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EXCELLENCE IN MOUSE PHENOGENOMICS

MODEL GENERATION TECHNICAL REPORT

Conditional anti-Otx2 scFv chain overexpression under the *Rosa*26 promoter / knock-in into the Rosa26 locus

Project code: K3959 / IR3959

Report finalized: 2024/11/18

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

1 PROJECT PROCESS & QUALITY CONTROL

2 GENETIC STRATEGY

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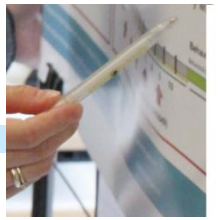
4 ES ELECTROPORATION & SCREENING OF RECOMBINANT CLONES

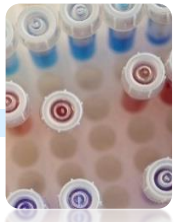
5 MICROINJECTION & BREEDING

6 SEQUENCE OF THE DELIVERED ALLELE

Project process & quality controls

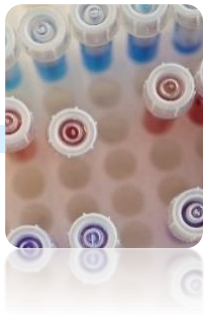


GENETIC STRATEGY

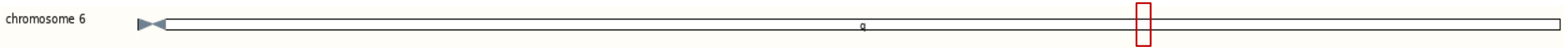


- Target locus structure
- Genetic strategy
- Sequence detail
- PRO & CONS evaluation of the strategy

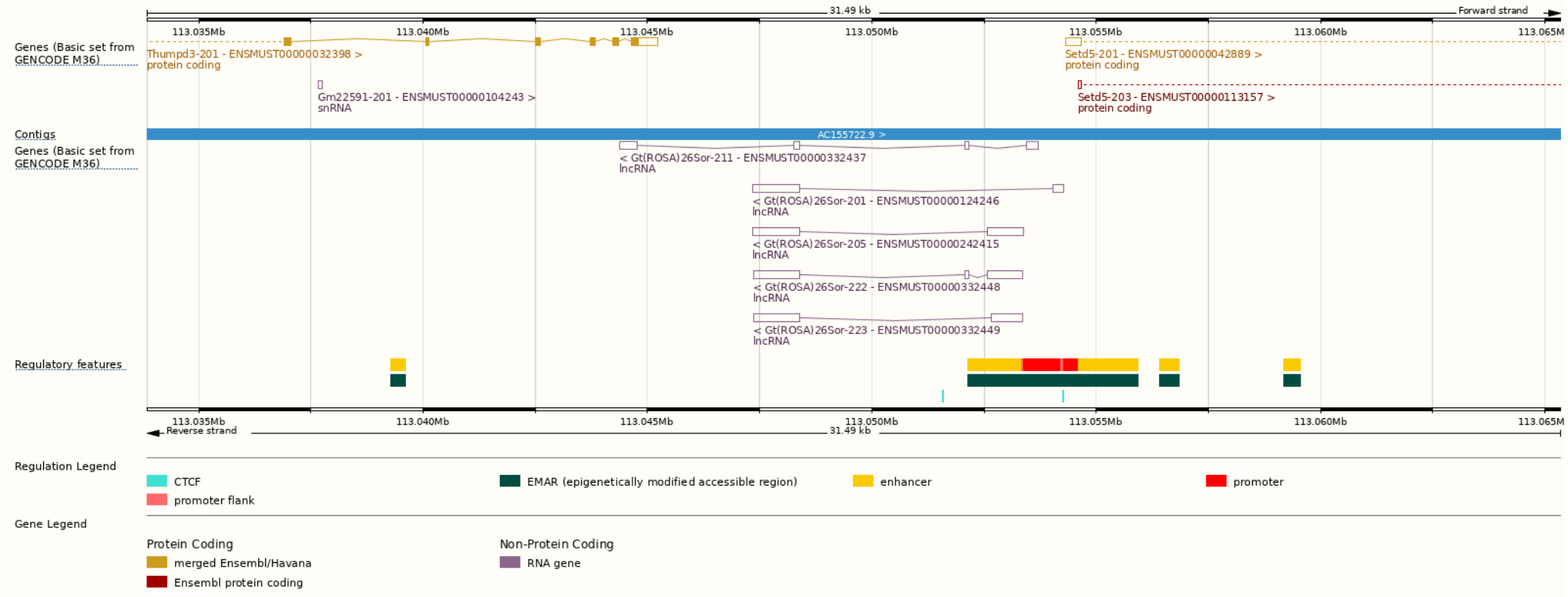
Rosa26 mouse genomic locus – structure



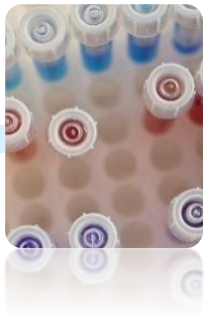
Location : Chromosome 6: 113,043,843-113,055,336

