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INVESTIR L'AVENIR

MODEL GENERATION TECHNICAL REPORT

Generation of mouse model : Tubg1 Y92C point mutation-conditional KO

Project code: G4583/ IR4583

Report finalized: 04/10/2023

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Université de Strasbourg
Instituts thématiques Inserm
Institut national de la santé et de la recherche médicale
CERBM gie
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MODEL GENERATION TECHNICAL REPORT

1 PROJECT PROCESS & QUALITY CONTROLS

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3 HOMOLOGOUS RECOMBINATION VECTOR CONSTRUCTION

4 ES ELECTROPORATION & SCREENING OF RECOMBINANT CLONES

5 MICROINJECTION & BREEDING

6 SEQUENCE OF THE DELIVERED ALLELE

PROJECT PROCESS & QUALITY CONTROLS

Quality controls on ES validation:

- **Long-Range PCR**
- **Southern Blot**
  - Internal Neomycin probe
  - External probes
- **ddPCR karyotyping**
- **Giemsa karyotyping**

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

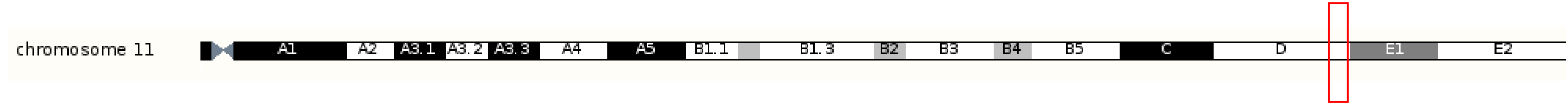
- Vector Construction ← **Sequencing**
- ES Screening ← **Long-Range PCR**
- Chimera breeding & amplification ← **Chimera genotyping** (confirm injection of the right clone & validate genotyping assays)

2 GENETIC STRATEGY

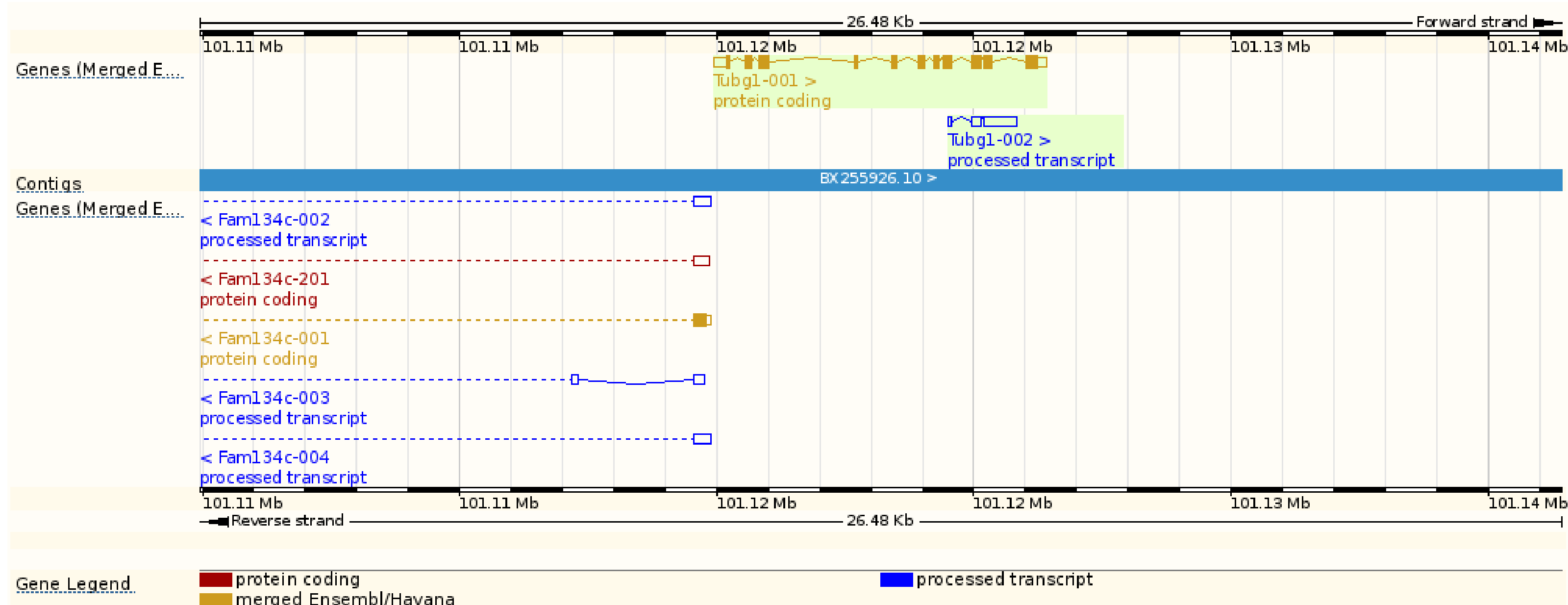
- Target locus structure
- mRNA(s) and protein(s)
- Genetic strategy
- PRO & CONS evaluation of the strategy

