



# MODEL GENERATION TECHNICAL REPORT

## Generation of a Fshr Cre-F3-ERT2-F3 KI line

Project code: Ros6278 / IR6278

Report updated: 16/05/2023

1 PROJECT DESCRIPTION

2 GENETIC STRATEGY

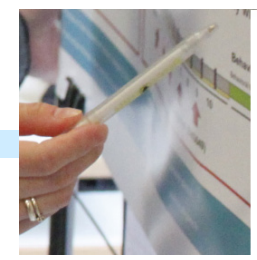
3 HOMOLOGOUS RECOMBINATION  
VECTOR CONSTRUCTION

4 ES ELECTROPORATION & SCREENING OF  
RECOMBINANT CLONES

5 MICROINJECTION & BREEDING

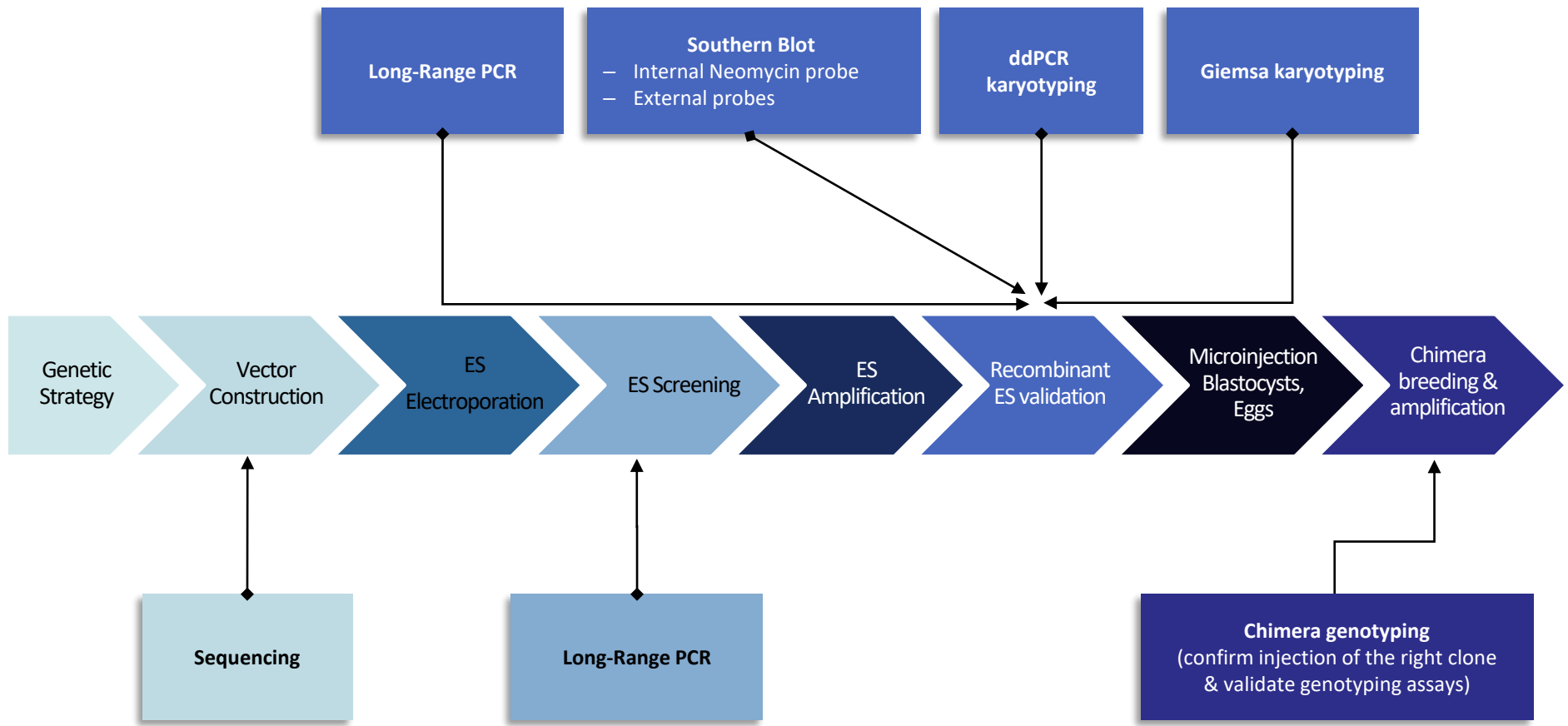
6 SEQUENCE OF THE DELIVERED ALLELE

# 1 PROJECT DESCRIPTION

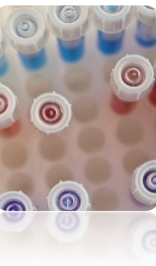


- Aim
- Project process & quality controls

# Project process & quality controls



## 2 GENETIC STRATEGY



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

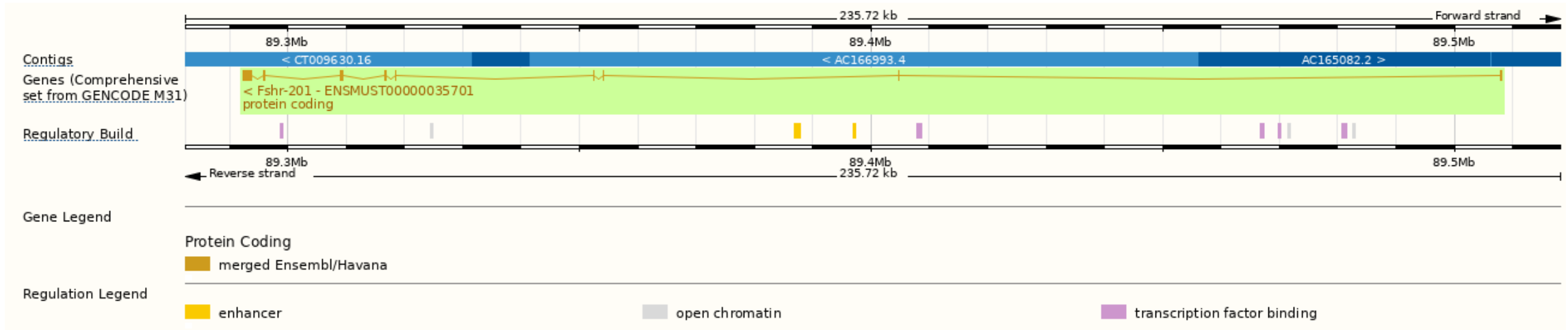
# ■ mouse genomic locus – structure



## Chromosome 17: 89,292,380-89,508,103



## Gene: Fshr ENSMUSG00000032937



## ■ mRNA(s) and protein(s)

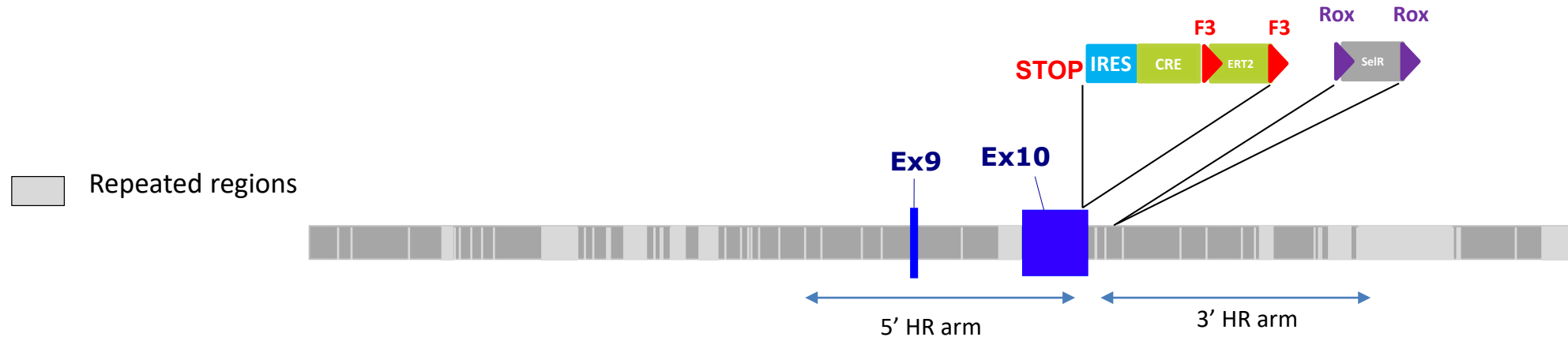


