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INVESTISSEMENTS D'AVENIR

MODEL GENERATION TECHNICAL REPORT

IRES-Cre-2A-TdTomato Knock-In into Mcpt8

Kos6459 / IR6459

Report finalized the 15/02/2019

cnrs

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UNIVERSITÉ DE STRASBOURG

Aix*Marseille université

MODEL GENERATION TECHNICAL REPORT

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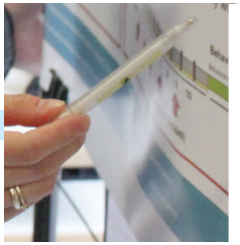
Project process & quality controls



ISO 9001 · NFX 50-900

Long-Range PCR

Southern Blot
- Internal Neomycin probe
- External probes

ddPCR karyotyping

Giemsa karyotyping

Genetic Strategy → Vector Construction → ES Electroporation → ES Screening → ES Amplification → Recombinant ES validation → Microinjection Blastocysts, Eggs → Chimera breeding & amplification

Sequencing

Long-Range PCR

Chimera genotyping
(confirm injection of the right clone & validate genotyping assays)

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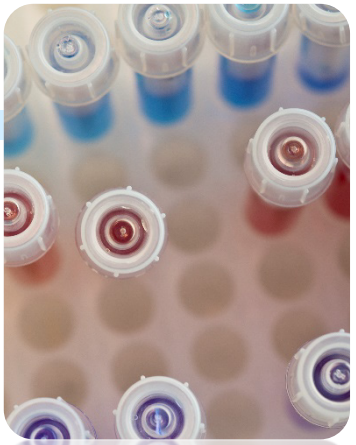
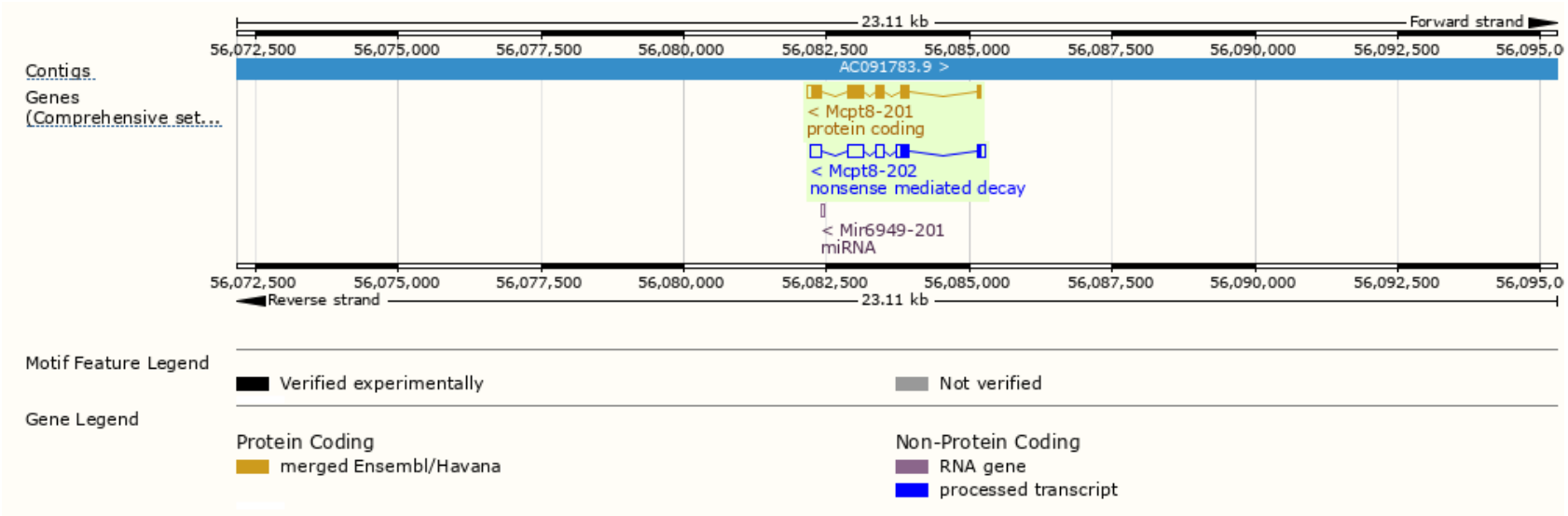
2 GENETIC STRATEGY