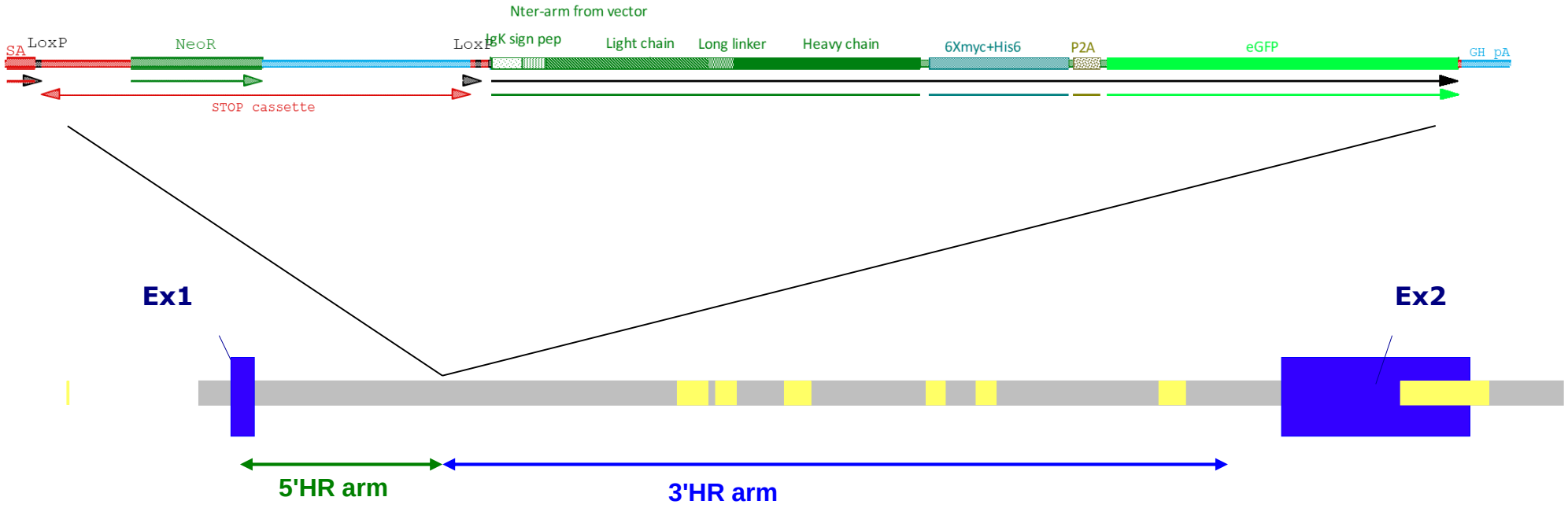


Gene: Gt(ROSA)26Sor ENSMUSG00000086429



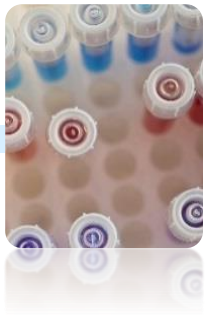
Strategy

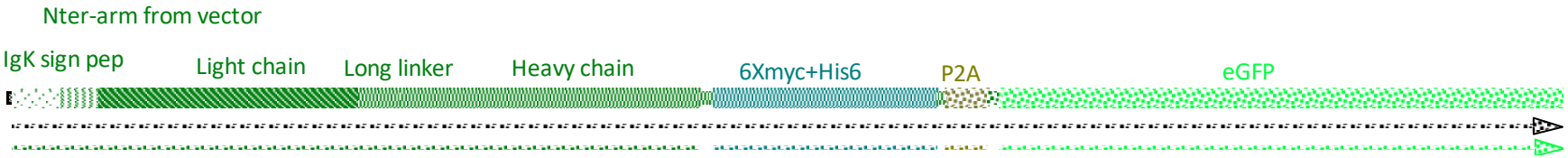




* Sequence of anti-Otx2 scFv single-chain see next slide

Sequence details of the cDNA to overexpress (after Cre mediated excision of the floxed STOP cassette)





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K G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T Y G K L T L K F I C T T G K L P V

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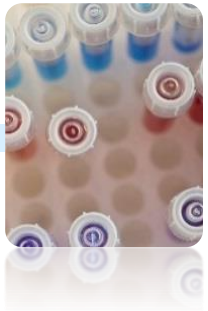
CGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCtgtacAagtGA
R D H M V L L E F V T A A G I T L G M D E L Y K

PROs& CONs evaluation of the strategy



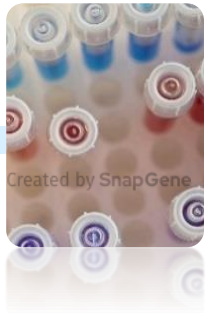
Pros

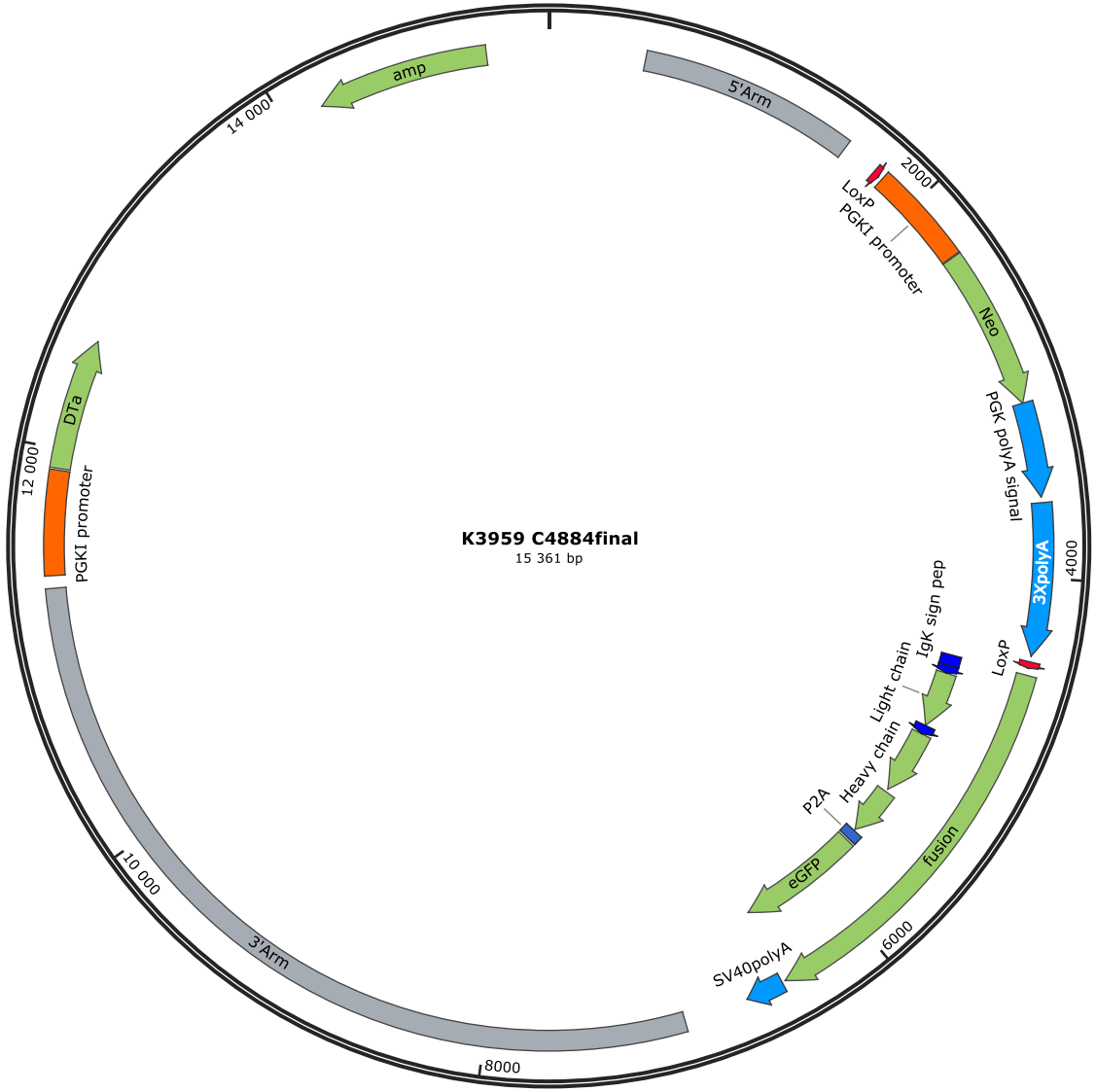
- As asked
- The STOP K7 will contain the Neo selection marker
- One complete copy of the transgene in the heterozygous mice
- Possibility to breed these mice with different Cre cell and/or tissue specific lines