Transcript ID	Name	bp	Protein	Biotype	CCDS	UniProt Match
<a href="#">ENSMUST00000035701.6</a>	Fshr-201	2360	<a href="#">692aa</a>	Protein coding	<a href="#">CCDS29026</a>	<a href="#">B2RQM3</a> <a href="#">P35378</a>

# ■ Strategy: insertion of a IRES-Cre-F3-ERT2-F3 cassette in the 3' of the Fshr gene



## Approach: Homologous recombination in C57BL/6N ES cells





## ■ PROs& CONs evaluation of the strategy



### ■ Pros

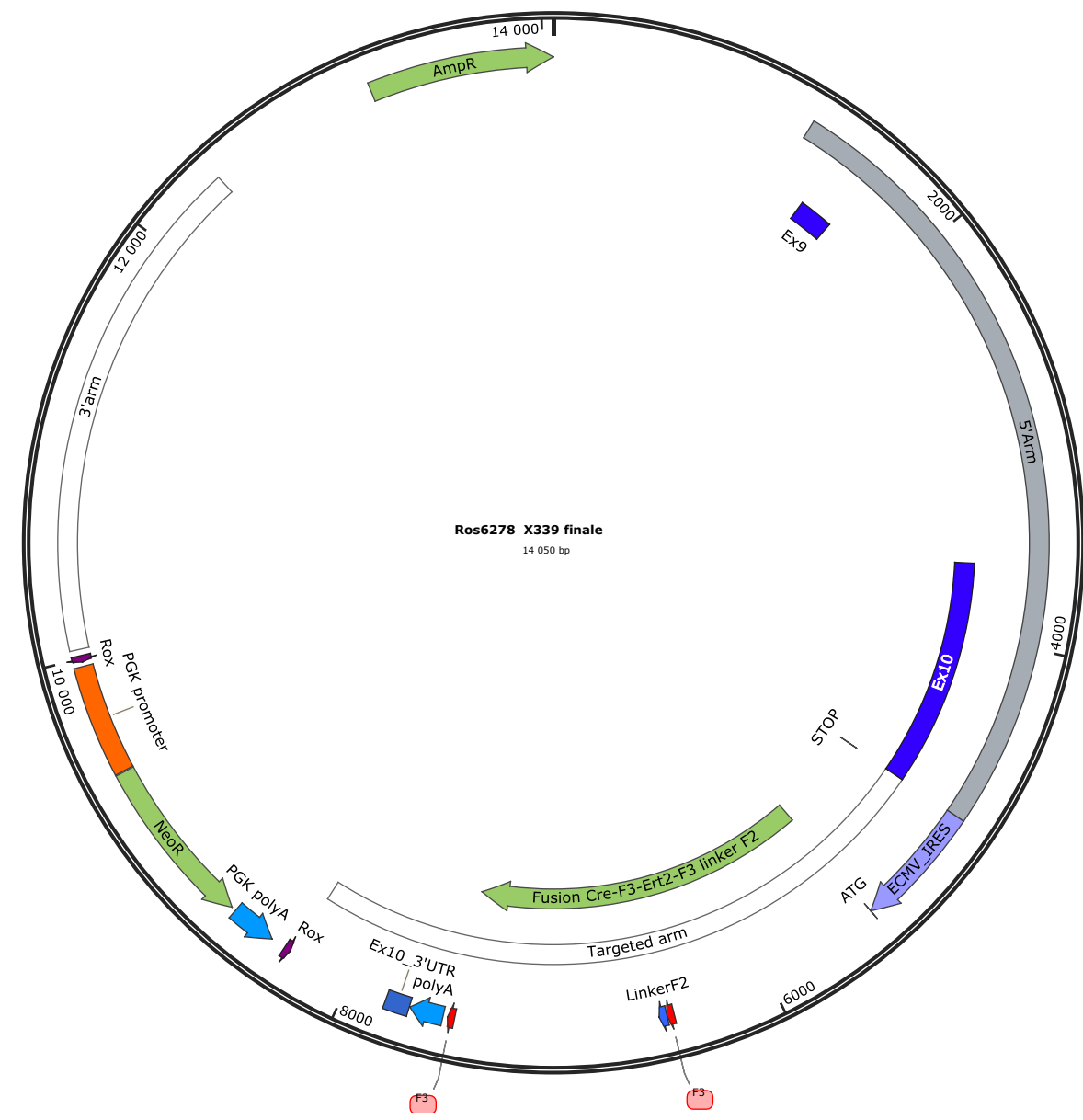
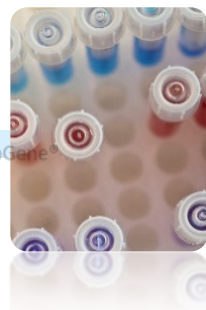
- Expression of CreER<sup>T2</sup> expected to follow the same pattern than the gene itself
- The endogenous Fshr expression should not be modulate (as WT)
- The ER<sup>T2</sup> sequence (F3-ER<sup>T2</sup>-F3) can be deleted *in vivo* by breeding males from the established line with Flp deleter females (see Birling et al. Genesis. 2012 Jun;50(6):482-9). So we will be able to obtain the non inducible Cre line also.