Tubg1 mouse genomic locus – structure

Location:



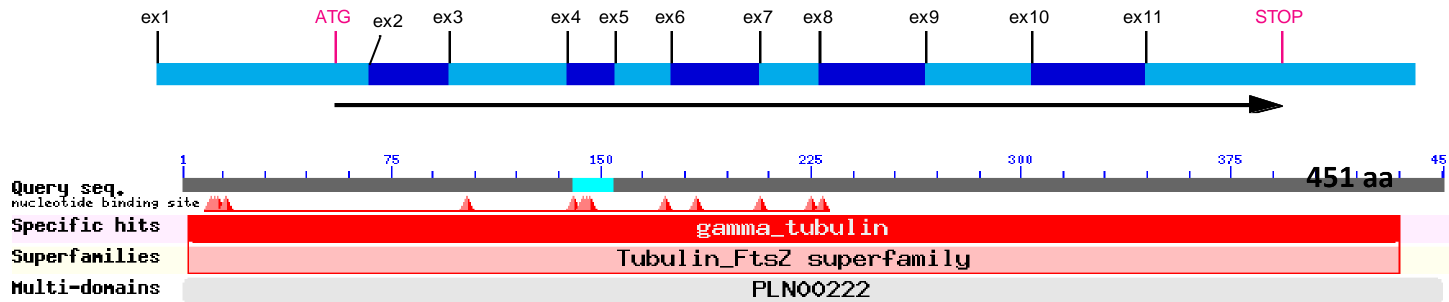
Tubg1 ENSMUSG00000035198



Tubg1 mRNAs and proteins

Name	Transcript ID	Length (bp)	Protein ID	Length (aa)	Biotype	CCDS
Tubg1-001	ENSMUST00000043680	1802	ENSMUSP00000048036	451	Protein coding	CCDS25451
Tubg1-002	ENSMUST00000137844	852	No protein product	-	Processed transcript	-

Tubg1-001 ENSMUST00000043680



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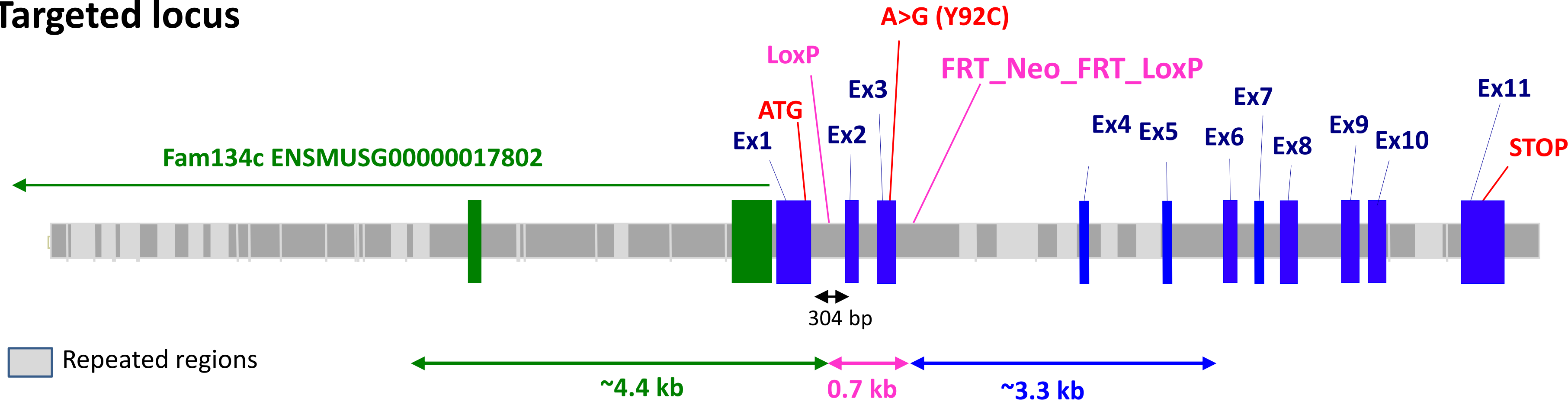
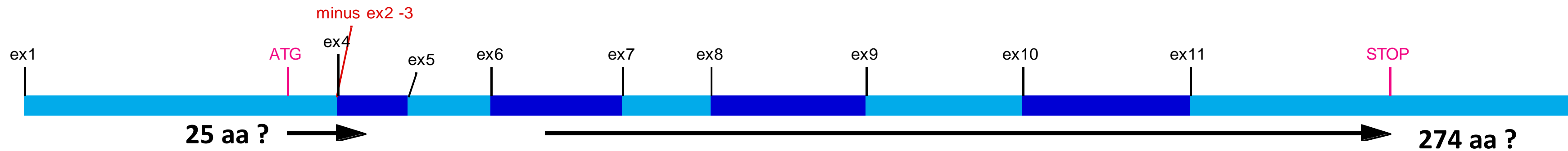
Proposition: introduction of the Y92C point mutation and flox exons 2 and 3 in Tubg1

