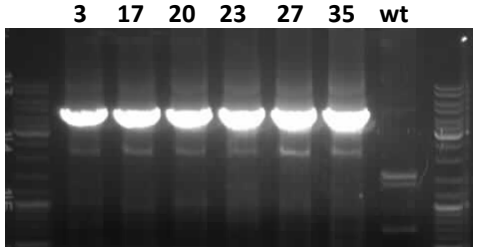
Pcr Fext – Rcre : 6 kb



Pcr Fext – Rki : 5.3 kb



Ladder pattern



Pcr Fneo – Rext : 4.2 kb

Six candidate clones identified by 3’ PCR screening were further analysed by 5’ PCR screening.
Six clones (clones #3, #17, #20, #23, #27 and #35) were confirmed.

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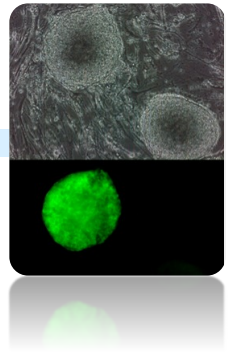
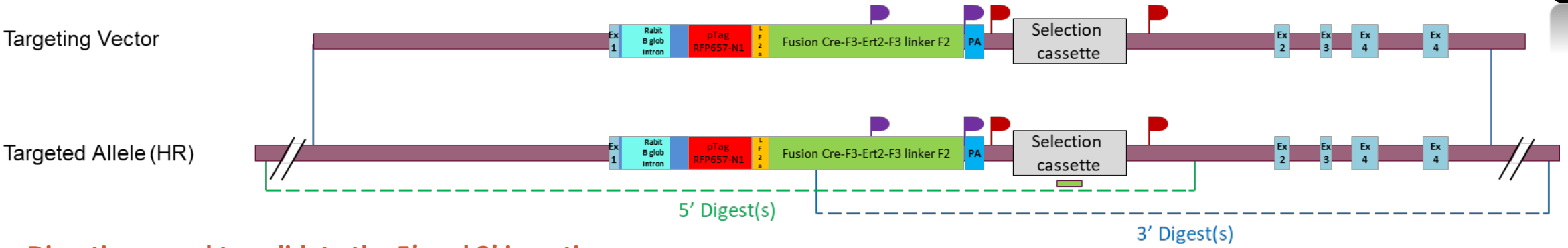
Recombinant ES clones validation by Southern Blot – Internal probe



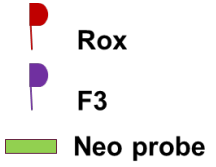
Schematic Southern Blot validation strategy

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.

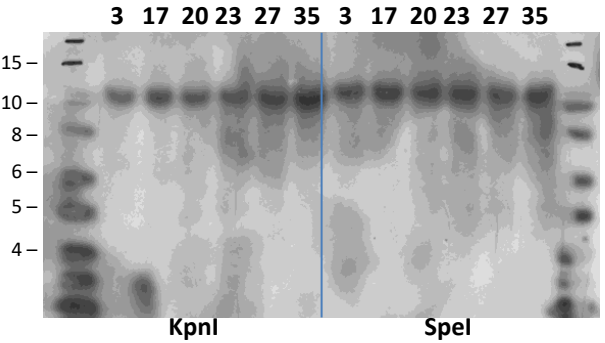


Digestions used to validate the 5' and 3' insertion

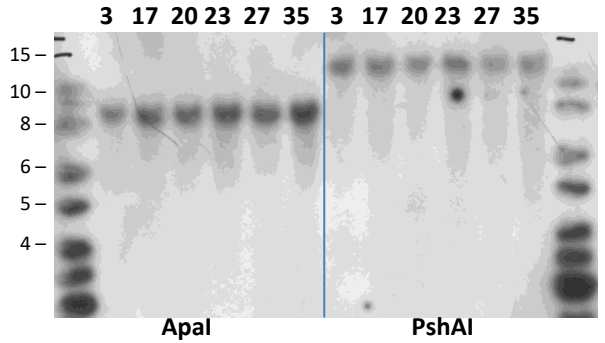
Probe		Genomic DNA digest	Targeted Allele (kb)
Neo	5' digest	KpnI	11.2
		SpeI	11.6
	3' digest	ApaI	9.1
		PshAI	13