### ■ Cons

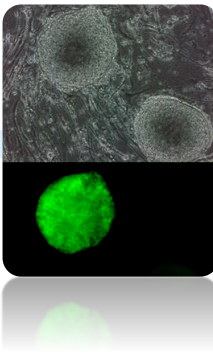
- Presence of repeated sequences in both homology arms might render PCR amplification or LR-screen difficult
- The expression level of Cre-F3-ERT-F3 might be different (lower) than Fshr as an IRES will be used

# 3 HOMOLOGOUS RECOMBINATION - VECTOR CONSTRUCTION

Created with SnapGene

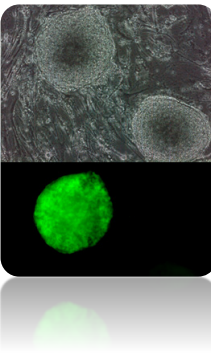


## 4 ES cell electroporation & Screening of recombinant clones



- 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.

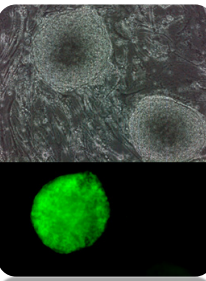
Transfected ES clones were submitted to neomycin selection (G418) and 93 resistant ES clones were isolated. The clones were then submitted to the screening process allowing secured identification of those harbouring the expected recombination events at both ends of targeting vector.

Screening process steps:

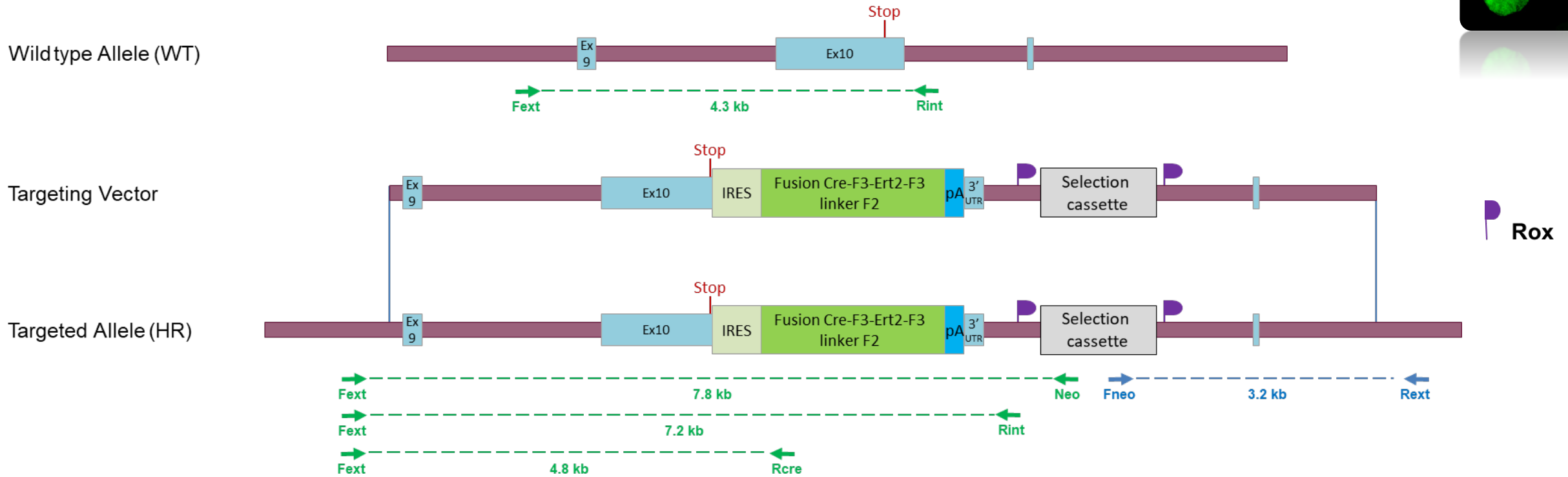
1. Identification of candidate recombinant clones by initial 5' Long-Range PCR
2. Eight of 5' PCR positive clones are confirmed for 3' recombination event by Long-Range PCR
3. Positive clones in step2 are further validated by Southern blot analysis using internal and external probes
4. The karyotype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

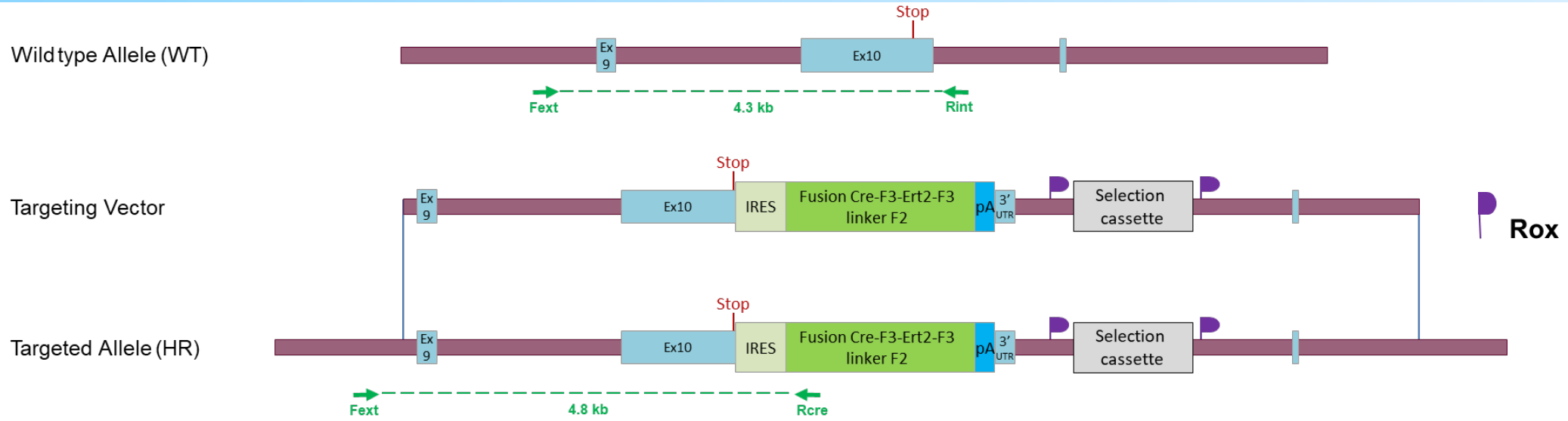
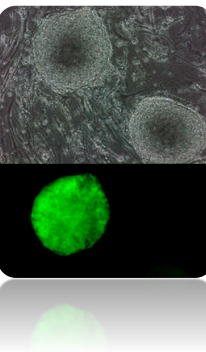