Cons

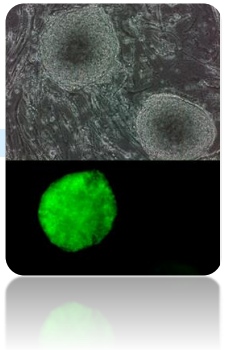
- /

3 HOMOLOGOUS RECOMBINATION - VECTOR CONSTRUCTION



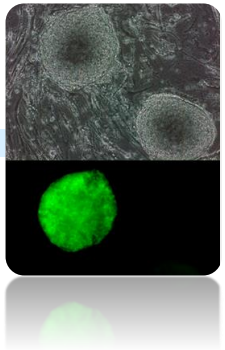


ES cell electroporation & Screening of recombinant clones



- 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



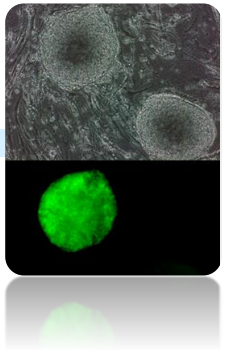
The targeting vector was electroporated in the proprietary C57BL/6NTac BD10 cell line.

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.

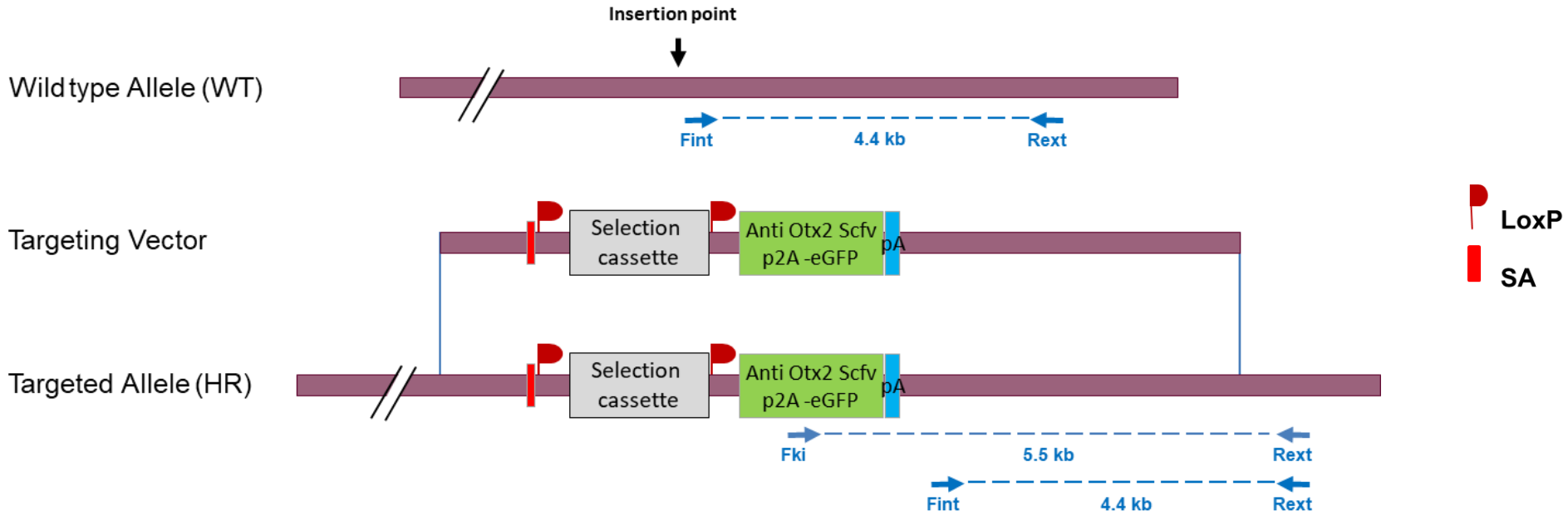
Screening process steps:

1. Identification of candidate recombinant clones by initial 3' Long-Range PCR
2. Ten of 3' 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 Giemsa staining