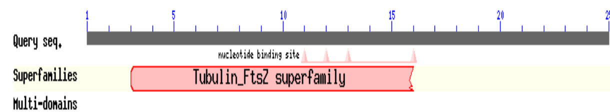
Targeted locus

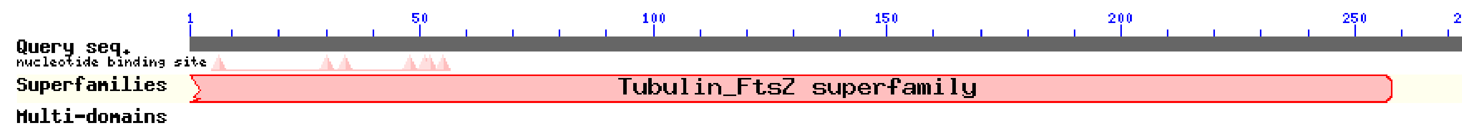


Ex2 and 3 Ensembl IDs:

mRNA and protein expressed after Cre mediated excision







Partial cDNA sequence

ATG

ex2

ATG CCG AGA GAA ATC ATC ACC CTA CAG TTG GGC CAG TGC GGC AAT CAG ATT GGG TTC GAG TTC TGG AAA CAG CTA TGT GCC
M P R E I I T L Q L G Q C G N Q I G F E F W K Q L C A

GAG CAT GGC ATC AGT CCC GAG GGC ATC GTA GAA GAG TTC GCC ACG GAG GGC ACT GAC CGA AAG GAC GTC TTT TTC TAC CAG
E H G I S P E G I V E E F A T E G T D R K D V F F Y Q

ex3

GCA GAT GAT GAG CAC TAC ATC CCC CGG GCT GTG CTG CTG GAC CTG GAG CCC CGG GTG ATC CAT TCC ATC CTA AAC TCC TCC
A D D E H Y I P R A V L L D L E P R V I H S I L N S S

A>G (Y92C)

TAT GCT AAG CTC TAC AAC CCA GAG AAC ATC TGC CTG TCG GAG CAT GGC GGA GGA GCT GGC AAC AAC TGG GCC AGC GGA TTC
Y A K L Y N P E N I C L S E H G G G A G N N W A S G F

ex4

TCC CAG GGA GAA AAA ATC CAC GAG GAC ATT TTT GAC ATC ATA GAT CGG GAG GCA GAT GGC AGT GAC AGT CTA GAG GGA TTT
S Q G E K I H E D I F D I I D R E A D G S D S L E G F

ex5

GTA CTG TGT CAT TCC ATT GCT GGG GGG ACA GGC TCT GGC TTG GGC TCC TAC CTC CTG GAA CGG CTA AAT GAC AGG TAC CCC
V L C H S I A G G T G S G L G S Y L L E R L N D R Y P

AAA AAA CTA GTG CAG ACA TAC TCT GTG TTC CCC AAC CAG GAT GAG ATG AGT GAC GTG GTG GTC CAA CCC TAC AAC TCC CTC
K K L V Q T Y S V F P N Q D E M S D V V V Q P Y N S L

CTC ACA CTA AAG AGG CTG ACC CAG AAC GCG GAC TGT GTG
L T L K R L T Q N A D C V

PROs& CONs evaluation of the strategy

Pros

- Y92C mutation introduced

Cons

- The insertion of the 5' LoxP site into a small intron (304 bps) might perturb the splicing from exon 1 to exon 2 before Cre mediated excision
- The expression of the Fam134c might be perturbed after Cre mediated excision (deletion of the promoter region)
- A peptide of 25 aa might be expressed after Cre mediated excision if RNA decay does not occur.
- A protein of at most 274 aa might be expressed after Cre mediated excision if reinitiation occurs at one of the 'in frame' ATG present in exon 6 or further exons (if RNA decay does not occur).
- Presence of repeated sequences might render PCR amplification and/or screening 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)

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.