## Schematic 5' and 3' PCR screening strategy

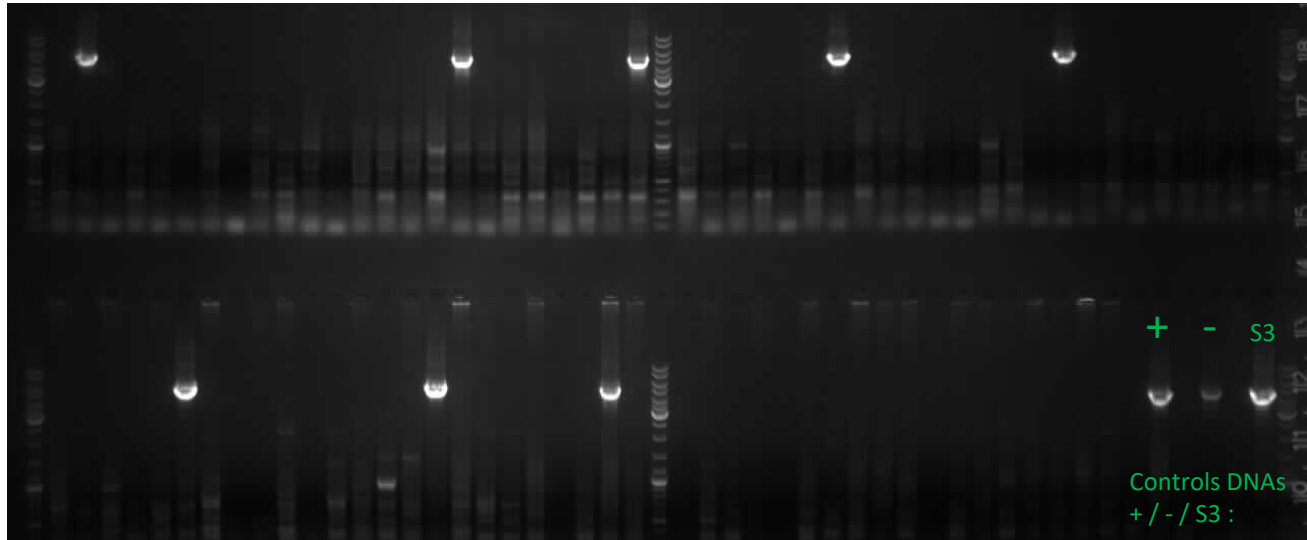


PCR	Primer Name	Primer sequences	PCR product size
5' PCR	Fext	GGCTGATTCTAATGCTCACCAGCAA	4.8 kb
	Rcre	CCAGATTACGTATATCCTGGCAGCG	
5' PCR	Fext	GGCTGATTCTAATGCTCACCAGCAA	7.8 kb
	Rneo	AGGGGCTCGGCCAGCCGAACTGTT	
5' PCR	Fext	GGCTGATTCTAATGCTCACCAGCAA	WT : 4.3
	Rint	GCTTCAGCAGCAGTGTGATCCACA	
3' PCR	Fneo	GCGGCCGAGAACCTGCGTGCAATC	3.2 kb
	Rext	GCTCTGCAGATGGACAGAGGTAGT	

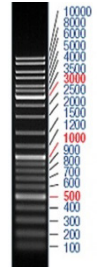
# Long-Range 5' PCR screening – results



PCRFext – Rcre : 4.8 kb



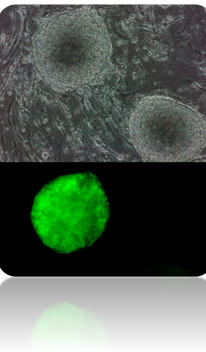
Fext – Rint : 4.3 kb



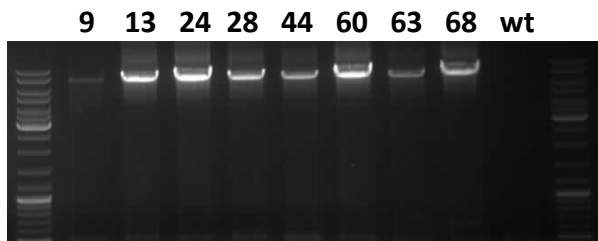
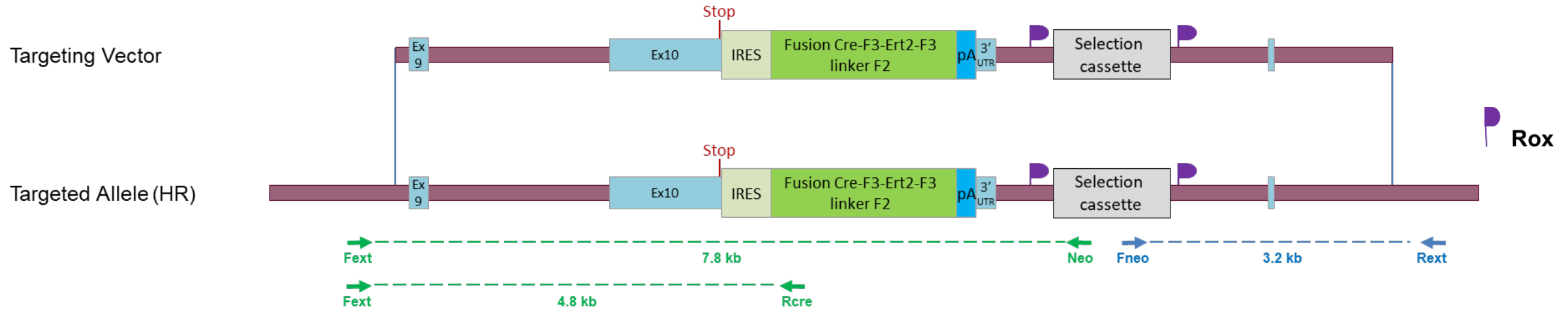
Ladder pattern

Eight candidate clones out of the 8 positive clones were selected for 3' Long-Range PCR and Southern blot validation.