Long range PCR screening – strategy

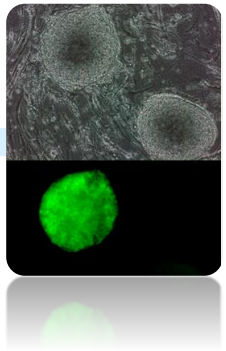


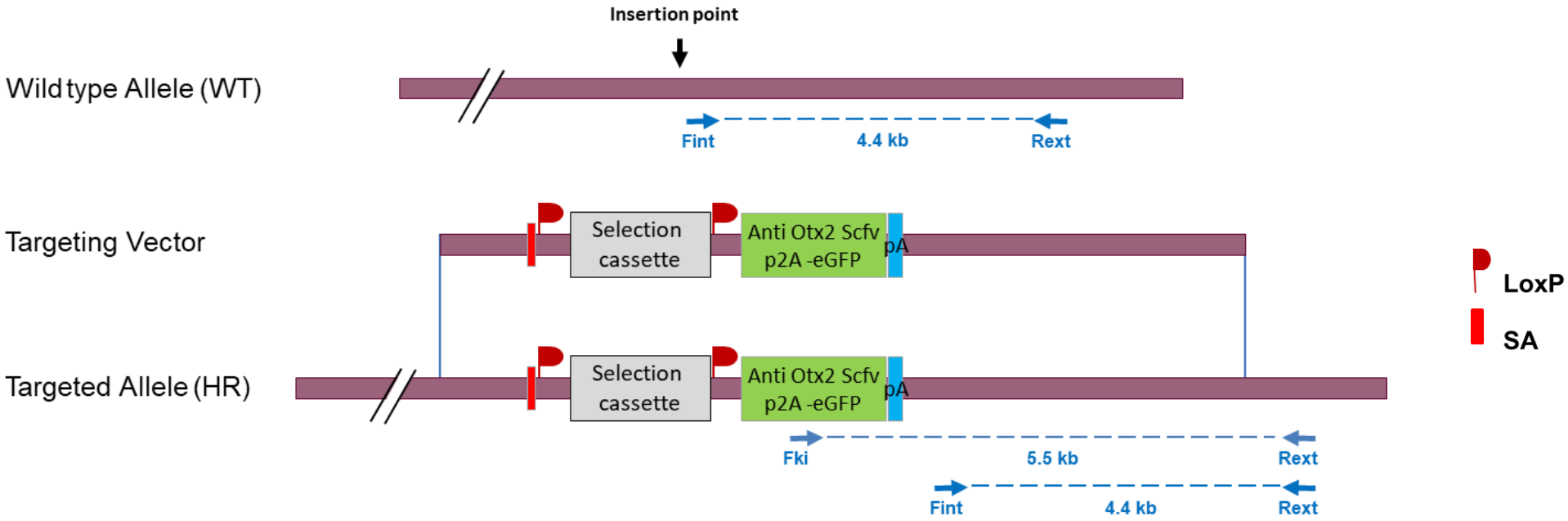
Schematic 5’ and 3’ PCR screening strategy



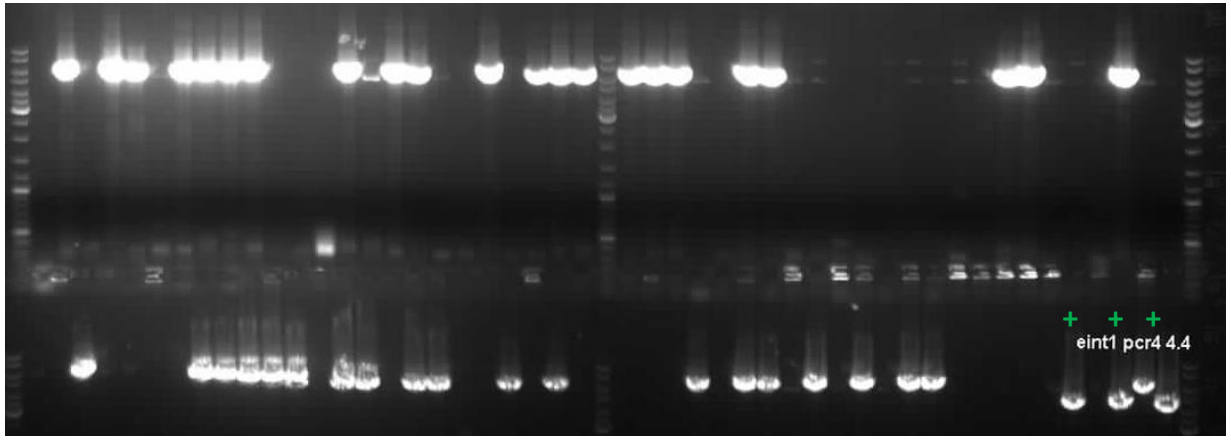
PCR	Primer Name	Primer sequences	PCR product size
3’ PCR	Fki	CCTACGGCGTGCAGTGCTTC	5.5 kb
	Rext	CTCAGTGGCTCAACAACACTTGGTC	
3’ PCR	Fint	CTGGTGTGTGGGCGTTGTCCTGCAG	4.4 kb
	Rext	CTCAGTGGCTCAACAACACTTGGTC	

