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EXCELLENCE IN MOUSE PHENOGENOMICS

INVESTISSEMENTS D'AVENIR

TECHNICAL REPORT

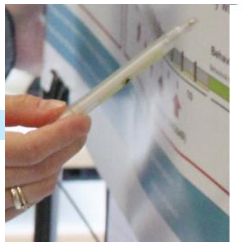
Humanization of TOMM40, APOE3 and APOC1

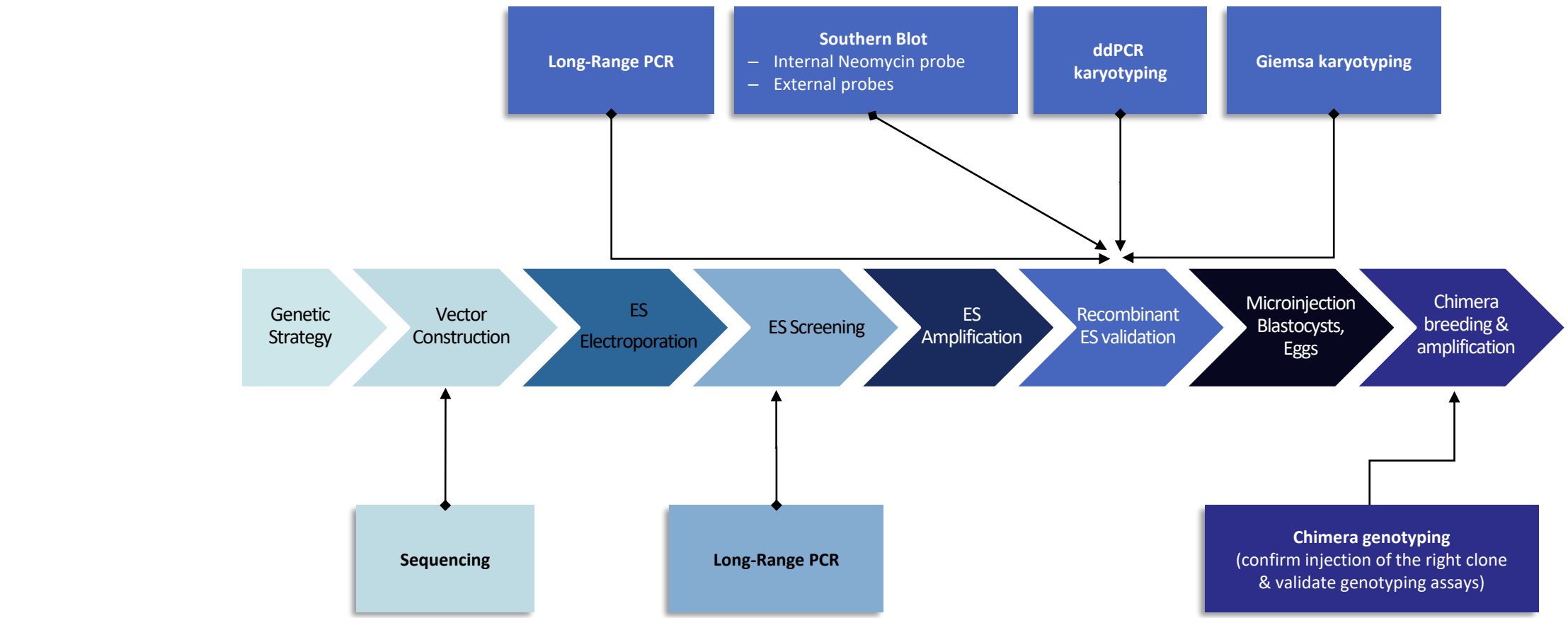
Project code: Kos6295

cnrs
centre européen de recherche en biologie et en médecine CERBMgie
Instituts thématiques Inserm Institut national de la santé et de la recherche médicale
UNIVERSITÉ DE STRASBOURG
Aix*Marseille université

MODEL GENERATION TECHNICAL REPORT

Project process & quality controls





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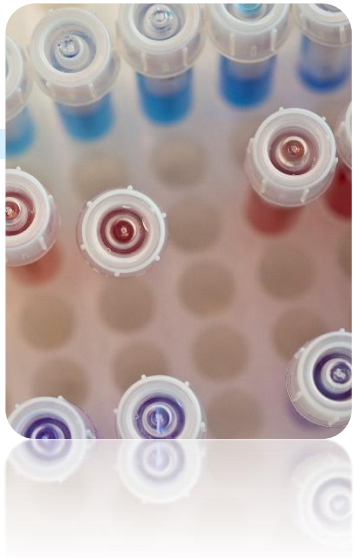
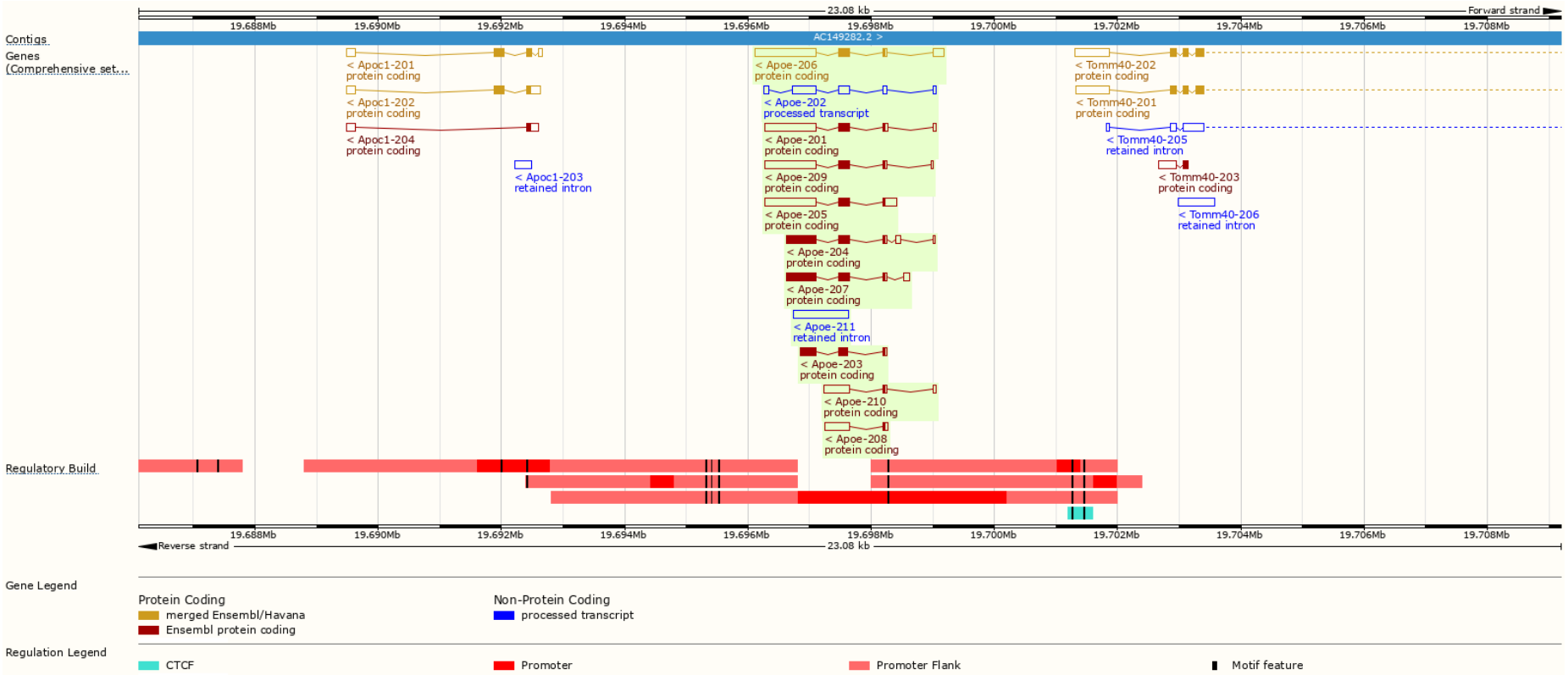
2 GENETIC STRATEGY