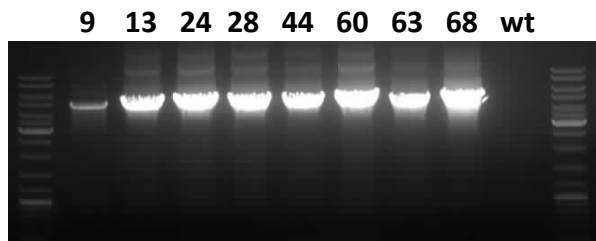
# Recombinant ES validation by Long Range PCR



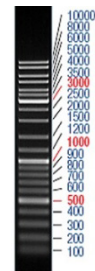
## Confirmation and Validation of candidate recombinant ES clones by 5' and 3' PCRs



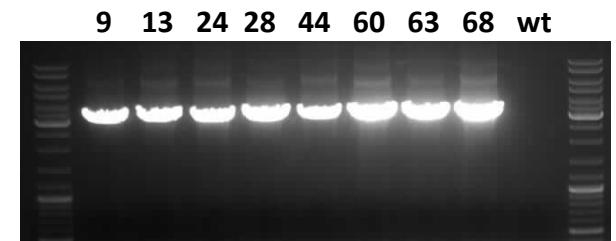
Pcr Fext – Rneo : 7.8 kb



Pcr Fext – Rcre : 4.8 kb



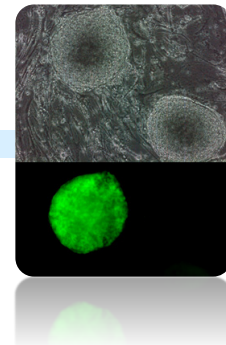
Ladder pattern



Pcr Fneo – Rext : 3.2 kb

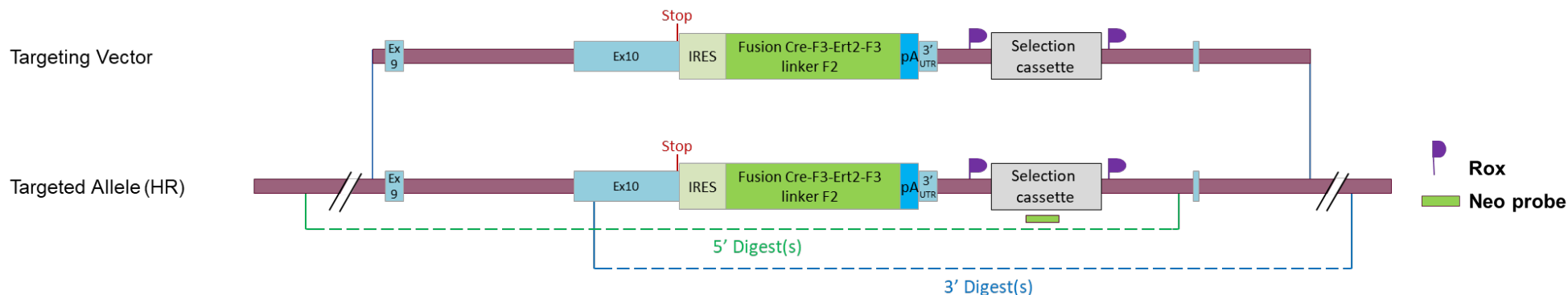
Eight candidate clones identified by 5' PCR screening were further analysed by 3' PCR screening. Eight clones (clones #9, #13, #24, #28, #44, #60, #63 and #68) were confirmed.

# 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	SpeI	9.1
		Swal	11.2
	3' digest	Dralll	7.2
		HindIII	10.9

Southern blot - Neo 5'

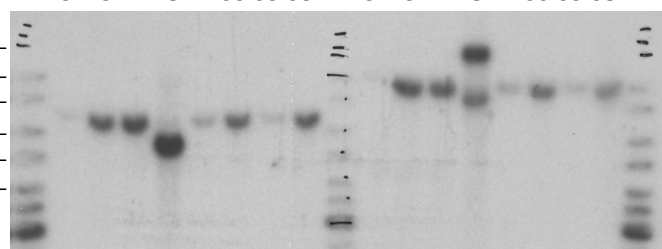
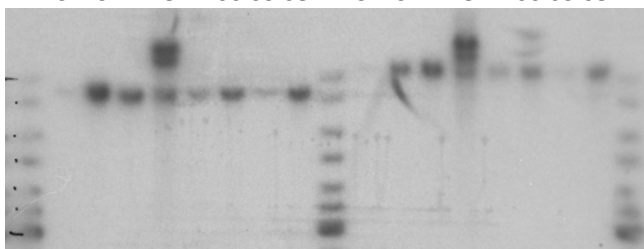
Southern blot - Neo 3'

## Neo probe sequence

```
AGACTTACAGCGGATCCCCTCAGAAGAAGCTCGTCAAGAAGGCGATAGAAGGCCGA
TGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTGAGCCC
ATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGAT
AGCGGTCGCCACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCAT
TTCCACCATGATATTCGCAAGCAGGCATCGCCATGGGTGACGACGAGATCCT
CGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCCGGTGGCGCGAGCC
CCTGATGCTCTTCGTCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAG
TACGTGCTCGCTCGATGCGATGTTTTGCTTGGTGGTGAATGGGCAGGTAGCCG
GATCAAGCGTATGCAGCCGCCGATTGCATCAGCCATGATGGATACTTTCTCGG
CAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCA
GCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGC
CCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCTGCG
```

9 13 24 28 44 60 63 68    9 13 24 28 44 60 63 68

9 13 24 28 44 60 63 68    9 13 24 28 44 60 63 68



SpeI

Swal

Dralll

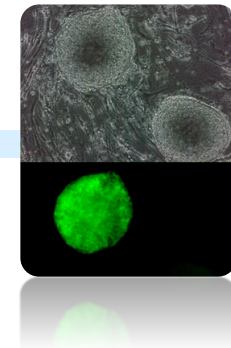
HindIII

=> All but clone #28 show a single band at the expected size with both restriction enzymes.

=> Clones #13 and #68 were further analysed by Southern blot with a 5' external probe.