Long-Range 3' PCR screening – results



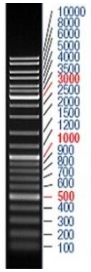


PCR Fki – Rext : 5.5 kb



+ : Controls DNAs

PCR Fint – Rext : 4.4 kb



Ladder pattern

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

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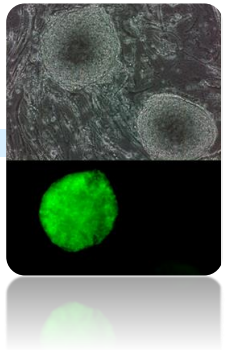
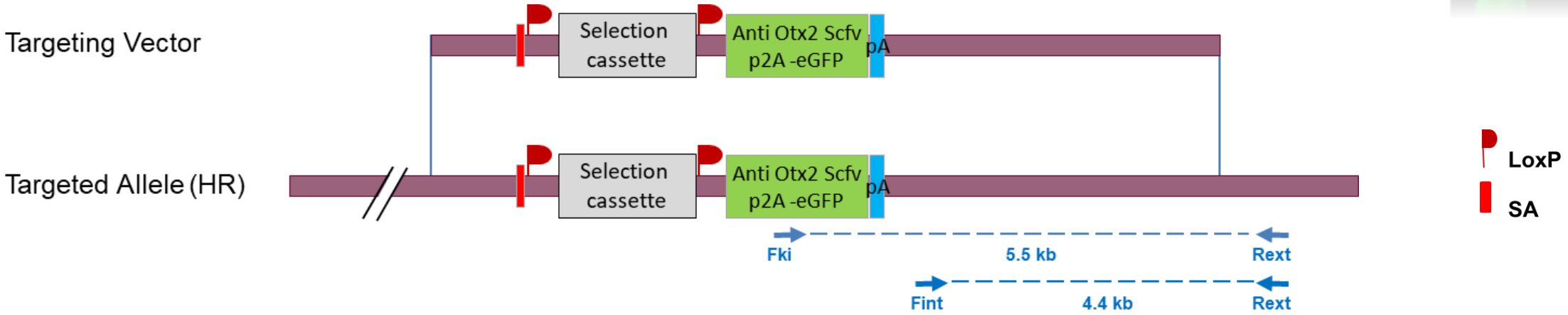
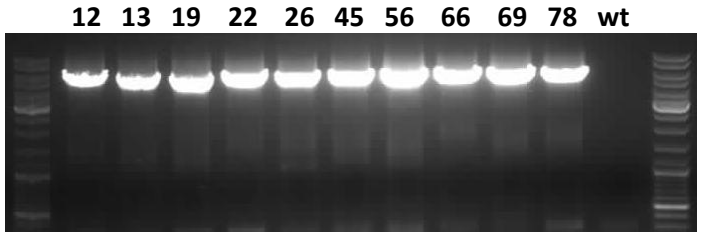
Recombinant ES validation by Long Range PCR

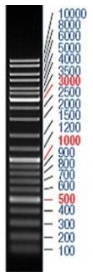


Confirmation and Validation of candidate recombinant ES clones by 5’ and 3’ PCRs





PCR Fki – Rext : 5.5 kb



Ladder pattern

Ten candidate clones identified by 3’ PCR screening were further analysed by 3’ Long-Range PCR screening.
Ten clones (clones #12, #13, #19, #22, #26, #45, #56, #66, #69 and #78) were further analysed.

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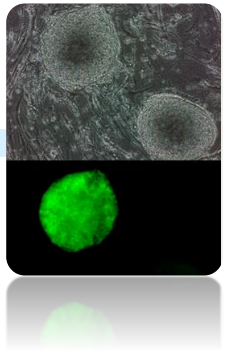
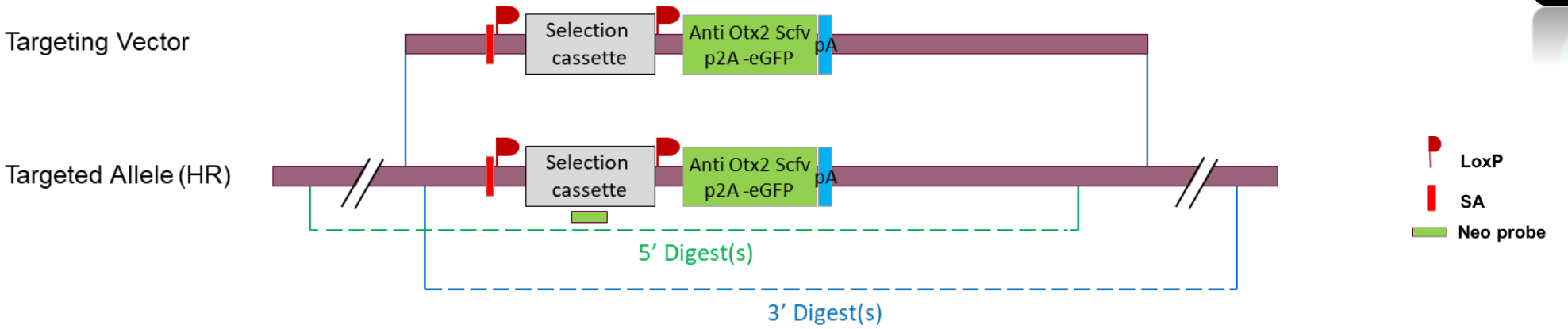
Recombinant ES clones validation by Southern Blot – Internal probe



Schematic Southern Blot validation strategy

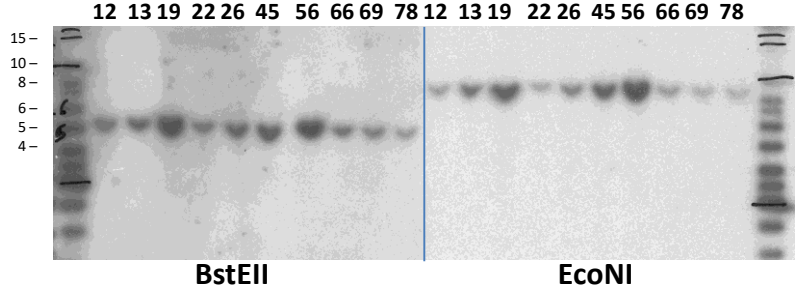
Digests on the scheme illustrate the position of the chosen restriction sites relative to the probe. They don't show the exact position of the restriction sites.



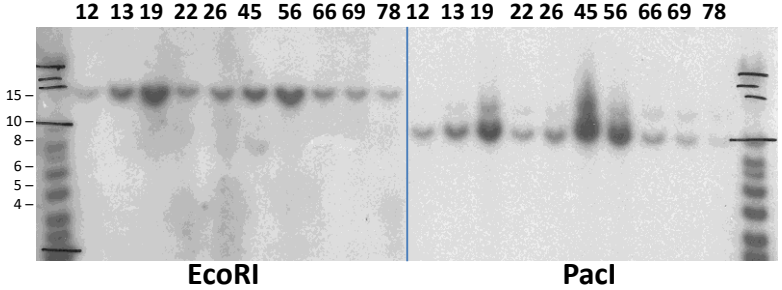
Digestions used to validate the 5' and 3' insertion

Probe	Digests	Genomic DNA digest	Targeted Allele (kb)
Neo	5' digest	BstEII	5.5
		EcoNI	9.2
	3' digest	EcoRI	16.8
		PacI	10

Southern blot - Neo 5'



Southern blot - Neo 3'