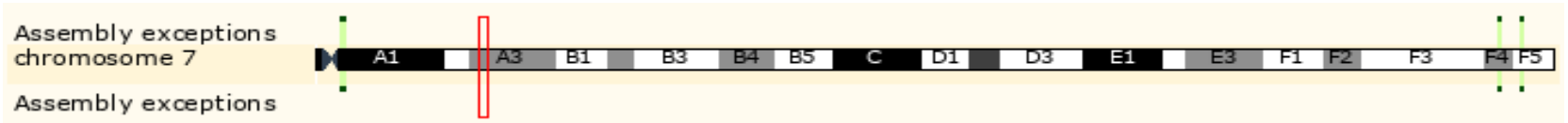
- Target locus structure
- Genetic strategy
- Sequence detail
- PRO & CONS evaluation of the strategy

ApoE mouse genomic locus – structure

Gene: ApoE ENSMUSG00000002985



Chromosome 7: 19,696,109-19,699,188



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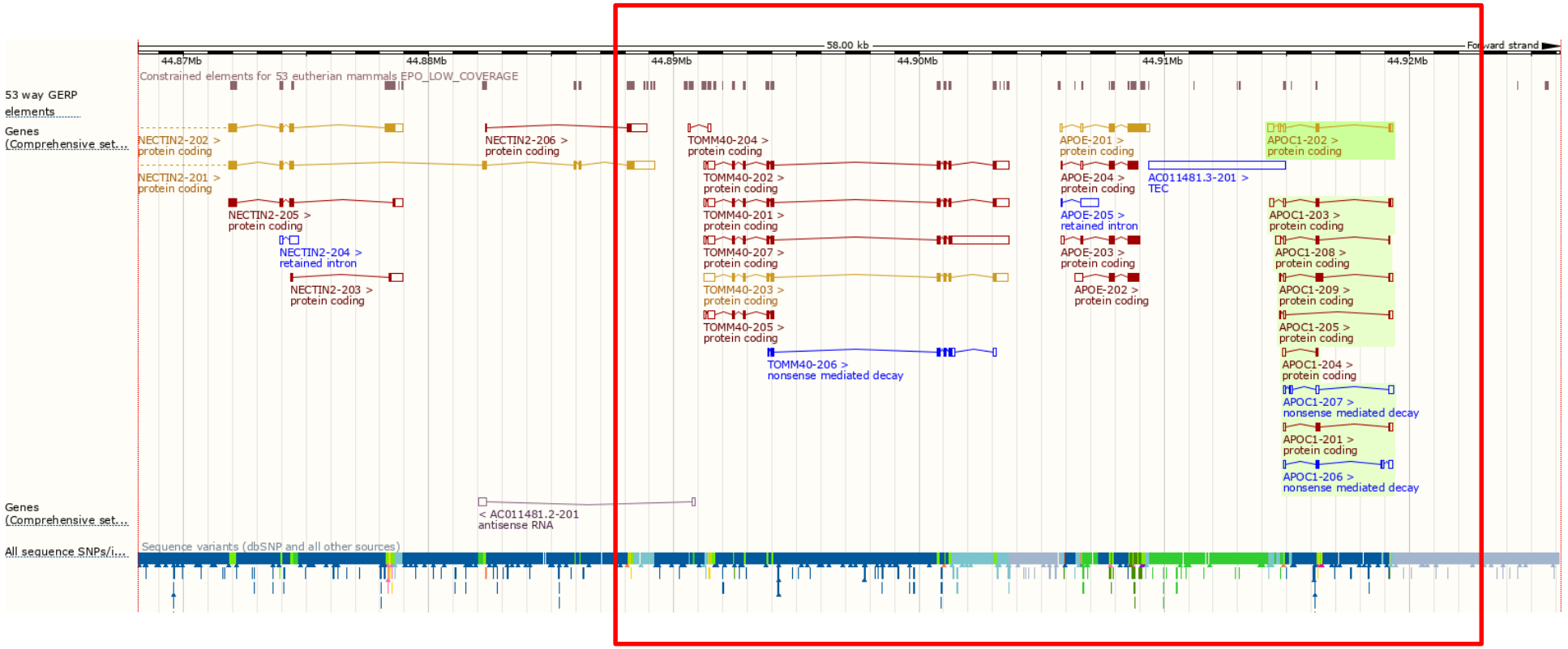
APOE human genomic locus – structure

Human region selected for humanization (34 kb)