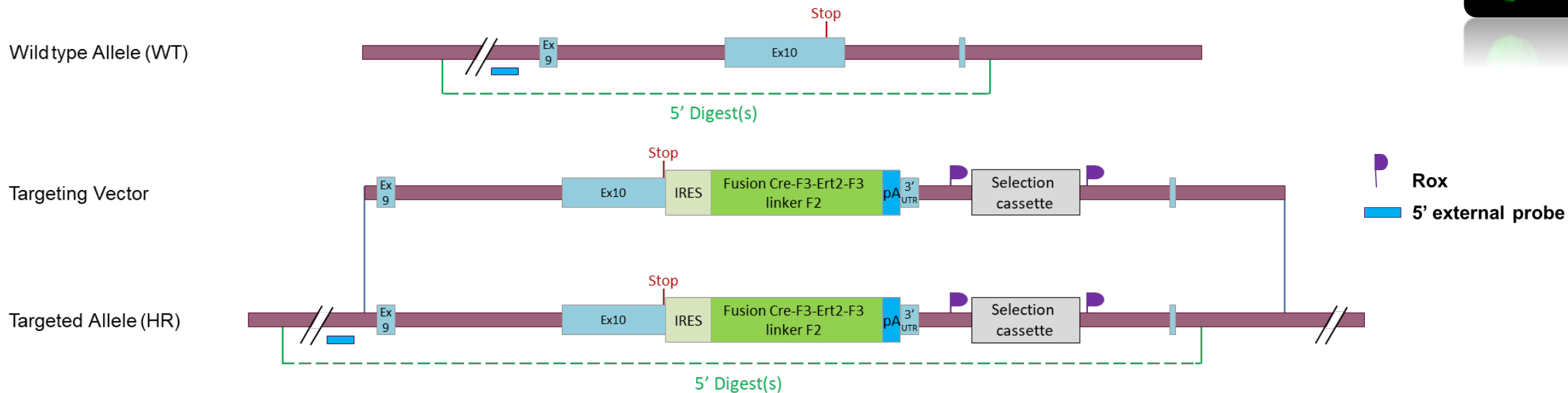


# Recombinant ES clones validation by Southern Blot – External probe

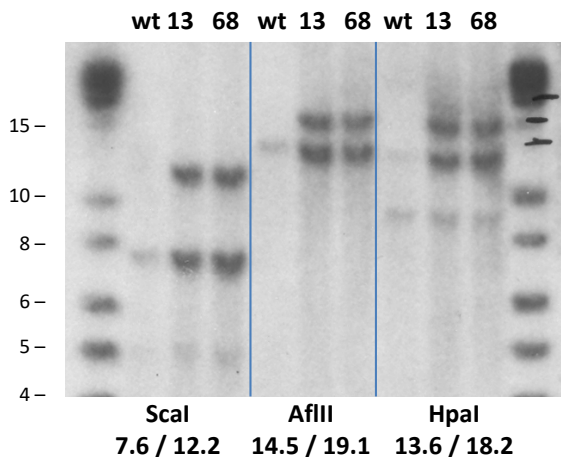


## Schematic Southern Blot validation strategy

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



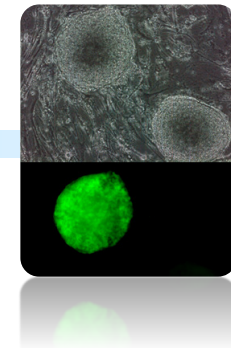
### 5' probe sequence

```
CCCTATTTATCTGCCATCTGGACTTCCAGTGAAGGCTGCCTCT
ATTGCTGCCAGATTCTTTTCCATGGCATGTTCCCTTCTCAT
CATTTTCTGTGATTGAGCCACAGAAACCACCATAGTTCATTA
GAGATATTTTTATTGCTTGCCTCACAGTGGCAGAAAATGGTTT
CAATCTCAGGCACTAATCAACTACAGAAGAGATGGCTGATT
CTAATGCTCACCAGCAAGGATTAAGCCCAAACCAGCCAGAATT
CAAGCTTATCCAGTTTTAGGAGCAGCCTGCTACCTAACTCCTC
TCATCAAGACCAGTAGAACC
```

### 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	Scal	7.6	12.2
	5' second digest	AflIII	14.5	19.1
	5' third digest	HpaI	13.6	18.2

## ■ Aneuploidy screening in ES recombinant clones



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

Clone ID	ddPCR	Giemsa
#9	Pass	Not done
<b>#13</b>	<b>Pass</b>	<b>Pass</b>
#24	Failed (loss of ChrY)	Not done
#44	Failed (loss of ChrY and Chr8 trisomy)	Not done
#60	Pass	Not done
#63	Failed (loss of ChrY and Chr8 trisomy)	Not done
#68	Pass	Not done

<sup>1</sup> 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



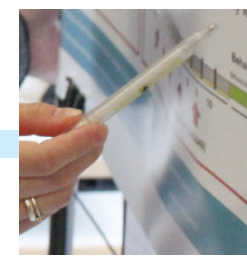
- The ES cells used in the injection experiment were originally derived from a C57BL/6 mouse strain (which have black coat colour). These cells were injected into blastocysts derived from an BALB/cN strain, which have a white coat colour. The resulting offspring are thus chimeras of two different cell 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 #13 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
#13	3	0	8	11

## ■ Breeding to F1 generation



- Four 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 04/10/2017
- Allele nomenclature (following MGI guidelines) : **Fshr<sup>tm1.1(cre/ERT2)lcs</sup>**



AGCTGTATGTTATGGCCCTCTCGTACTCAACGCCCTGGCCTTTGTGGTCATCTGTGGTTGCTACACCCACATCTACCTCACAGTGAGGAATCCTAACATTGTGTCCTCGTCAAGAGACACCAAGATTGCCAAGCGCATGGCCACACTCATCTTCACGGACTTTCTCTG  
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**TTGGCAGAACGAAAACGCTGGTTAGCACCCGAGGTGTAGAGAAGGCACTTAGCCTGGGGTAACTAACTGGTTCGAGCGATGGATTTCCGTCTCTGGTGTAGCTGATGATCCGAATAACTACCTGTTTTGCCGGTCCAGAAAAATGGTGTTCGCGCCCATCTG**  
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**TCCTACCAGACCCTTCAGTGAAGCTTCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATCAACTGGGCGAAGAGGGTGCAGGCTTTGTGGATTTGACCCTCCATGATCAGGTTCCACCTTCTAGAATGTGCCTGGCTAGAGATCCTG**  
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**ATGTGACTCATTAAGAAGTGAATATTGGTAACCGCAATTAATAAGTGAACACTGCTGCATGCTTGTATGTTTCTGTTTGTATTATCTCAACAGAGTTGGTAAAAAATAATCATGTTTCAGTAGGCTAGACAGGAATTTTTCTGGAGTTTTACAAATGAGG**  
**AAATTAAGACCCAAAGATCTCAGTGAATTTTTCGACATTGCTCGTAGATAGAGGCAGAGCCAGGATGAAGTATCCTGACTTTTCAAAATTTCCATGAGAAATTTACACCACTCATGCACATGTTTACCAGGATGCCAGTGTGTTTTGAAATGGGTACCATGTA**  
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**TGAGGCTGACTCAGCTCAAGGCAGCCCTCCTCTGGCTGGATCCTCCTCAGGCCACCTGAGGGTTTTATAGCATGACTAGGAAGCACAAAATTTCTACAGGATGTTTACCACCAAGGTCAGCGCAAGGACAGAAGTGGGGCTCATCACTTGGTCTCCCTG**  
**GGTTGCATTCTGTGTTGCTGAGTGCATGCTTGTGATCTTTCTTTATAAAGGACATATGCCCTGAGGACACTGACAACATCCTTACACATATTGCTTAGCCAGCCTCTCAAATGTGGGAGAGACAATCCAGTAACAAATATTTACCACCTATACATATTTGCATTC**  
**ATACATATGTATGATTGCTTCTGCTGTTCTGGCTGAACTCTAATGGAAAATACACCTATTATATGGTATGCATATAGATTACAATGTTAAGAATGCAGCAGCTTTGAAGTCAAGTGTAAAGATTTTTGTTTCTAGATTCTGTAATACACCAGTTCAAGCTCTCCAGCTCAC**  
**CT**