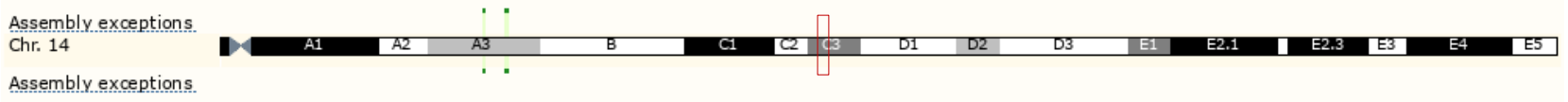


- Target locus structure
- Genetic strategy
- PRO & CONS evaluation of the strategy

Mcpt8 mouse genomic locus

Gene: Mcpt8 ENSMUSG00000022157





Name	Transcript ID	bp	Protein	Biotype	CCDS
Mcpt8-201	ENSMUST00000015594.8	836	247aa	Protein coding	CCDS27141
Mcpt8-202	ENSMUST00000225107.1	945	68aa	Nonsense mediated decay	-

Transcript: Mcpt8-201 ENSMUST00000015594.8

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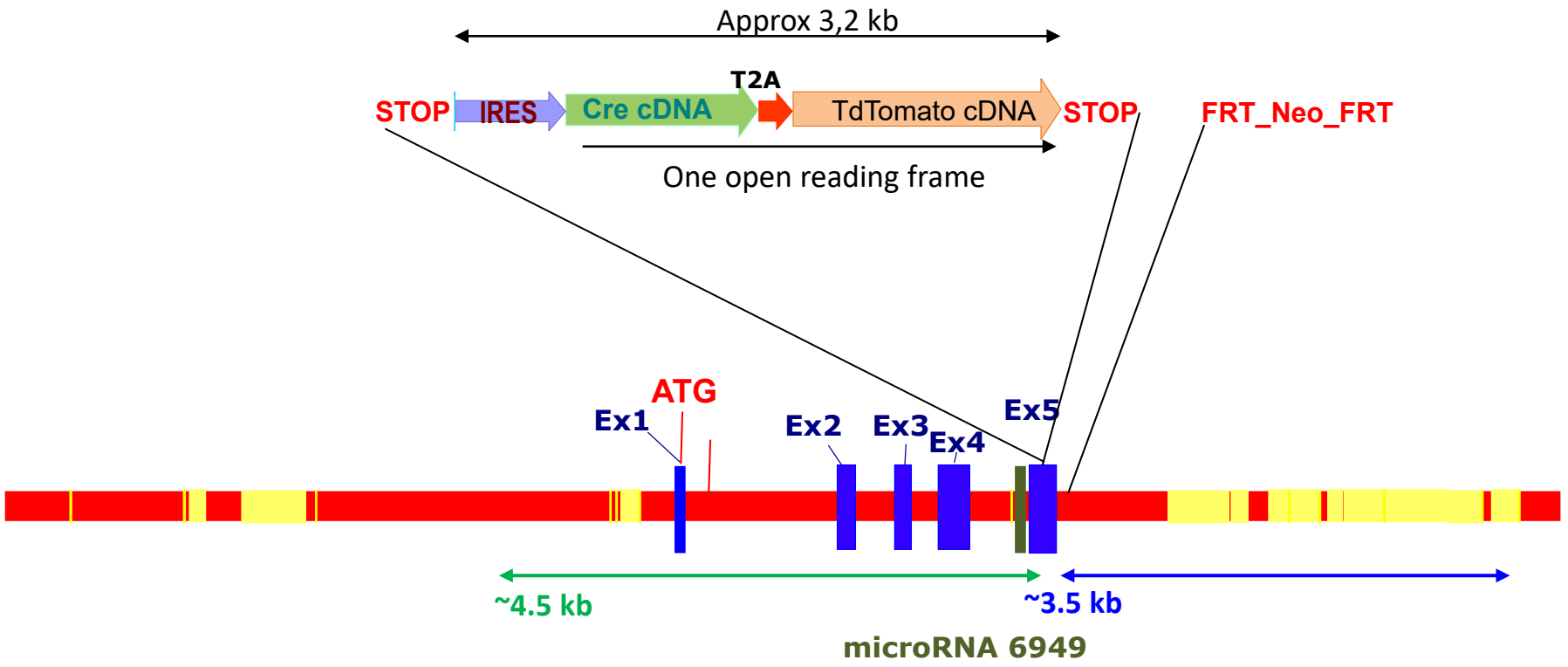
Strategy accepted

IRES-Cre-T2A-TdTomato Knock-In just after the Mcpt8 STOP codon



Repeated regions

PROs & CONs evaluation of the strategy

PROs

- As asked
- Expression of Mcpt8 should not be affected by the knock-in of IRES-Cre-T2A-TdTomato
- The endogenous 3'UTR will be kept
- The flipped Neo selection marker will be inserted 3' of the gene and should not interfere with its expression