Southern blot - Neo 5'



Southern blot - Neo 3'



Neo probe sequence

AGACTTACAGCGGATCCCCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCG
AATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTT
CAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGCCAC
AGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCGCCAT
GGGTCACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTG
GCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAG
TACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCG
TATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATG
ACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAA
CGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGT
CCTGCAG

=> All 6 clones showed only one band at the expected size with all 4 restriction digestions

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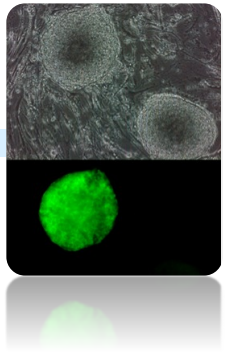
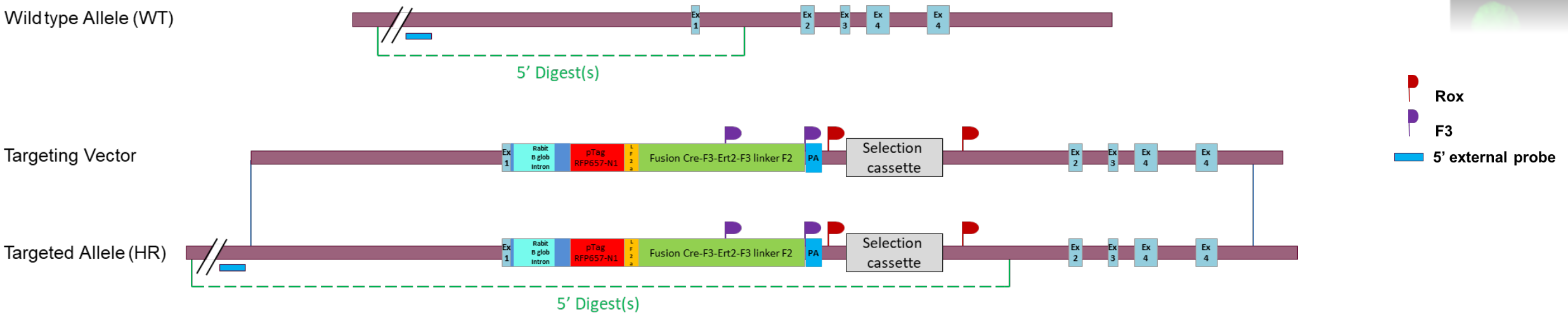
Recombinant ES clones validation by Southern Blot – External probe

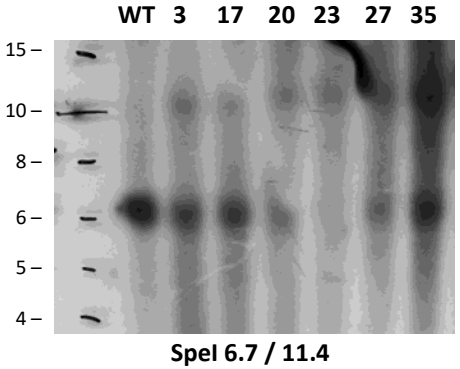


Schematic Southern Blot validation strategy

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.