IRES

Cre

Ert2

F3

Linker F2

Rox



## REPORT REDACTION & VALIDATION

Protocol finalized on 2023/02/13

Prepared by Romain LORENTZ, IE

Verified by Marie-Christine BIRLING, PhD

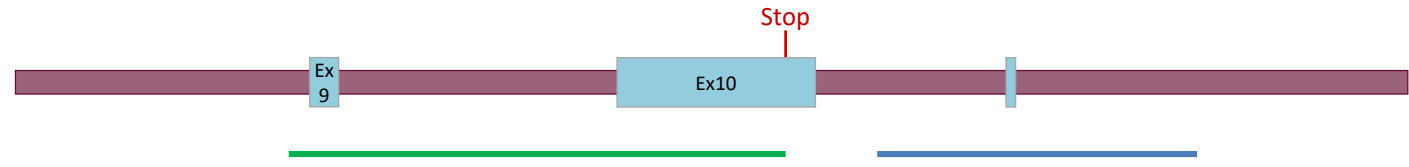
## CONTACT US

By email at [mutagenesis@igbmc.fr](mailto:mutagenesis@igbmc.fr)

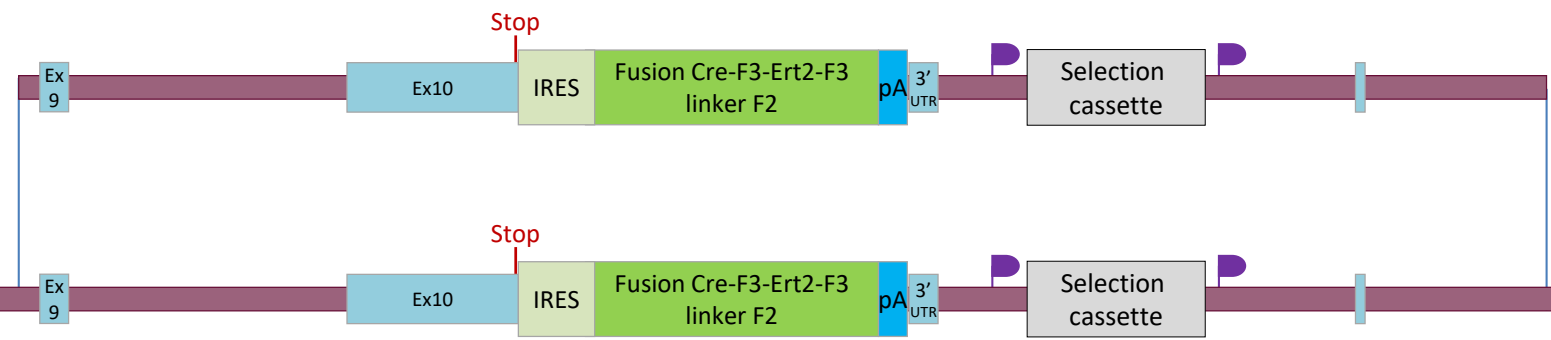
By phone at +33 (0)3 88 65 56 57

[www.phenomin.fr](http://www.phenomin.fr)

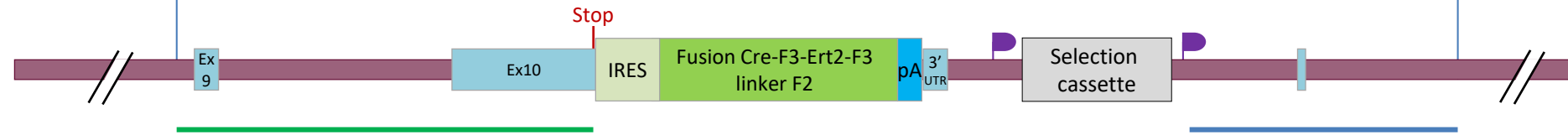
Wild type Allele (WT)



Targeting Vector

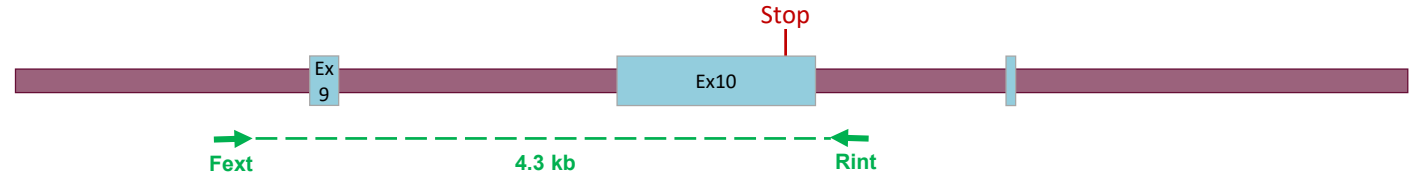


Targeted Allele (HR)