Chromosome 19: 44,868,129-44,926,130

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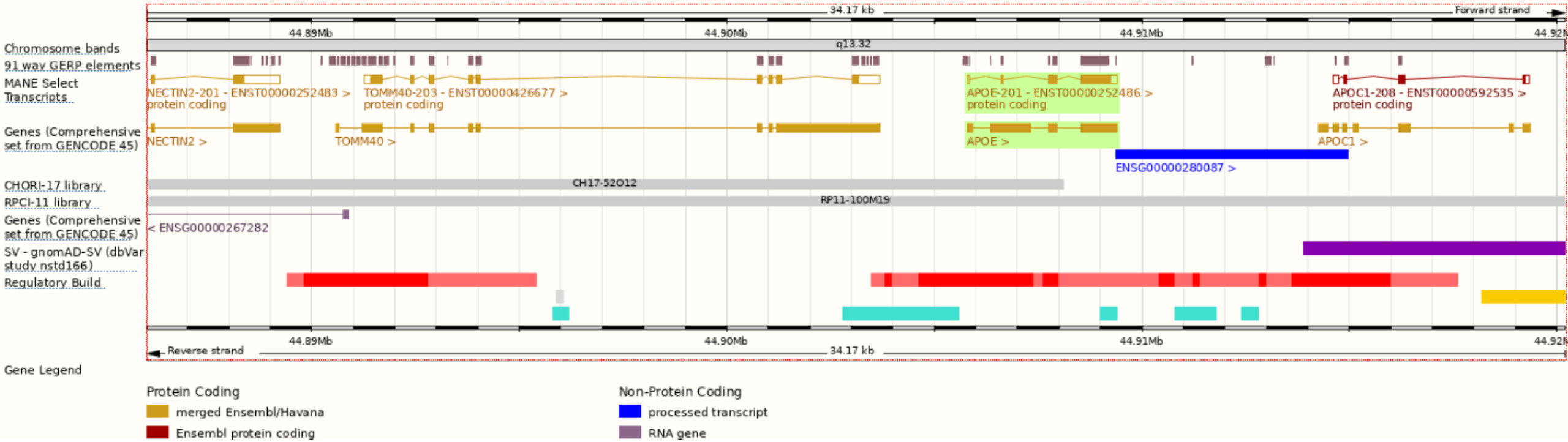
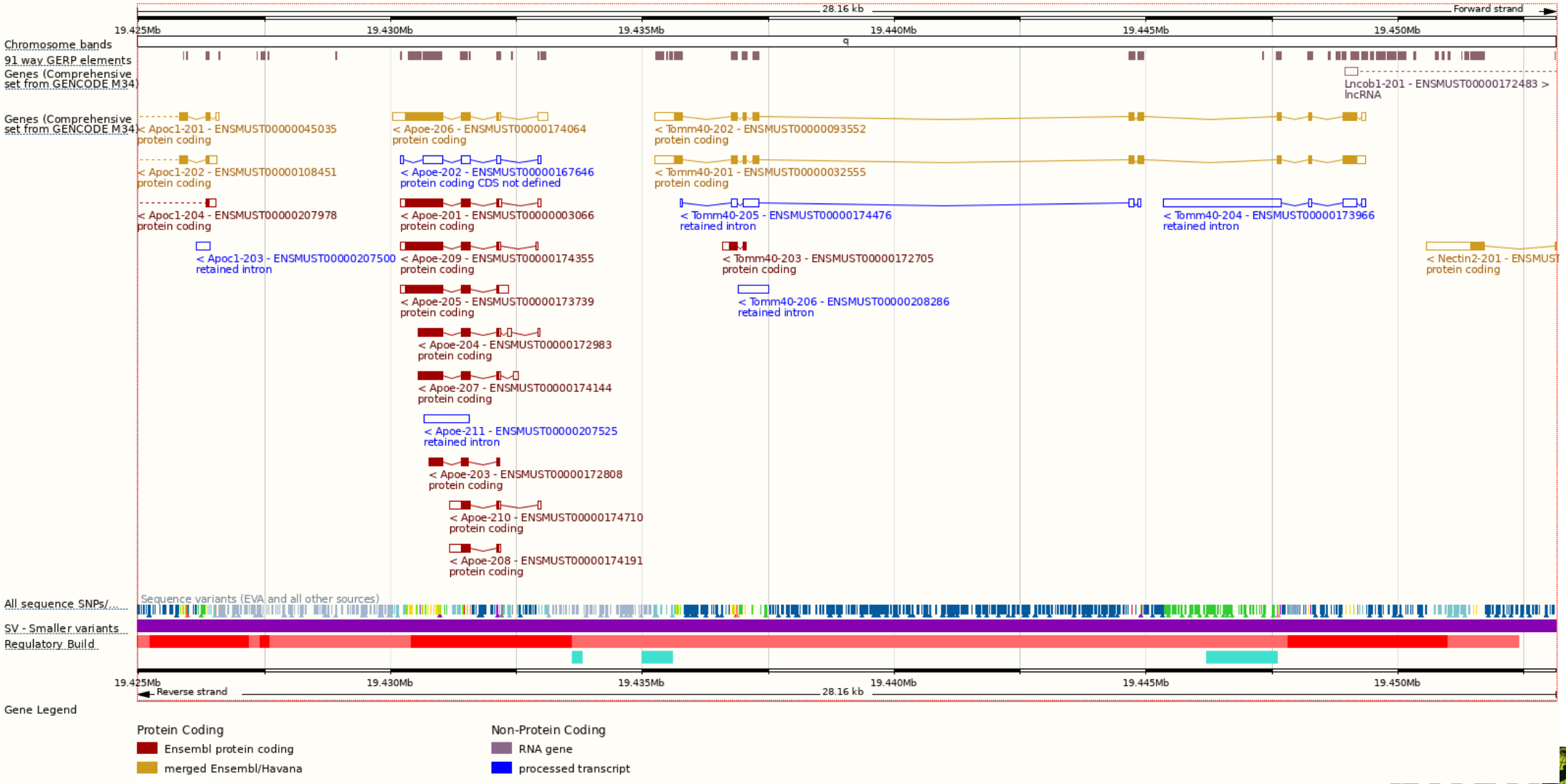
Sequence humanized (19:44886017-44920186; GRCh38.p14)

19:44886017-44920186

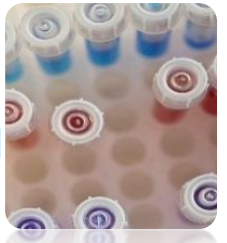


Murine sequence deleted and replaced by the human sequence

7:19424966-19453126 (GRCm39)