CONs

- Expression of Cre-T2A-TdTomato might be less efficient than Mcpt8 as an Internal ribosome entry site (IRES) will be used. Expression after an IRES is always lower than the upstream gene in vivo if using a strong promoter proteins (see http://www.oxfordgenetics.com/SiteContent/Store/internal-ribosome-entry-site-ires-information)
- Presence of repeated sequences in both homology arms might render PCR amplification or Long Range PCR screen difficult

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

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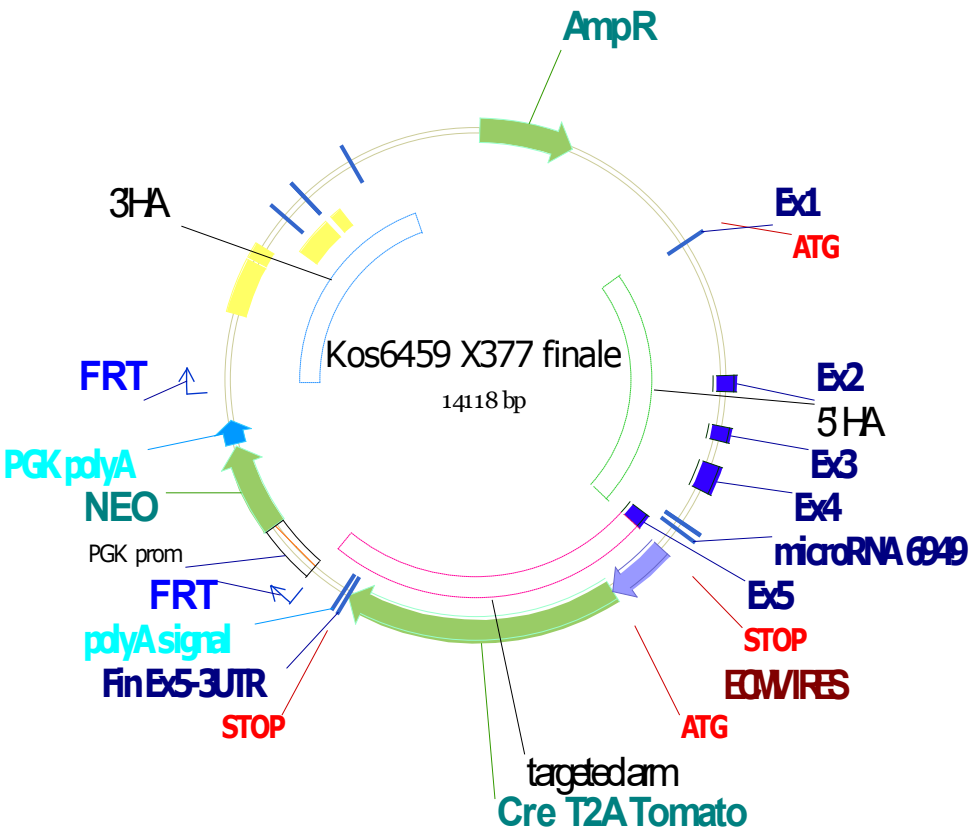
3 HOMOLOGOUS RECOMBINATION

VECTOR CONSTRUCTION

- Vector - structure
- Vector - sequence

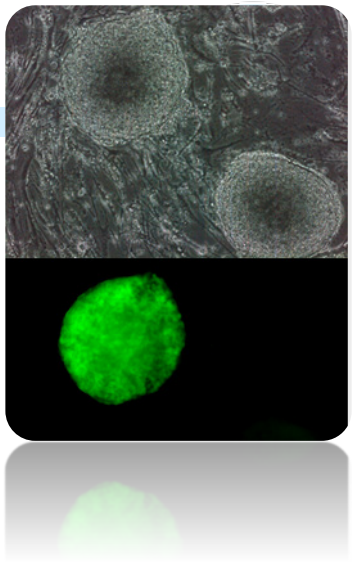
Vector - structure



Vector - sequence

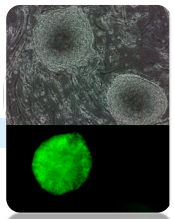
4 ES cell transfection &

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 – 3’ external probe

Electroporation and screening process



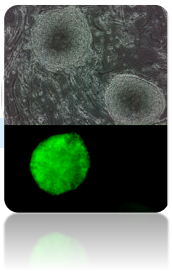
The targeting vector was electroporated in the proprietary C57BL/6NCrl S3 cell line.

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.