Southern blot – 5' probe



SpeI 6.7 / 11.4

5' probe sequence

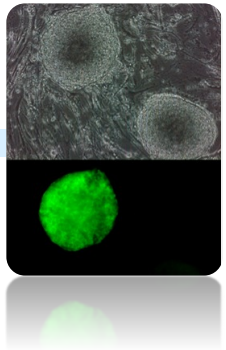
GCTCATTTCATTCTATAATAATCCTTGGACAAGCTGCTACCTTCTCCAGA
ATGCCCTTCTTTCCCTCAGGTGCCAATAGAATGGACTCCAGTCAAACTTG
ACATTCATTCTTACCACAGACACTGTTTACTGGTGGATCCTTACCACAAA
AGGGTTAAAGCACATGGCAATGAGGATTCTCTTACCAAGCAGCTCCAGAA
GACTGTTGGATTCCTAGGCCCCAGGACTTTCTGTTGCATGGTATCTACAG
GGCTGTCCTGCCAGACCAGTAACTTGCCACTCAGATCCCATCATGTCCAA
GGGCATTCGAATTGTGGAACAGTCTAAATGGGTTAGAGGGAGAAAACGGA
ATCAAGATACCTACCCCATACCACTACATCAGAGAAAACCATGAGTATTT
GCTATGGC

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	SpeI	6.7	11.4

All 6 clones are correct.

Aneuploidy screening in ES recombinant clones

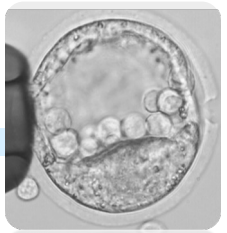


Selected recombinant ES cells clones were karyotyped by ddPCR as described in Codner *et al.* [1] and by Giemsa metaphase staining. Results of aneuploidy analysis are presented in the table below.

Clone ID	ddPCR	Giemsa
#3	Pass	Not done
#17	Pass	Not done
#20	Pass	Not done
#23	Pass	Not done
#27	**Pass**	**Pass**
#35	Pass	Not done

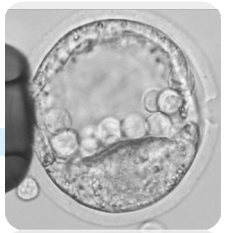
[1] Codner, G.F., Lindner, L., Caulder, A., Wattenhofer-Donzé, M., Radage, A., Mertz, A., Eisenmann, B., Mianné, J., Evans, E.P., Beechey, C.V., Fray, M.D., Birling, M.-C., Hérault, Y., Pavlovic, G., Teboul, L
Aneuploidy screening of embryonic stem cell clones by metaphase karyotyping and droplet digital polymerase chain reaction.
BMC Cell Biology 2016 **doi:10.1186/s12860-016-0108-6**

5 MICROINJECTION & BREEDING



- Microinjection
- Breeding to F1 generation

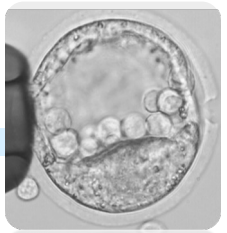
Microinjection



- 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 clone #27 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
#27	0	1	13	14

Breeding to F1 generation



- Five 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 29/05/2019

- Allele nomenclature (following MGI guidelines) : **Mcpt8$^{tm2.1(RFP657/cre/ERT2)Ics}$**

6 SEQUENCE OF THE DELIVERED ALLELE

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

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F3 pTagRFP657-N1 LF2A rabbit b- globin intron
Rox Fusion Cre-F3-Ert2-F3 linker F2 PM Exon LinkerF2

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REPORT REDACTION & VALIDATION