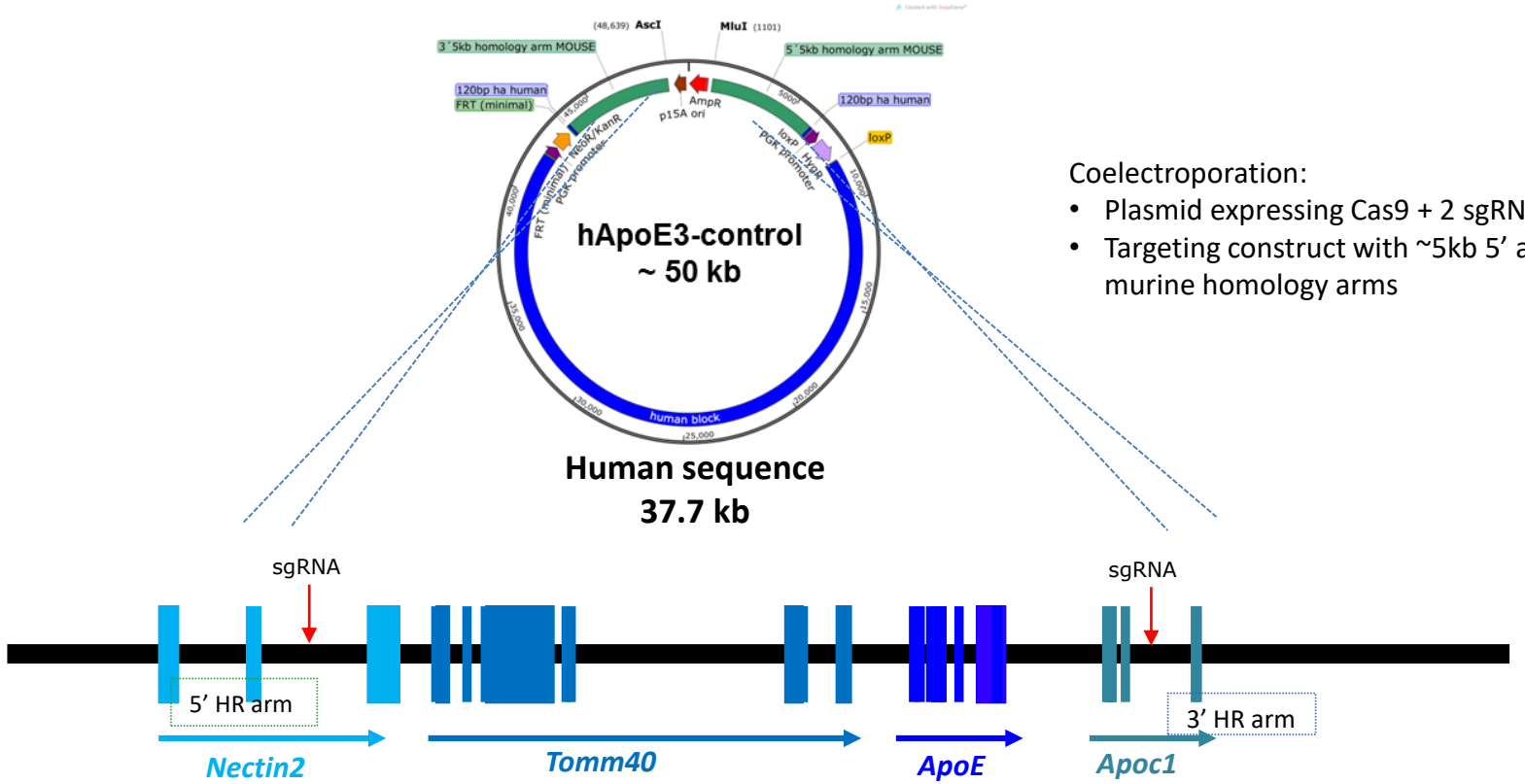


Approach selected; Humanization of a mouse locus with the help of CRISPR/Cas9



Aim :

1) to design a humanized APOE mouse model (APOE3), TOMM40 and APOC1
2) to generate APOE2 and APOE4 variants



Coelectroporation:

- Plasmid expressing Cas9 + 2 sgRNAs
- Targeting construct with ~5kb 5' and 3' murine homology arms

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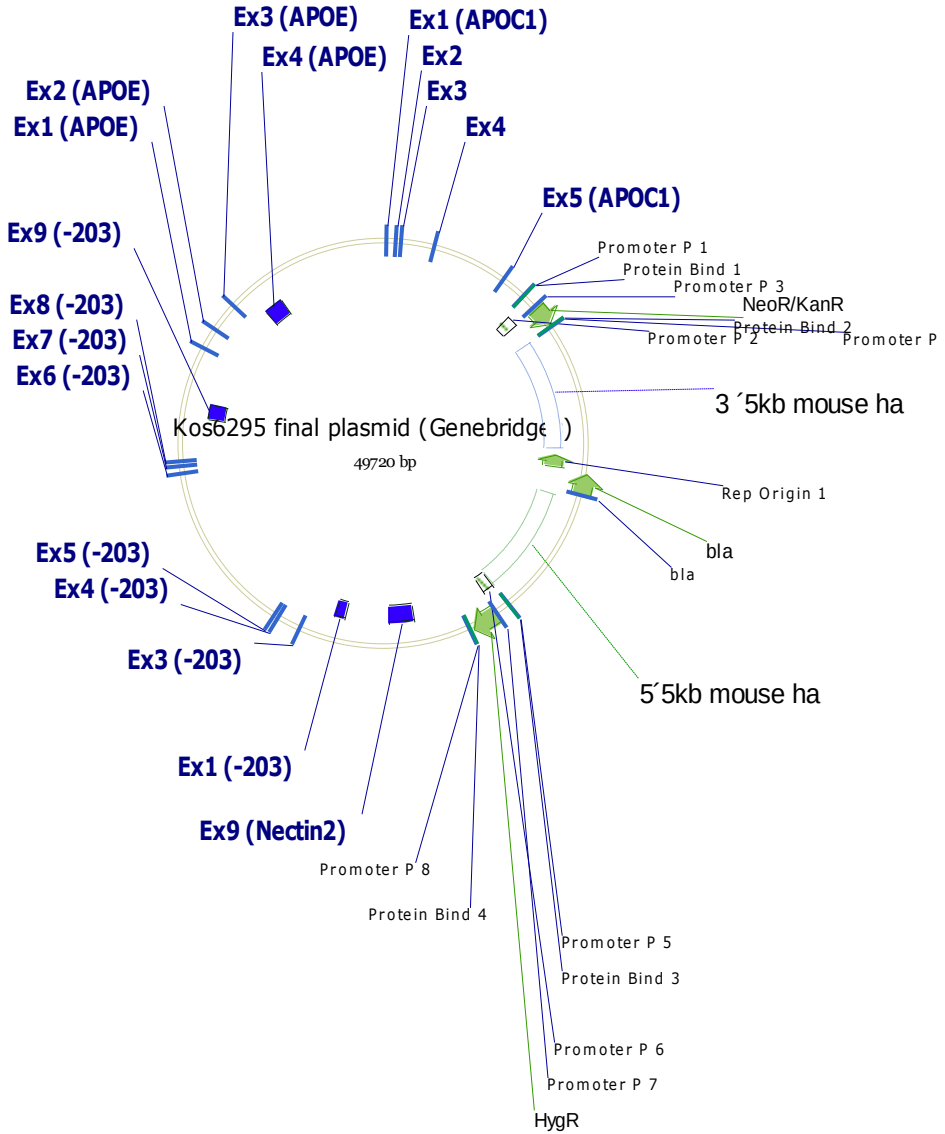
HOMOLOGOUS RECOMBINATION

VECTOR MAP

Vector build by:

THE RECOMBINEERING COMPANY
GENE BRIDGES



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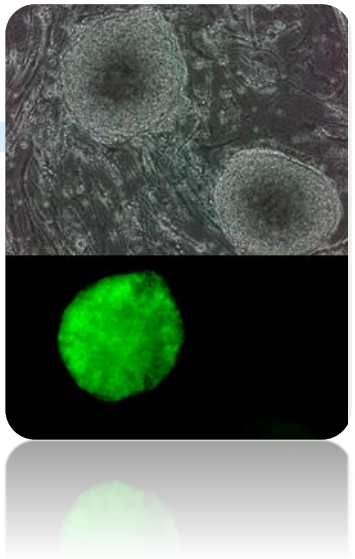
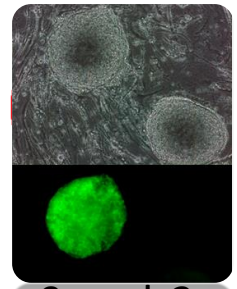
4 ES cell transfection &

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
- Aneuploidy screening in ES recombinant clones

Electroporation and screening process



The targeting vector was co-electroporated with a plasmid derived from pX330 that co-expressed the WT spCas9 and 2 guides RNA (gR83 –aagacgccatactttgatgc and gR92-gaatggccttctagactcgg directed against the mouse 5’ and 3’ extremity of the sequence to replace in the proprietary C57BL/6NCrl S3 cell line.

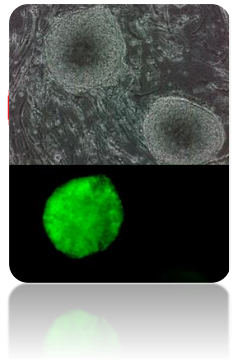
Transfected ES clones were submitted to neomycin (G418), hygromycin, and neomycin/hygromycin selection 186 resistant ES clones were isolated.
The clones were then submitted to the screening process allowing secured identification of those harbouring the expected recombination events at both ends of targeting vector (see Erbs et al., 2023*).