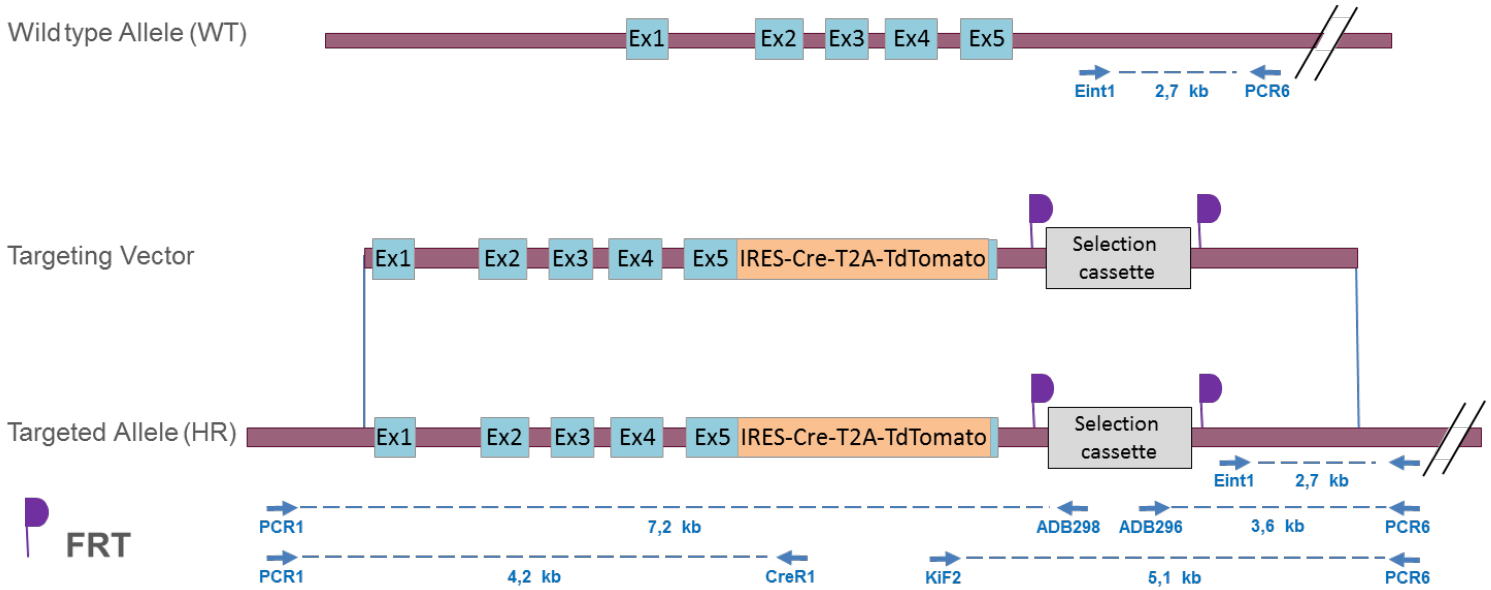
Screening process steps:

1. Identification of candidate recombinant clones by initial 3' Long-Range PCR
2. 8 of 3' PCR positive clones are confirmed for 5' and 3' recombination event by Long-Range PCR
3. Positive clones in step 2 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

Long range primary PCR screening – strategy



Schematic 5’ and 3’ PCR screening strategy



PCR	Primer Name	Primer sequences	PCR product size
5' PCR	PCR1	GGTCATGCTCACCAGTAACTGACAG	7,2 kb
	ADB298	GCGGCCGGAGAACCTGCGTGCAATC	
5’ PCR	PCR1	GGTCATGCTCACCAGTAACTGACAG	4,2 kb
	CreR1	CCAGATTACGTATATCCTGGCAGCG	
3’ PCR	ADB296	AGGGGCTCGCGCCAGCCGAACTGTT	3,6 kb
	PCR6	GGTAAGTGGAACTGTTTAGAAAGGT	
3’ PCR	KiF2	CCTGTTCCTGTACGGCATGGACGAG	5,1 kb
	PCR6	GGTAAGTGGAACTGTTTAGAAAGGT	
3’ PCR	Eint1	GCCTTTACCTCCCACTCTCTTCATT	2,7 kb
	PCR6	GGTAAGTGGAACTGTTTAGAAAGGT	