Protocol finalized on 2023/03/31

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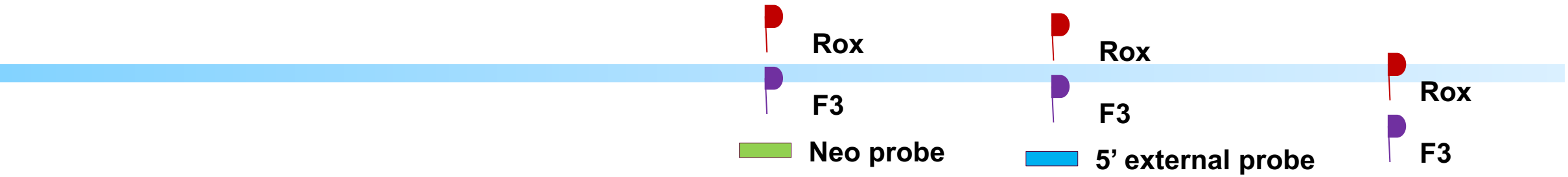
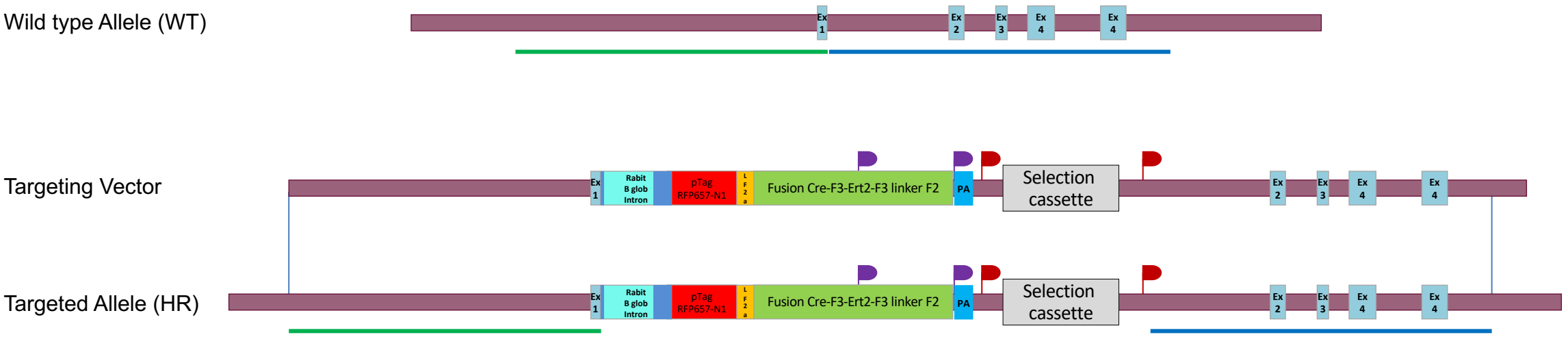
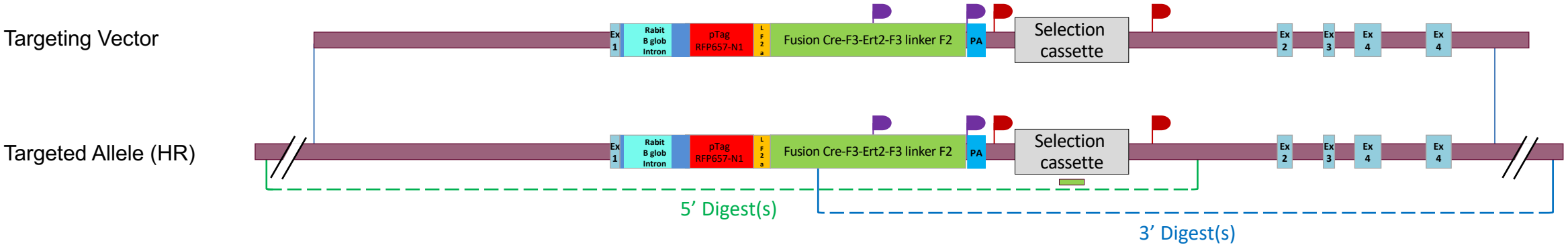