Screening process steps:

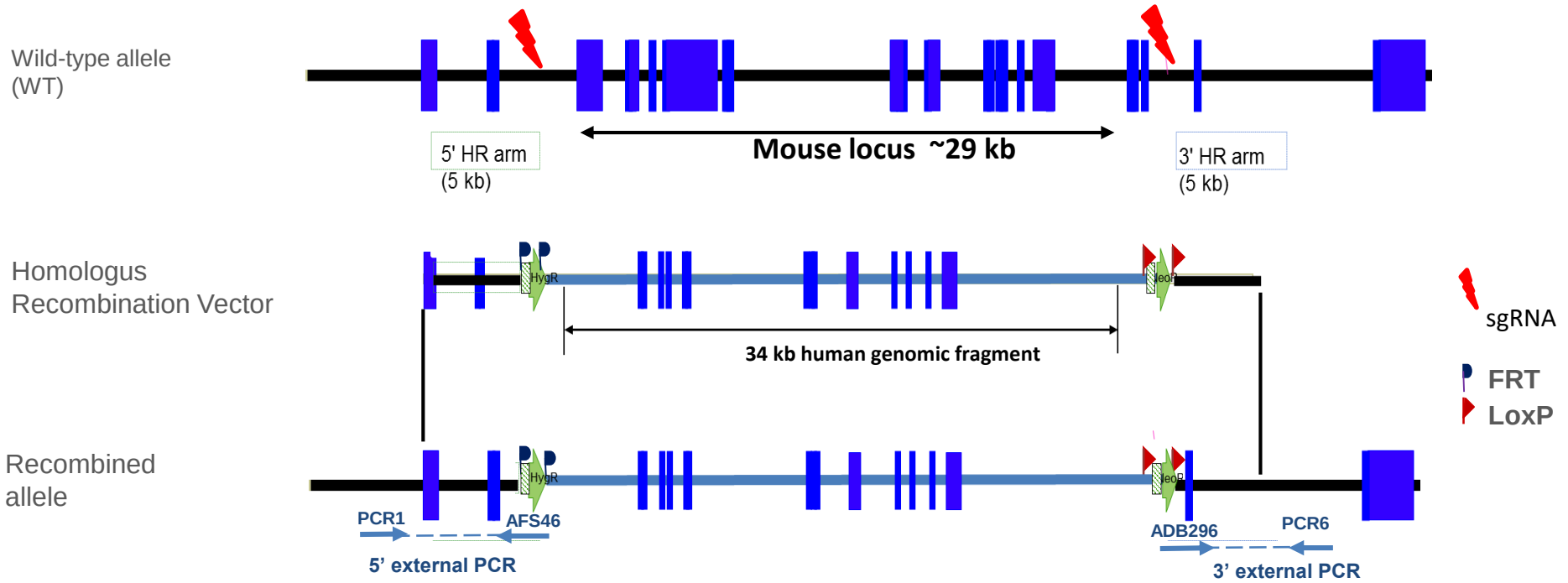
1. Identification of candidate recombinant clones by initial 3’ Long-Range PCR
2. 3’ LR-PCR positive clones are confirmed for 5’ recombination event by Long-Range PCR
3. Positive clones in step2 are further validated by Southern blot analysis using internal and external probes
4. The karyotype of at least 2 validated clones is verified using Giemsa staining

*__Increased On-Target Rate and Risk of Concatemerization after CRISPR-Enhanced Targeting in ES Cells.__ Erbs V, Lorentz R, Eisenman B, Schaeffer L, Luppi L, Lindner L, Hérault Y, Pavlovic G, Wattenhofer-Donzé M, Birling MC. Genes (Basel). 2023 Feb 3;14(2):401. doi: 10.3390/genes14020401. PMID: 36833328

Long range PCR screening – strategy

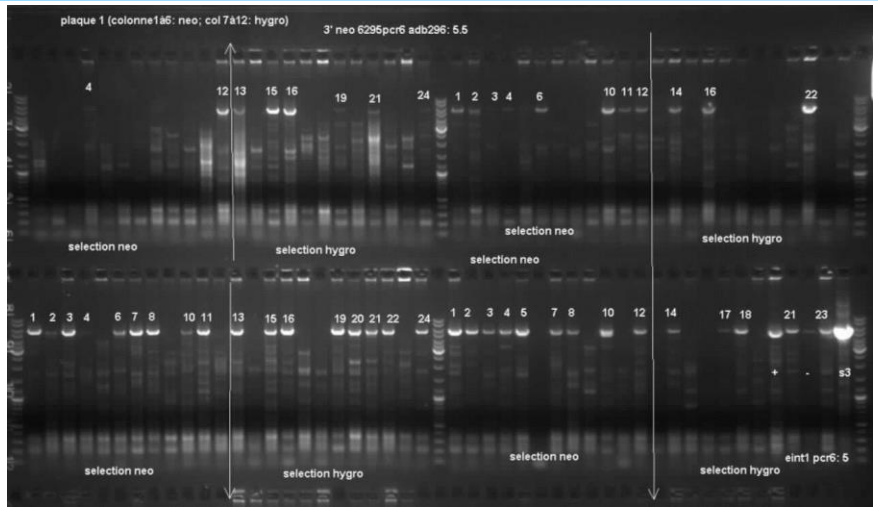


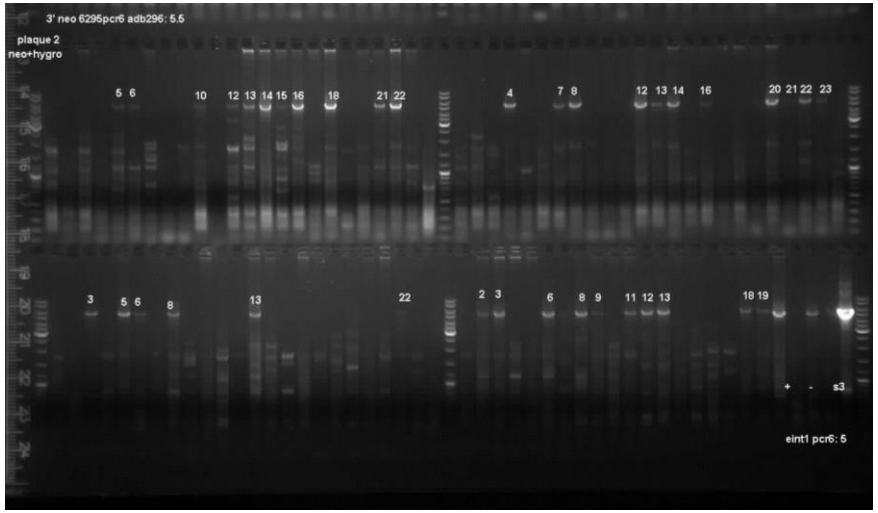
Schematic 5’ and 3’ PCR screening strategy