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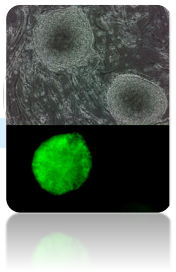
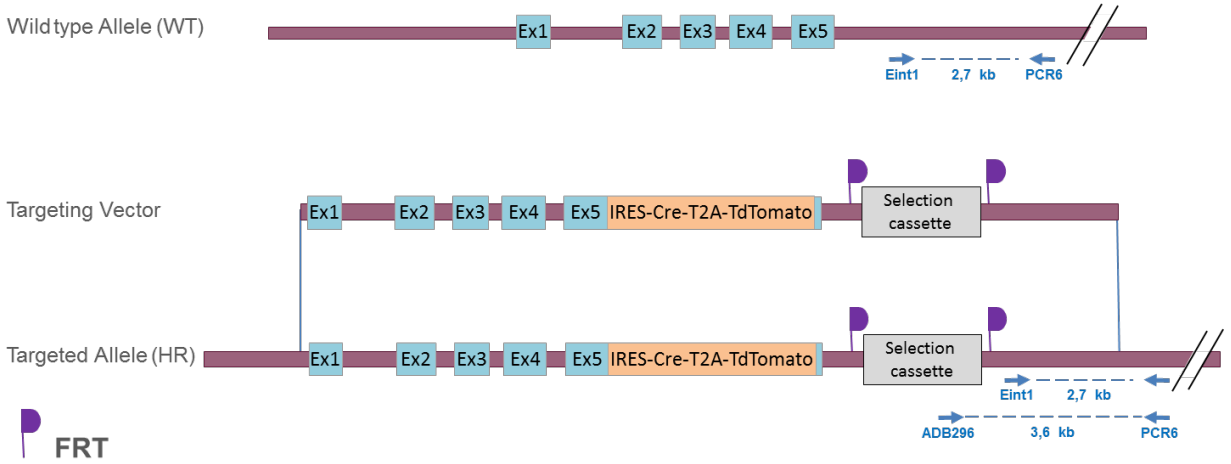
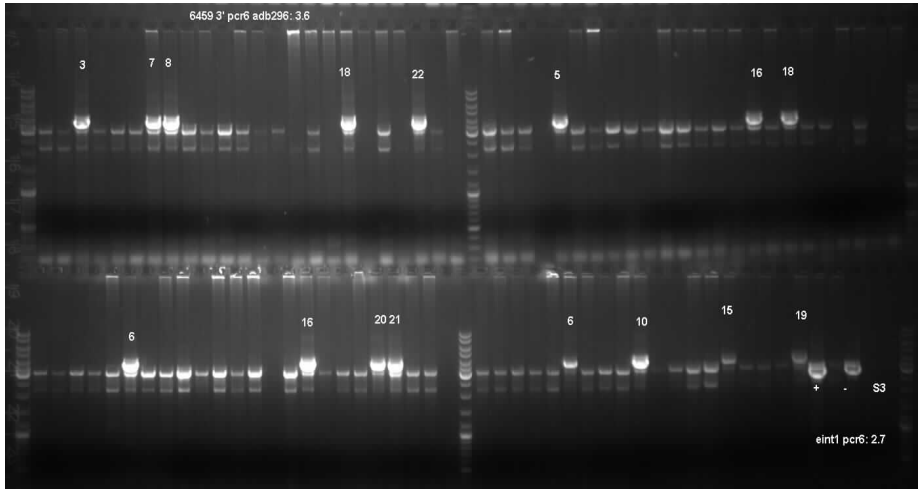
Long-Range 3’ PCR primary screening - results





ADB296-PCR6 : 3.6 kb



PCR Control
Eint1-PCR6 : 2.7 kb

Eight candidate clones out of the 16 positive clones were selected for 5’ Long-Range PCR and Southern blot validation.

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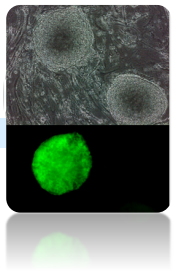
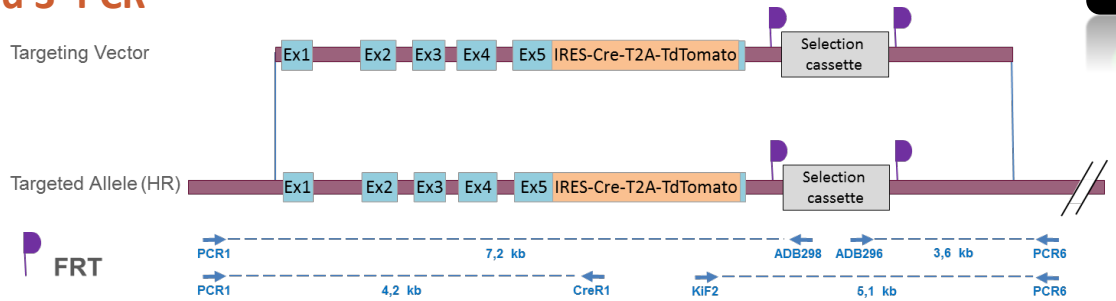
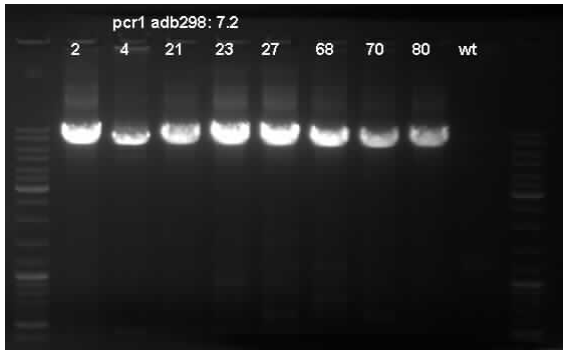
Recombinant ES validation by Long Range PCR