Neo probe sequence

```
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CCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATG
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GATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGGGGATCCG
CTGTAAGTCT
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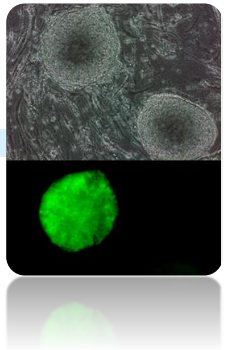
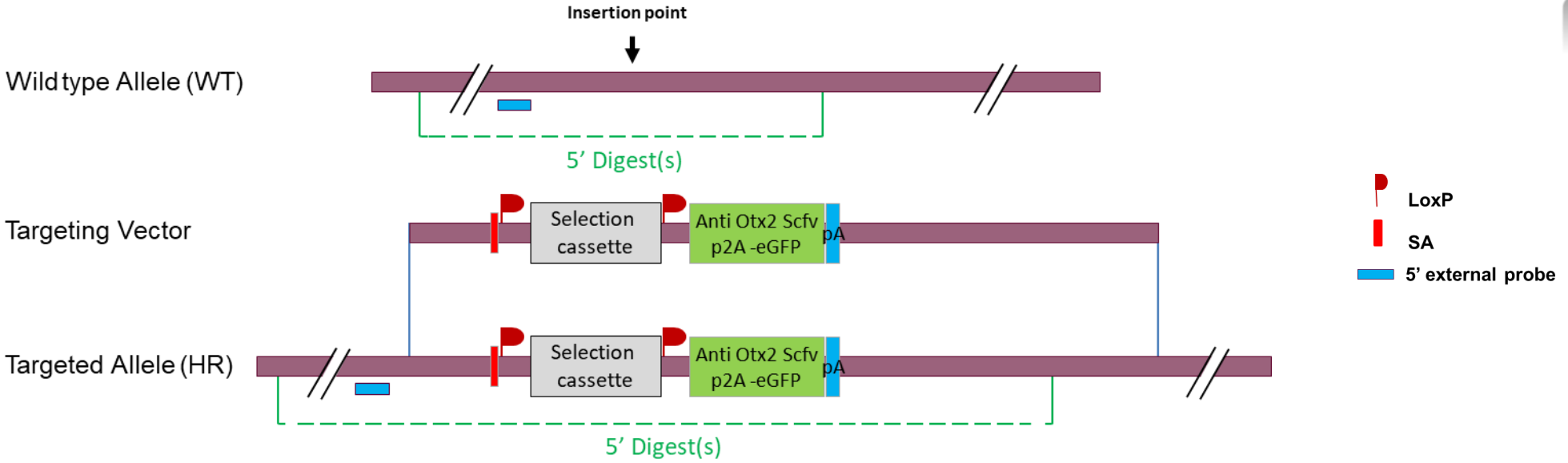
Recombinant ES clones validation by Southern Blot – External probe

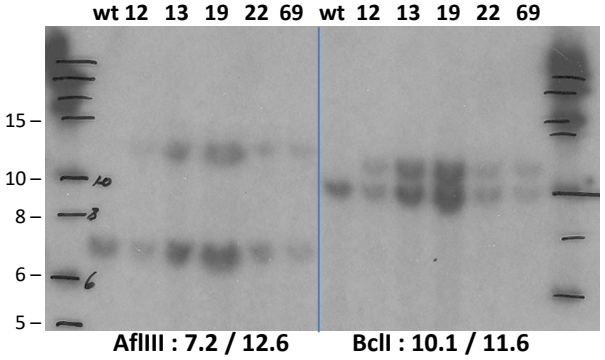


Schematic Southern Blot validation strategy

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



5' probe sequence

TATGTGTATTTTGAGAGCAGGGTTGGGAGGCCTCTCCTGAA
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AAGACATTAAAAAGGTAGTGGGGTCGACTAGATGAAGGAGA
GCCTTTCTCTCTGGGCAAGAGCGGTGCAATGGTGTGTAAAG
GTAGCTGAGAAGACGAAAAGGGCAAGCATCTTCCTGCTACC
AGGCTGGGGAGGCCCAGGCCCACGACCCCGAGGAGAGGGAA
CGCAGGGAGACTGAGGTGACCCTTCTTTCCCCCGGGGCCCG
GTCGTGTGGTTCGGTGTCTCTTTTCTGTTGGACCCTTACCT
TGACCCAGGC

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	AflIII	7.2	12.6
	5' second digest	BclI	10.1	11.8

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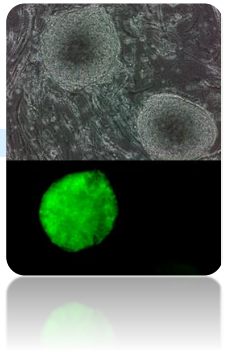
Aneuploidy screening in ES recombinant clones



Selected recombinant ES cells clones were karyotyped by Giemsa metaphase staining.

Results of aneuploidy analysis are presented in the table below.

Clone ID	Giemsa
#12	**Pass**
#13	**Pass**
#19	Not done
#22	Not done
#69	Not done

MICROINJECTION & BREEDING

- Microinjection
- Breeding to F1 generation

Microinjection

- The ES cells used in the injection experiment were originally derived from a C57BL/6NTac 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 #12 and #13 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
#12	0	0	1	1
#13	**2**	**5**	**1**	**8**

Breeding to F1 generation

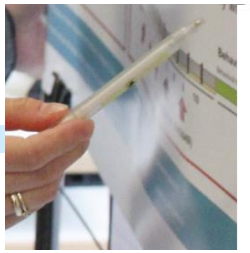
- Five highly chimeric males generated in the previous phase by blastocyst injection of ES clone #13 were mated with wild-type C57BL/6N Taq females (health status SPF – Specific Pathogen Free) to investigate whether the recombined ES cells have contributed to the germ layer.