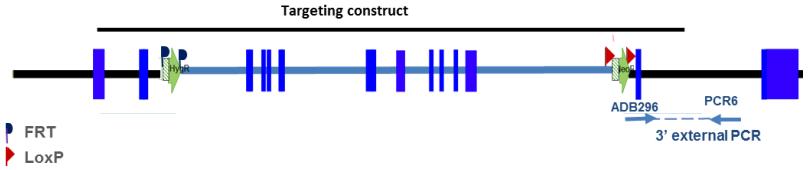


PCR	Primer Name	Primer sequences	PCR product size
5’PCR	AFS46	CTGCATCAGGTCGGAGACGCTGTCG	5,7 kb
	PCR1	CCCTACGATATAGACACTGGACACA	
3’ PCR	ADB296	AGGGGCTCGCGCCAGCCGAACTGTT	5,5 kb
	PCR6	GGCTGTTTGCTATTTGCTGCCTGCA	

Long-Range 3’ PCR screening - results







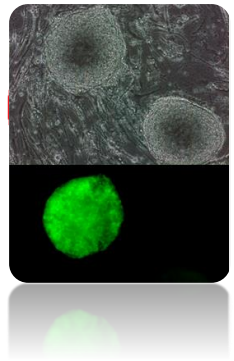
Selection	Neo	Hygro	Neo+ hygro
Number of clones	48	45	93
3' LR-PCR neo	28	22	36
5' LR-PCR hygro	4	6	21
Backbone PCR -	0	4	7
Positive clones	/	47_58_70_81	107_116_134_170_175_184_189

Data not shown

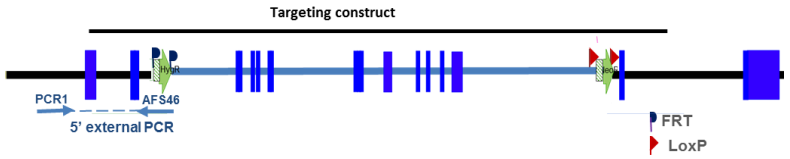
11 candidate clones were amplified for Southern blot validation and confirmed by LR-PCR .

Eleven clones were selected and amplified for further validation

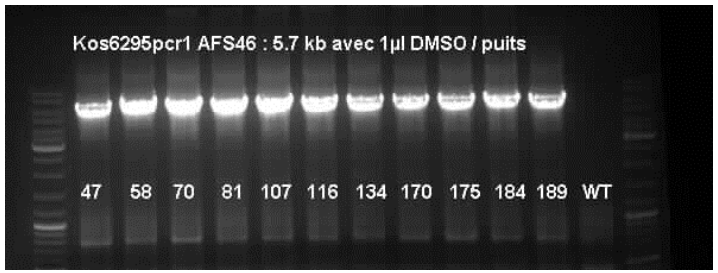
Recombinant ES validation by Long Range PCR



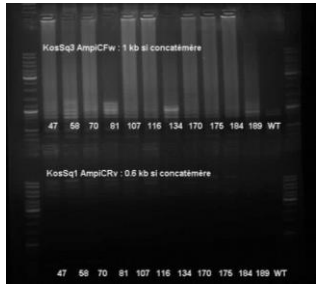
Validation of candidate recombinant ES clones by 5’ LR-PCR



Kos6295 – external 5’ PCR-hygro – 5,7 kb

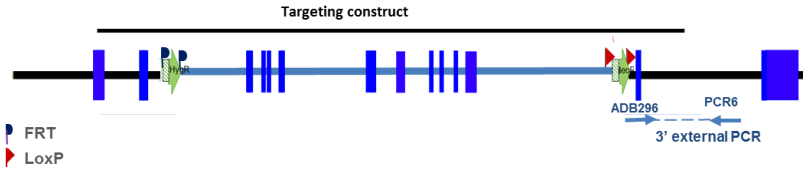


Backbone PCR

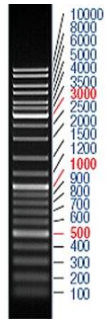


Confirmation of candidate by 3’ LR-PCR

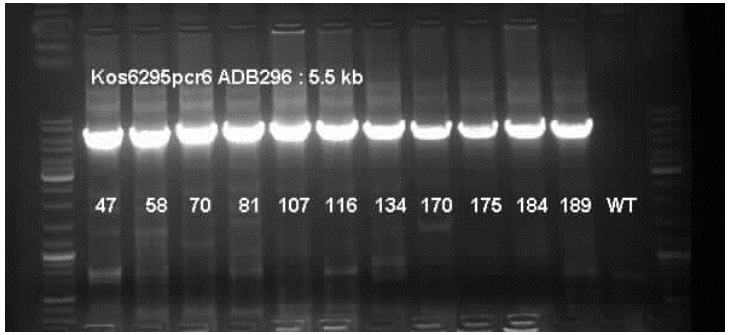
Kos6295 – external 3’ PCR-Neo – 5,5 kb



Kos6295 – external 3’ PCR-hygro – 5,5 kb



Ladder pattern



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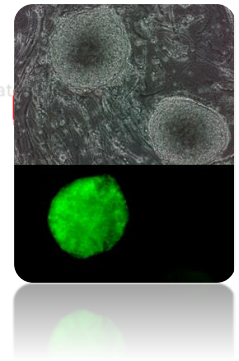
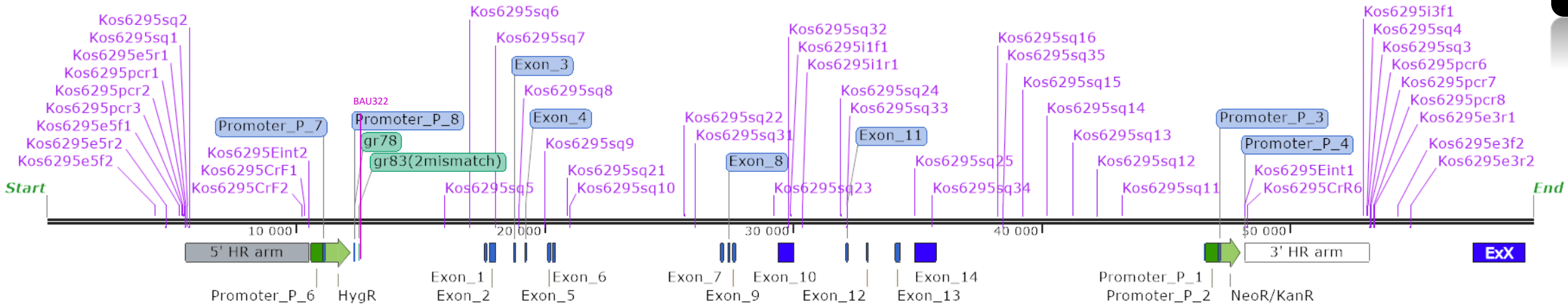
Recombinant ES validation by Long Range PCR