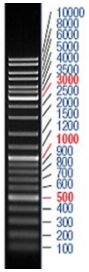


Confirmation of ES clones by 5’ and 3’ PCR

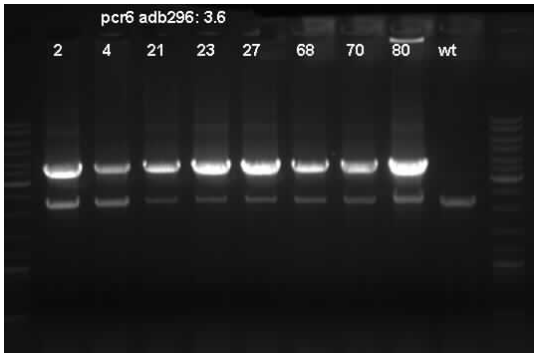




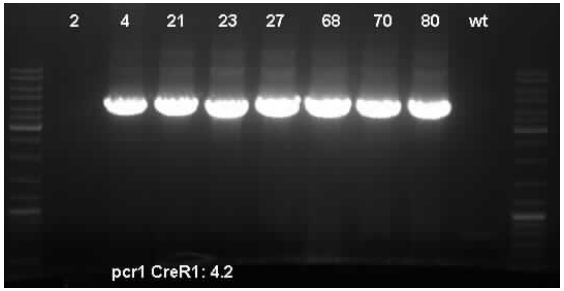
PCR1 – external 5’ PCR / ADB298-pCAG – 7,2 kb



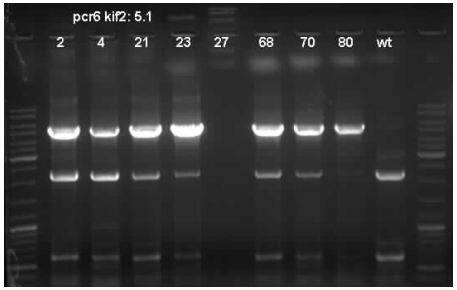
Ladder pattern



ADB296 – Neo / PCR6 - external 3’ 3,6 kb



PCR1 – external 5’ / CreR1 – 4,2 kb



Kif2 / PCR6 - external 3’ – 5,1 kb

Eight candidate clones identified by 3’ PCR screening were further analysed by 5 and 3’ PCR screening. Eight clones (clones 2, 4, 21, 23, 27, 68, 70 and 80) were confirmed.

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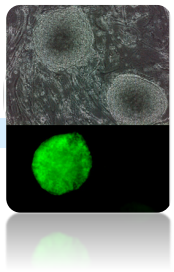
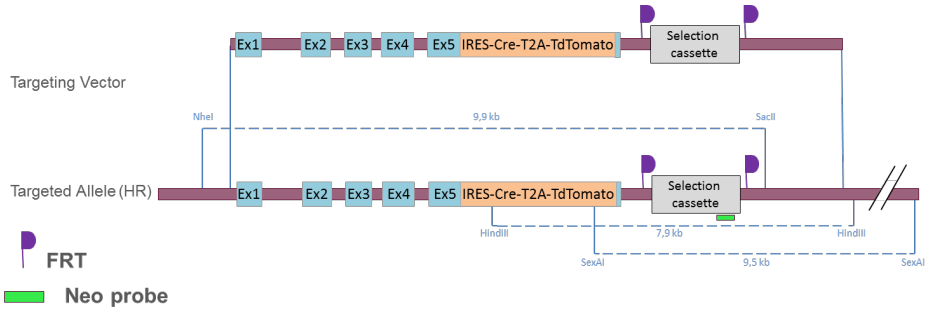
Recombinant ES clones validation by Southern Blot – internal probe