- Germ line transmission was obtained the : 27/02/2013

- Allele nomenclature (following MGI guidelines) : **Gt(ROSA)26Sor<tm8(Igk-V/Igh-V)Ics>**

- MGI allele ID: MGI:7788217

SEQUENCE OF THE DELIVERED ALLELE



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GCATTTT

LoxP Splice Acceptor Neo/STOP cassette Anti Otx2 Scfv p2A eGFP SV40pA

phenomin

EXCELLENCE IN MOUSE PHENOGENOMICS

REPORT REDACTION & VALIDATION

Protocol finalized on 2024/11/18

Prepared by Romain LORENTZ, IE

Verified by Marie-Christine BIRLING, PhD

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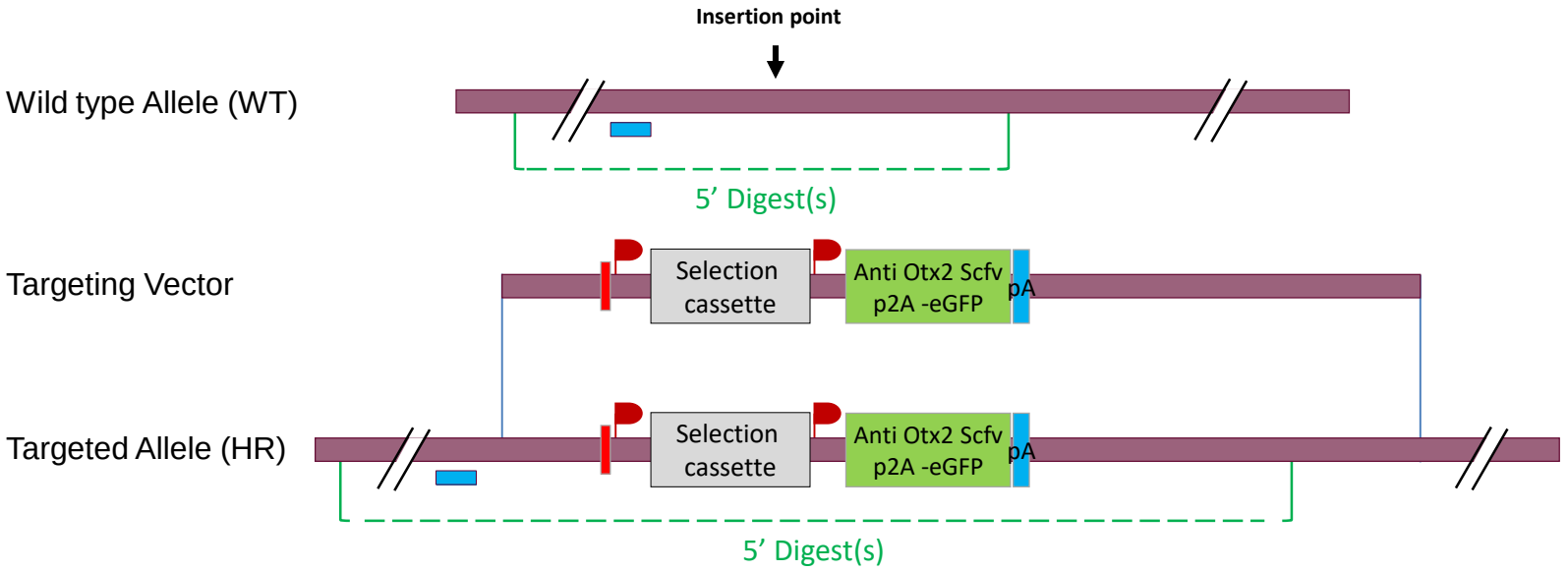
LoxP

SA

5’ external probe



Genotyping protocol

anti-Otx2 scFV conditionnel dans Rosa

IR00003959 / K3959

(ICS internal reference)

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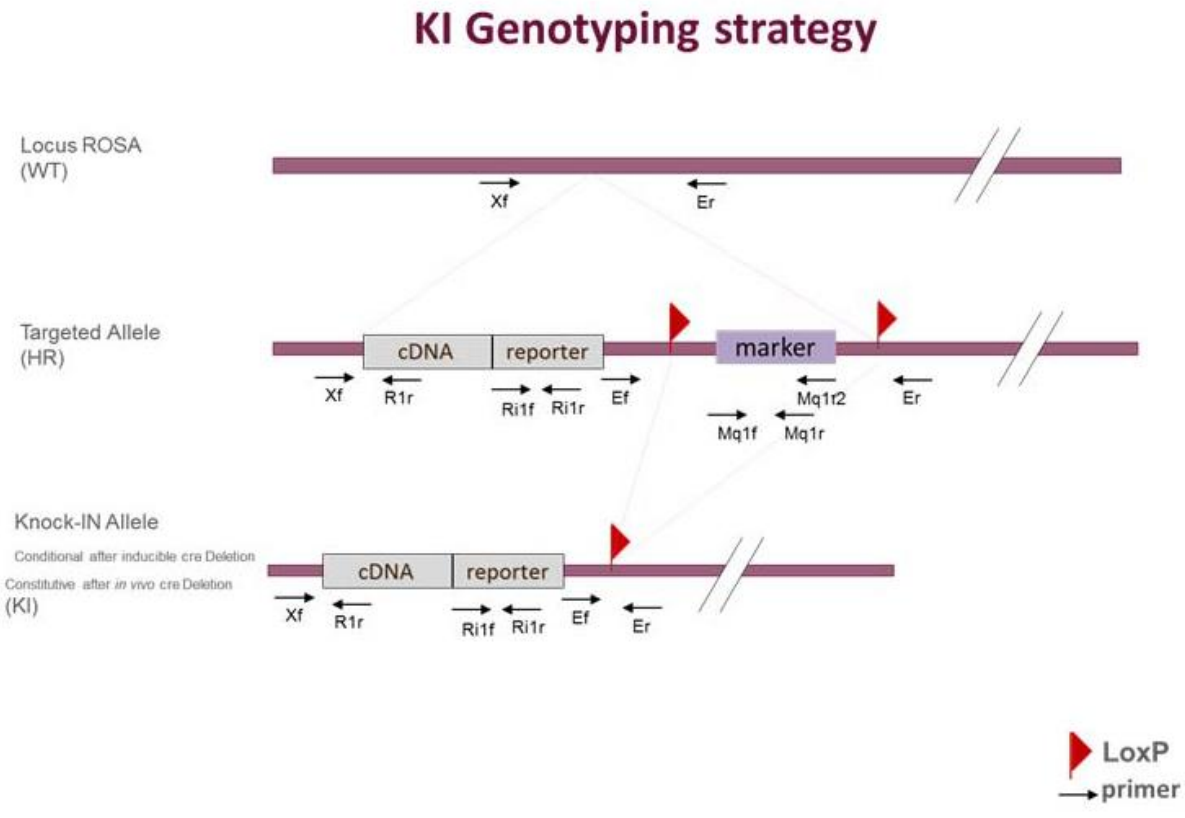
TABLE OF CONTENTS

1. Genotyping protocol and data

This section describes the condition used at the Mouse Clinical Institute (ICS) to genotype your **anti-Otx2 scFV conditionnel dans Rosa** Knockin (KI) project.

1.1. Genotyping strategy

The map below describes the position of the primers used for genotyping for each possible allele.



Sequence of primers used for genotyping:

Position	Primers	Sequence
Ef	6862	TGGAAGGGATTCCAGAGATAGA
Er	4036	AAAGTCGCTCTGAGTTGTTAT
Mq1f	4122	GCTATGACTGGGCACAACAGACAATC
Mq1r	4123	CAAGGTGAGATGACAGGAGATCCTG
Mq1r2	2687	CTGCATTCTAGTTGTGGTTTGTC
R1r	2242	CTCGGCATGGACGAGCTGTACAAG
Ri1r	2148	CCGAAGGCTACGTCCAGGAG
Ri1f	2149	GGTGTTCTGCTGGTAGTGGTCG
Xf	4035	CCTTTAAGCCTGCCCAGAAG

PCR fragments expected size (bp):

Region analyzed	Primers used	Position on the primer *(see the map above)*	l3 (L3)	Targeted allele (L2)	KI allele (L-)	WildType allele (WT)
WildType allele specific PCR (5' part of the targeted locus)	4035-4036	Xf / Er		---	---	239
Excision of the selection marker	6862-4036	Ef / Er		3426	757	---
5' part of the selection marker	6862-2687	Ef / Mq1r2		454	---	---