Validation of candidate recombinant ES clones by human specific PCR



BAU322-sq10
Exp 9 kb

BAU322-sq5
Exp 4 kb

Sq21-sq31
Exp 5.1 kb

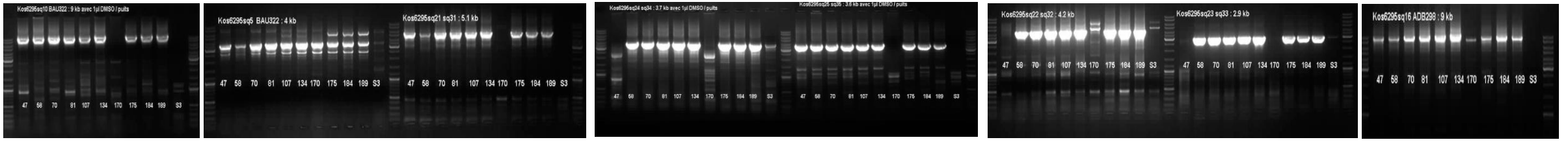
Sq24-sq34
Exp 3.7 kb

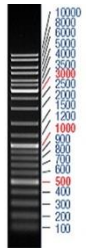
Sq25-sq35
Exp 3.6 kb

Sq22-sq32
Exp 4.2 kb

Sq23-sq33
Exp 2.9 kb

Sq16-ADB298
Exp 2.9 kb





Ladder pattern

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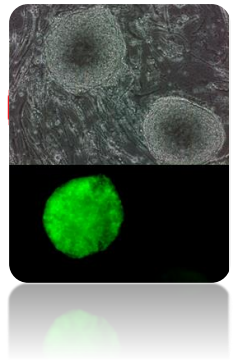
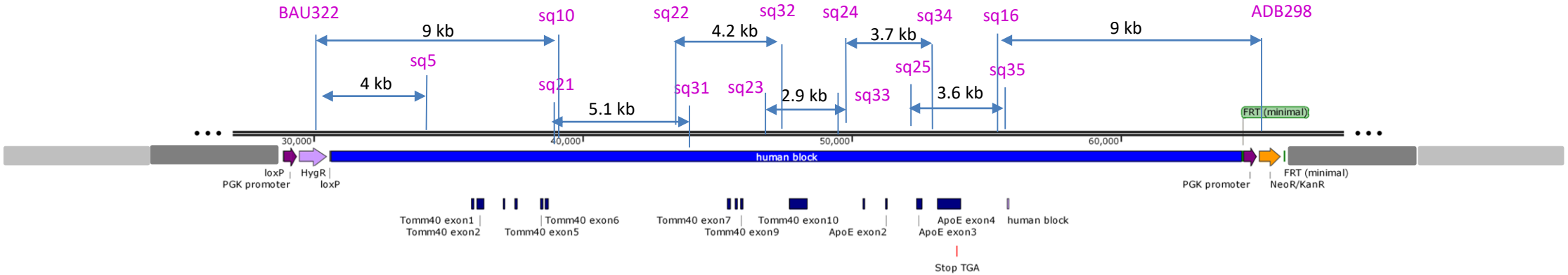
Recombinant ES validation by Long Range PCR



Validation of candidate recombinant ES clones by human specific PCR



BAU322-sq10
Exp 9 kb

BAU322-sq5
Exp 4 kb

Sq21-sq31
Exp 5.1 kb

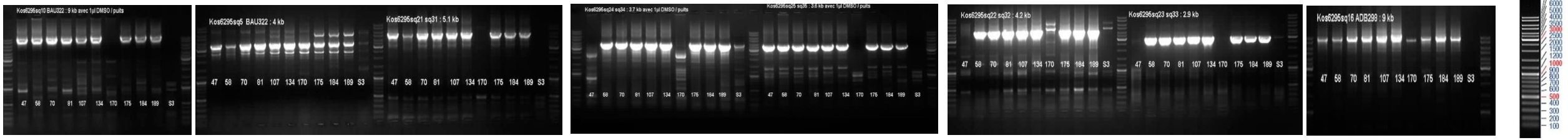
Sq24-sq34
Exp 3.7 kb

Sq25-sq35
Exp 3.6 kb

Sq22-sq32
Exp 4.2 kb

Sq23-sq33
Exp 2.9 kb

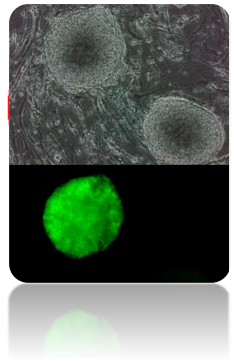
Sq16-ADB298
Exp 2.9 kb



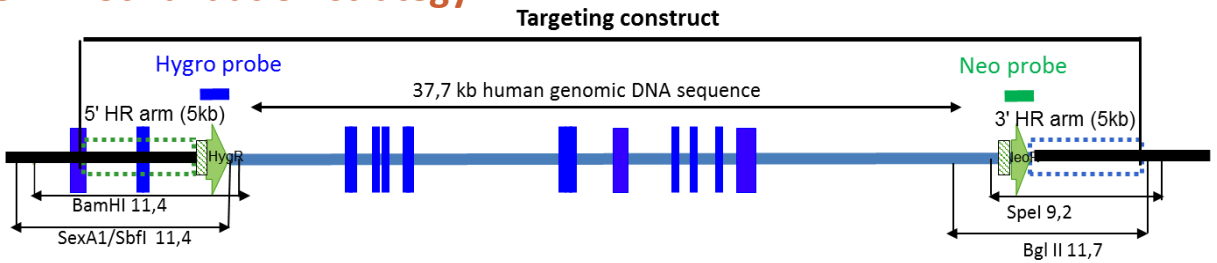
Ladder pattern

Ladder pattern

Recombinant ES clones validation by Southern Blot –internal probe



Schematic Southern Blot validation strategy



Internal probe : validation of the correct insertion of a single copy at the ApoE locus

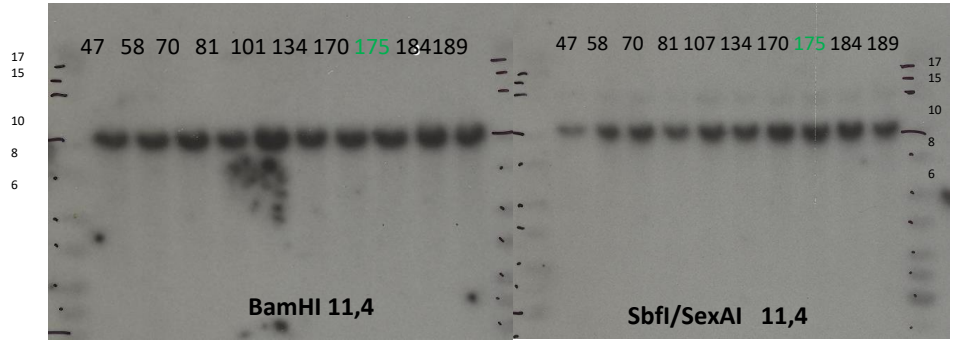
Digestions used to validate the 5'insertion

Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
Hygro	5' first digest	BamH1	/	11,4
	5' second digest	SbfI/SexAI	/	11,4