Schematic Southern Blot validation strategy



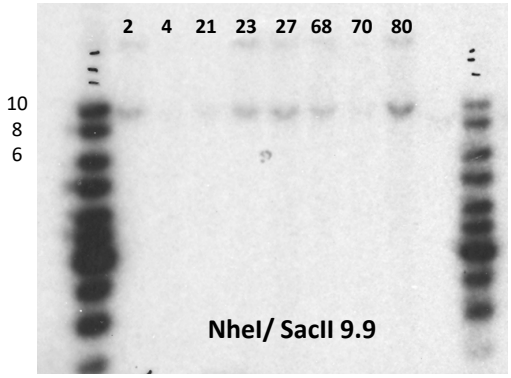
Digestions used to validate the 5' and 3' insertion

Probe	Name	Targeted Allele (kb)
Neo	5' NheI/SacII digest	9,9
	3' HindIII digest	7,9
	3' SexAI digest	9,5

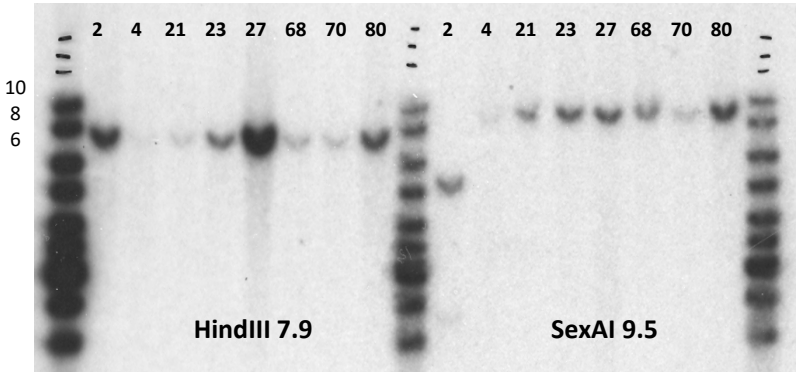
Neo probe sequence

agaagaactcgtcaagaaggcgatagaaggcgatgcgctgcgaatcgggagcggcgataccgtaaagcacgaggaagc
ggtcagcccattcgccgccaagctcttcagcaatatcacgggtagccaacgctatgtcctgatagcggtccgccacacccagcc
ggccacagtcgatgaatccagaaaagcggccattttccaccatgatattcggcaagcaggcatcgccatgggtcacgacgag
atcctcgccgtcgggcatgcgcgccttgagcctggcgaacagttcggctggcgcgagcccctgatgctcttcgtccagatcatcct
gatcgacaagaccggcttccatccgagtacgtgctcgctcgatgcgatgtttcgcttggtggtcgaatgggcaggtagccggatc
aagcgtatgcagccgccgcattgcatcagccatgatggatactttctcggcaggagcaaggtgagatgacaggagatcctgcc
ccggcacttcgcccaatagcagccagtcccttcccgcttcagtgacaacgtcgagcacagctgcgcaaggaacgcccgtcgtg
gccagccacgatagccgcgctgcctcgtcctgcag

Southern blot - Neo 5'

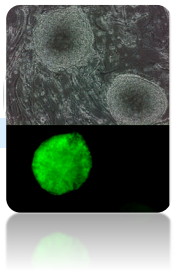


Southern blot - Neo 3'