internal marker	4122-4123	Mq1f / Mq1r	---	262	---	---
3' reporter	2242-4035	R1f / Xf		597	597	---
Internal reporter	2149-2148	Ri1r / Ri1f		293	293	---

*: this PCR product will not be observed using our PCR genotyping conditions (see description below)
**: this PCR is only verified if mice are generated
---: no Amplicon should be obtained

1.2. PCR protocol

This section describes the composition of the mix and cycling conditions used for genotyping.

Reagents:	Volume:
- FastStart PCR Master (Roche)	7.5µl
- DNA (50ng/µl)	1.5µl
- 5' primer (100 µM)	0.06µl
- 3' primer (100 µM)	0.06µl
- Sterile H_2O	up to 15 µl

Cycling conditions:

Temp	Time	#Cycles
95°C	4min	1
94°C	30s	
62°C	30s	34
72°C	1min	
72°C	7min	1
20°C	5min	1

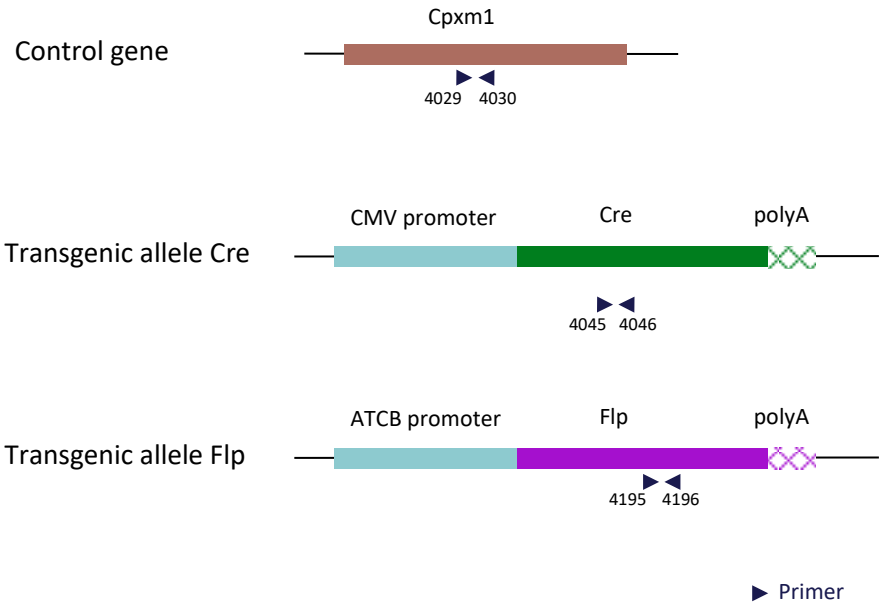
NB: These PCR conditions have been optimized for high-throughput genotyping. Adaptation to small-scale may be required.

2. Cre and Flp genotyping method

The protocol used to segregate the cre and/or flp transgene is indicated below.
Detection of cre transgene and flp transgene is done using a multiplex assay: primer pairs were designed for each gene and for a positive control (Cpxm1 gene).

2.1. Cre and Flp genotyping

Schematic representation of the genotyping strategy



Sequence of primers used for genotyping:

Primers	Sequence
4029	ACTGGGATCTTCGAACTCTTTGGAC
4030	GATGTTGGGGCACTGCTCATTCACC
4045	CCATCTGCCACCAGCCAG
4046	TCGCCATCTTCCAGCAGG
4195	TCTTTAGCGCAAGGGGTAGGATCG
4196	GTCCTGGCCACGGCAGAAGC

PCR fragments expected size (bp):

Primer pair	4045-4046	4195-4196	4029-4030
Region analyzed	Middle part of Cre transgene	Middle part of Flp transgene	Cpxm1 control gene
Control gene	/	/	397
Tg allele	281	328	/

2.2. PCR Protocol

This section describes the composition of the mix and cycling conditions used for genotyping.

Reagents	Volume
FastStart PCR Master (Roche)	7.5µl
DNA (50ng/µl)	1.5µl
5' primer (100 µM)	0.05µl
3' primer (100 µM)	0.05µl
Sterile H_2O	up to 15 µl

Cycling conditions are identical to those described in chapter 1.2