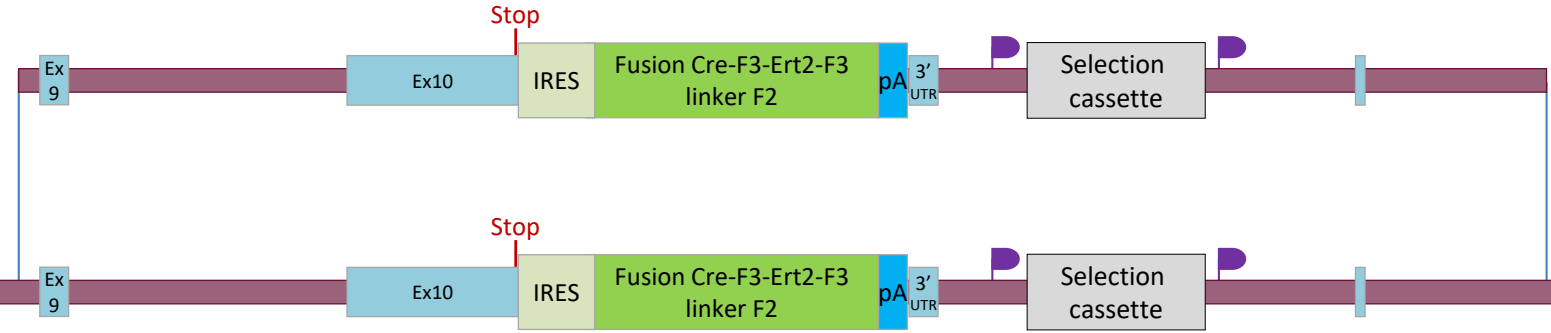




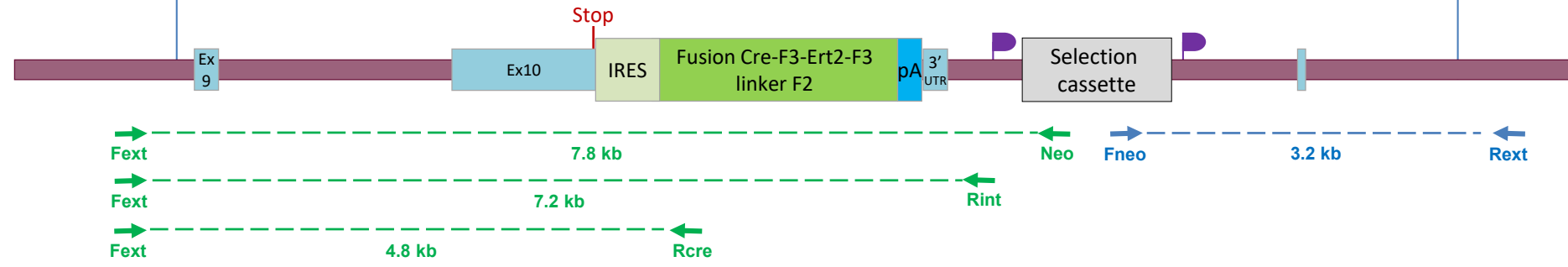
Wild type Allele (WT)





Targeting Vector



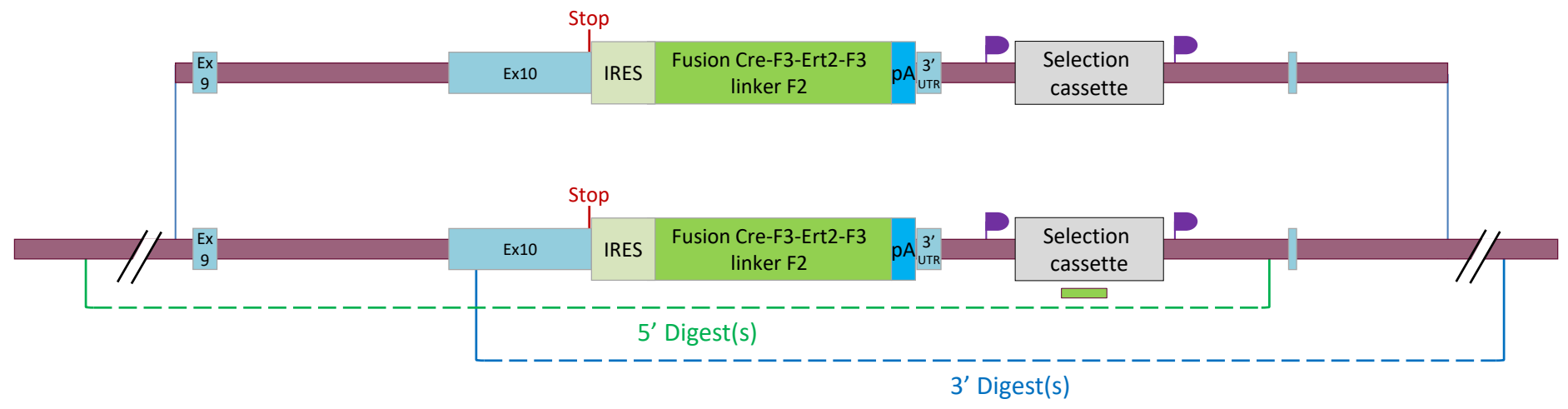
Targeted Allele (HR)



 **Rox**  
 **Neo probe**

Targeting Vector

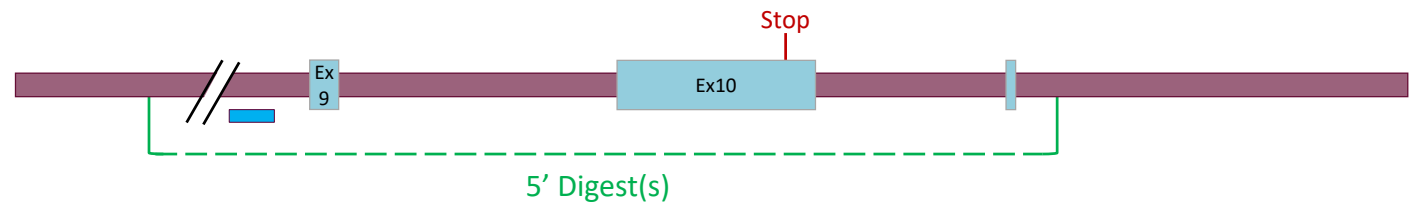
Targeted Allele (HR)



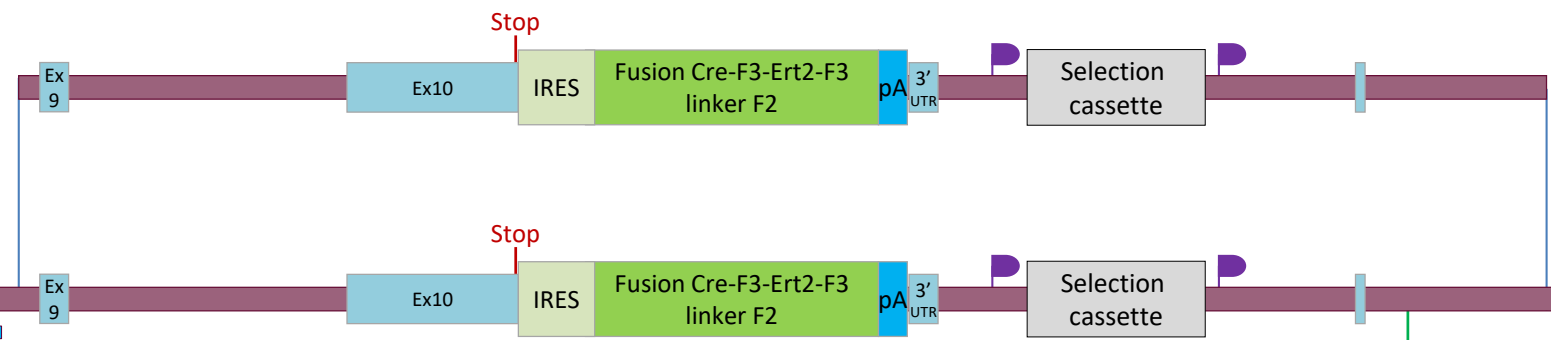
Rox

5' external probe

Wild type Allele (WT)



Targeting Vector



Targeted Allele (HR)