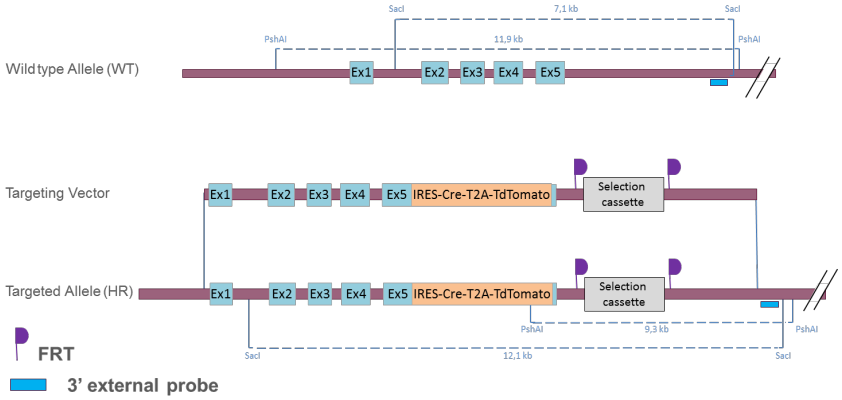


Recombinant ES clones validation by Southern Blot –

3’ external probe



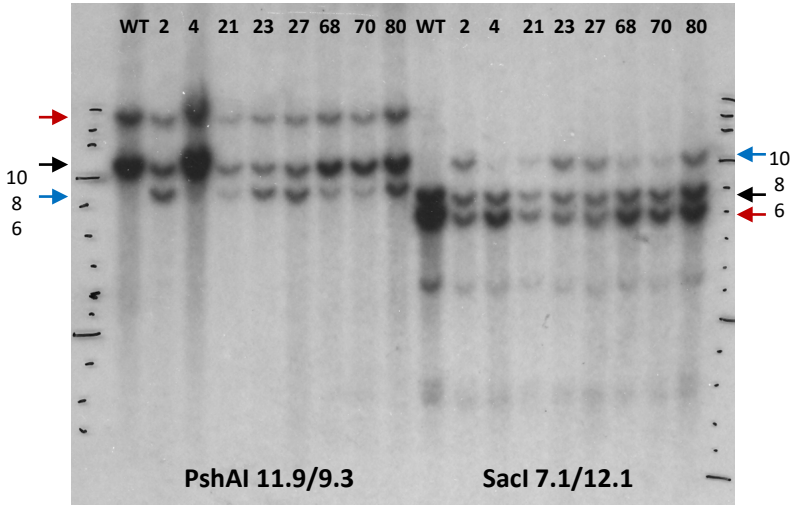
Schematic Southern Blot validation strategy



Digestions used to validate the 3’ insertion

Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
3’ external probe	3’ digest	PshAI	11.9	9.3
		SacI	7.1	12.1

Southern blot – 3’ probe



Wild Type Band
Targeted Band
Supplementary band also visible in the WT control

3’ probe sequence

GGAGGTCACCTAACCCATCTCAGGCACAGAAGGCCACCCTTAGTAT
TTAATCAATGGTGGGAATGGAATTAACCAAGATATGTTCAACATGACT
CTGCCAGTTCTGGTGAGTTCTCTCAACCAGGCAAGATCCTGGCCTG
CCATGGCTGACCTCTGGTTCCTTACCACAATGCTACCCTGAGACAA
GTGATCTTCTCCCAGAAATCTAAGCCTGCCCCACCTCCAGTTTCCTA
TCACATTGCTCCTCTACTCAGGTGGGTTTTCCACACCATCATGACCT
CACCCCACCCCAAATGTGATACCATCTCACAACCCTCCTCTAACCTC
AGACCCAGCCACTCCACAGGAGTGCTGTGGGCCTAGAACAGACTT
GAATCTAGTAAGACACTCTATCCTAAACCCAGATTCTCAGCCAGAGC
TTCATCC

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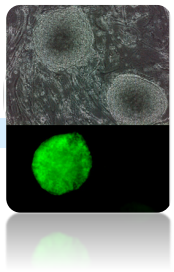
Aneuploidy screening in ES recombinant clones

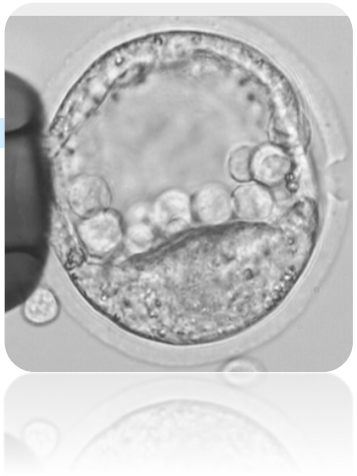


Selected recombinant ES cells clones were karyotyped by ddPCR as described in Codner *et al.* [1] and by Giemsa metaphase staining. Results of aneuploidy analysis are presented in the table below.

Clone ID	ddPCR	Giemsa
27	Pass	Pass
68	Pass	Pass
70	Not Done	Pass
80	Pass	Pass

[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 color. 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 27, 68, 70 and 80 validated in previous project phase were injected into blastocysts to generate chimeric males.

- Clone ES 80 went germ line.

Breeding to F1 generation

- **Eight highly chimeric** males generated in the previous phase by blastocyst injection of the **ES clone 80** were mated with Flp deleter C57BL/6NCrl females (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 26/12/2018

- Allele in MGI: **Mcpt8$^{tm1.1(cre)Ics}$** (MGI:7327249)

- Publication:

Tchen J, Simon Q, Chapart L, Pellefigues C, Karasuyama H, Miyake K, Blank U, Benhamou M, Daugas E, Charles N. CT-M8 Mice: A New Mouse Model Demonstrates That Basophils Have a Nonredundant Role in Lupus-Like Disease Development. Front Immunol. 2022 Jun 29;13:900532. doi: 10.3389/fimmu.2022.900532. PMID: 35844602; PMCID: PMC9277511.

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REPORT REDACTION & VALIDATION

Protocol finalized on 15/02/2019

Prepared by Romain LORENTZ,

Verified by Marie-Christine BIRLING, PhD

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