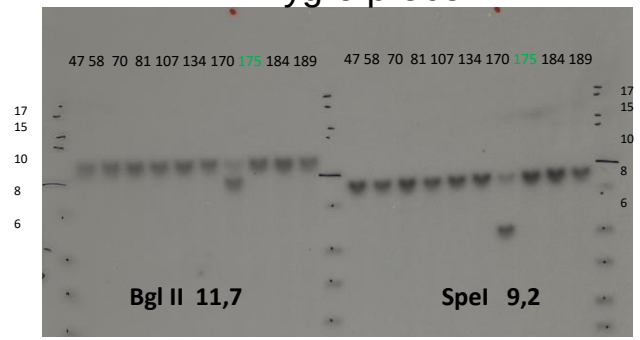
Digestions used to validate the 3'insertion

Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
Neo	3' first digest	Bgl II	/	11,7
	3' second digest	Spe I	/	9.2

Neo probe



Hygro probe



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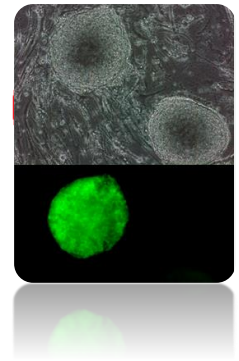
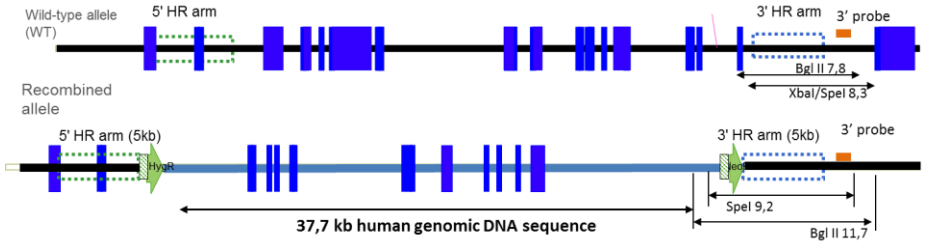
Recombinant ES clones validation by Southern Blot –External probe

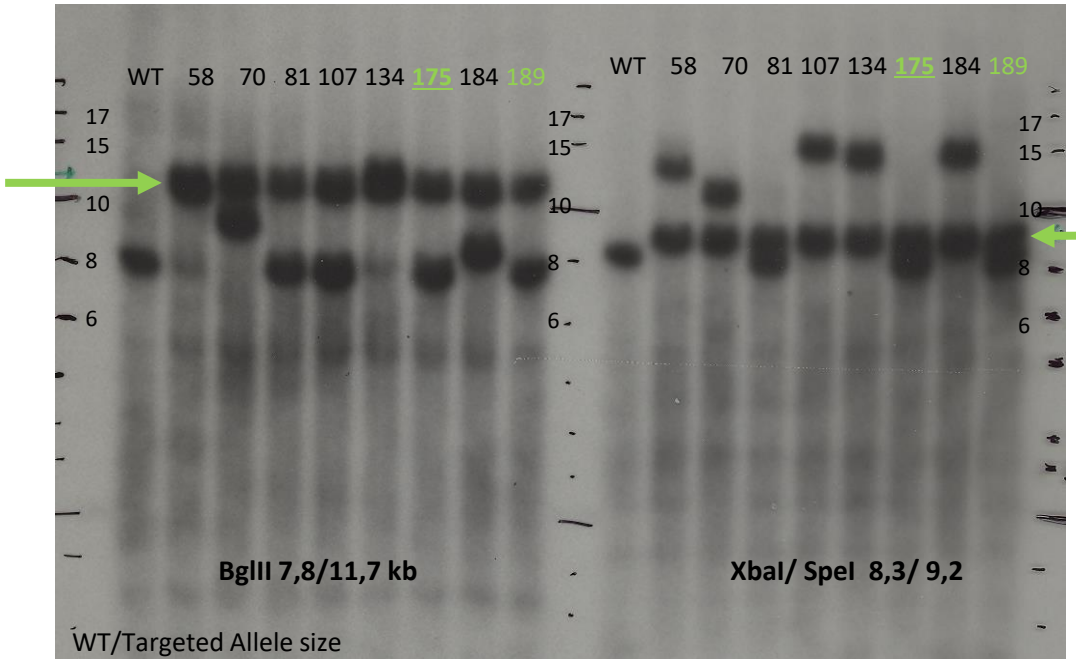


Schematic Southern Blot validation strategy



Digestions used to validate the 3' insertion

Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
3'external probe	3' first digest	BglII	7,8	11,7
	3' second digest	Xba/SpeI	8,3	9,2

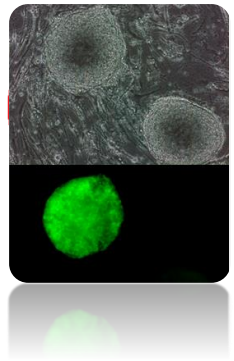


3' external probe sequence

3'probe sequence.gb

Note: As CRISPR/Cas9 was used to help to humanize the ES cells, the untargeted allele (previously wild type) could also be edited. This explains the differences in the sizes of the untargeted allele.

Aneuploidy screening in ES recombinant clones

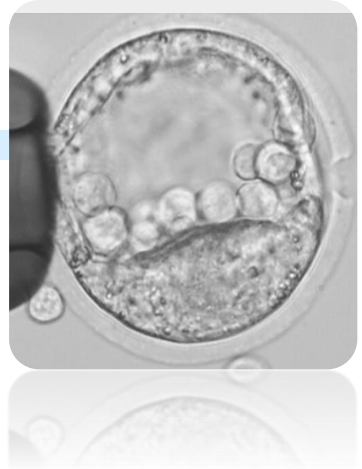


Selected recombinant ES cells clones were karyotyped by ddPCR as described in Codner *et al.* [1] and by Giemsa metaphase staining. Results of aneuploidy analysis are presented in the table below.

Clone ID	ddPCR	Giemsa
81	Pass	Pass
107	Failed	Not done
175	Pass	Pass
189	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/6N 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.
- Recombinant ES clones #81, #175 and #189 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			
	5%-40%	45% -55%	60% - 100%	Total
#81	0	0	0	0
#175	1	2	12	15
#189	5	0	1	6

Breeding to F1 generation

- Eight highly chimeric males generated in the previous phase by blastocyst injection of the ES clones were mated with Flp deleter females showing maternal contribution* to investigate whether the recombined ES cells have contributed to the germ layer.

- Black F1 pups were genotyped and F1 pups scored positive for the presence of the humanized knock-In (KI) allele were analysed by PCR and droplet digital PCR.

- Germ line transmission was obtained the : 07/05/2014

- Clone #175 was the first clone giving germ line transmission. The breeding of the chimeras issued from the 2 other clones was stopped.

- F1 males were further bred with Cre deleter females showing maternal contribution* to obtain the final allele

***Highly-efficient, fluorescent, locus directed cre and FlpO deleter mice on a pure C57BL/6N genetic background.**
Birling MC, Dierich A, Jacquot S, Hérault Y, Pavlovic G. Genesis. 2012 Jun;50(6):482-9. doi: 10.1002/dvg.20826.

ddPCR on F1 (het) animal issued from clone #175 after Flp excision of the HygroR cassette

phenomin
EXCELLENCE IN MOUSE PHENOGENOMICS

INVESTISSEMENTS D'AVENIR

REPORT REDACTION

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