Rox

F3

Neo probe



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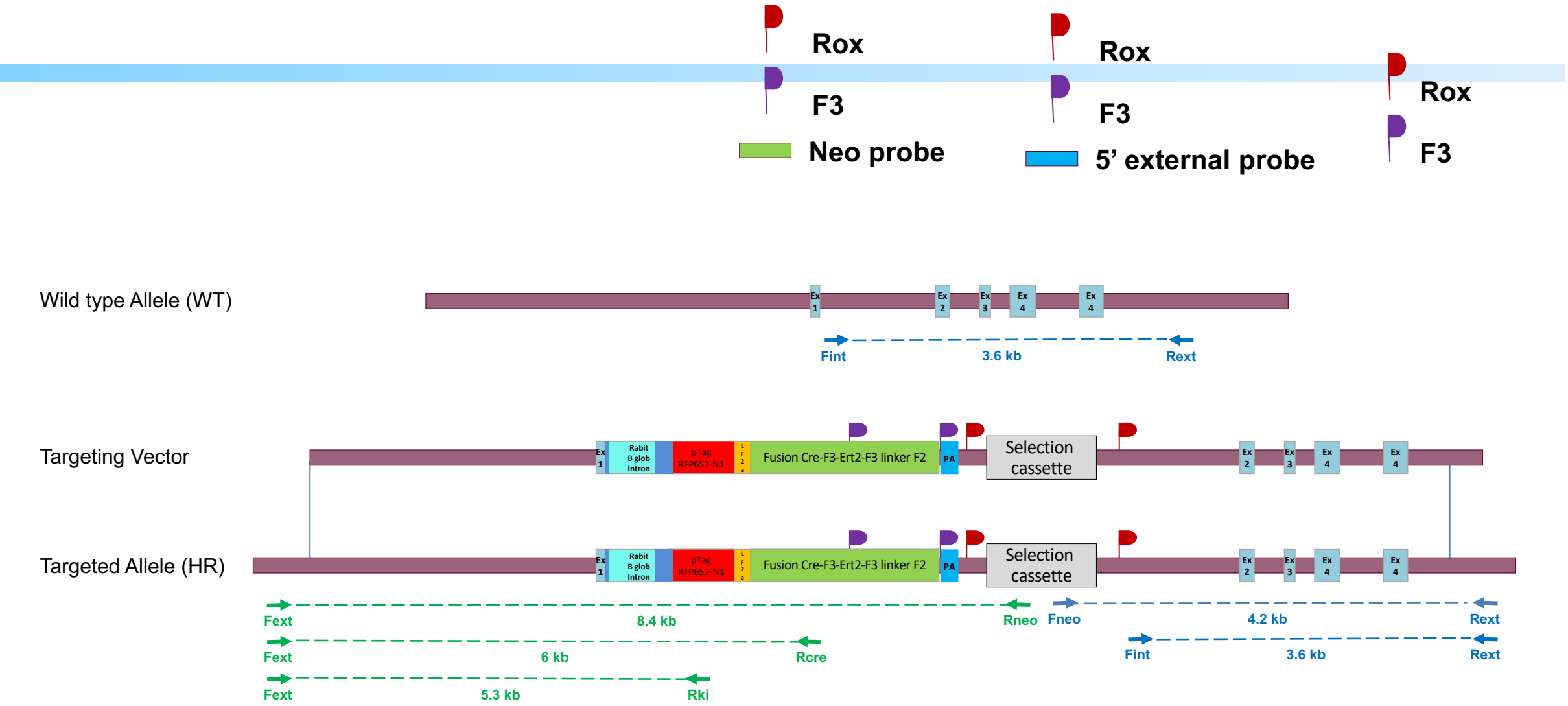

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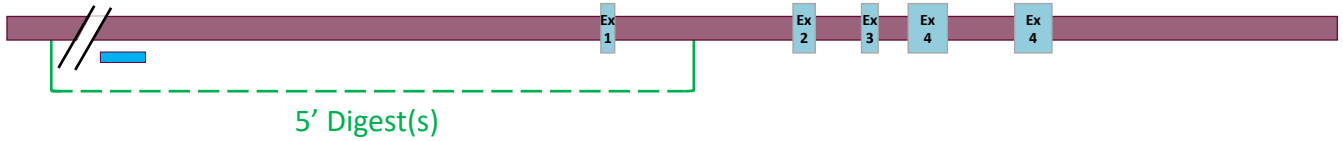
5’ external probe

Wild type Allele (WT)



Targeting Vector

Targeted Allele (HR)