3 HOMOLOGOUS RECOMBINATION - VECTOR CONSTRUCTION

Created by SnapGene

G4583 step3 final C5121
12 730 bp

AmpR
12 000
ExX
2000
5' arm
ExX
Ex1
ATG
4000
LoxP
Ex2
A>G (Y92C)
Ex3
Floxed arm
FRT
NeoR
6000
LoxP
FRT
8000
3' arm
Ins C
Ex4
Ex5
Ex6
10 000
Ex7
Ex8

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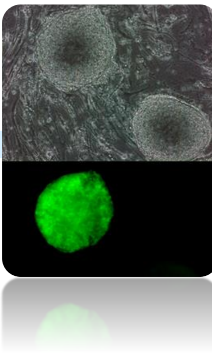
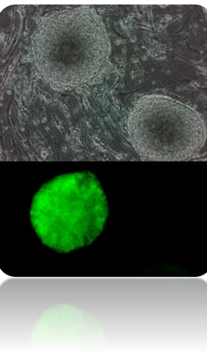
4 ES cell electroporation & Screening of recombinant clones



- 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



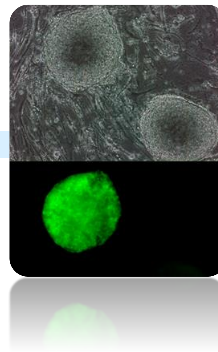
The targeting vector was electroporated in the proprietary C57BL/6N TB1 cell line.

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. Six 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

Long range PCR screening – strategy



Schematic 5’ and 3’ PCR screening strategy

Wildtype Allele (WT)

ATG

Ex X, Ex X, Ex 1, Ex 2, Ex 3, Ex 4, Ex 5, Ex 6, Ex 7, Ex 8

Fext 3.53 kb Rint

Targeting Vector

ATG

Selection cassette

Targeted Allele (HR)

ATG

Selection cassette

Fext 5.1 kb Rneo

Fneo 4.4 kb Rext

Fext 3.59 kb Rlox

Fext 3.53 kb Rint

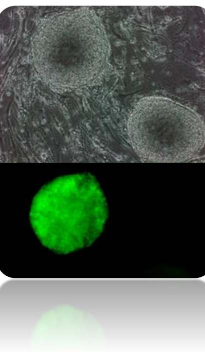
LoxP

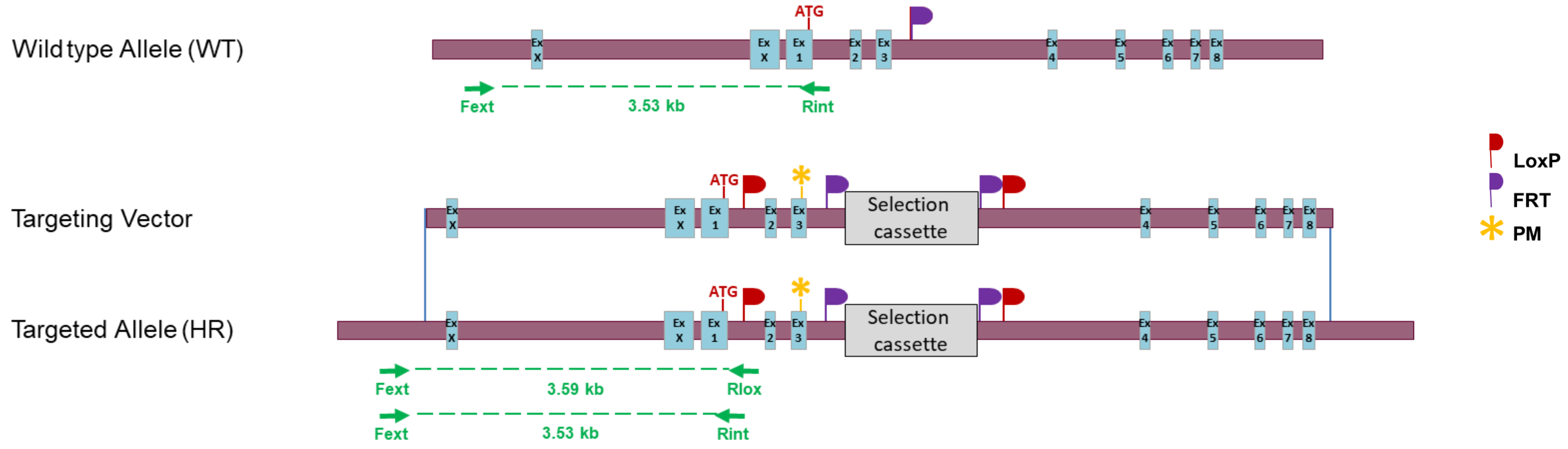
FRT

PM

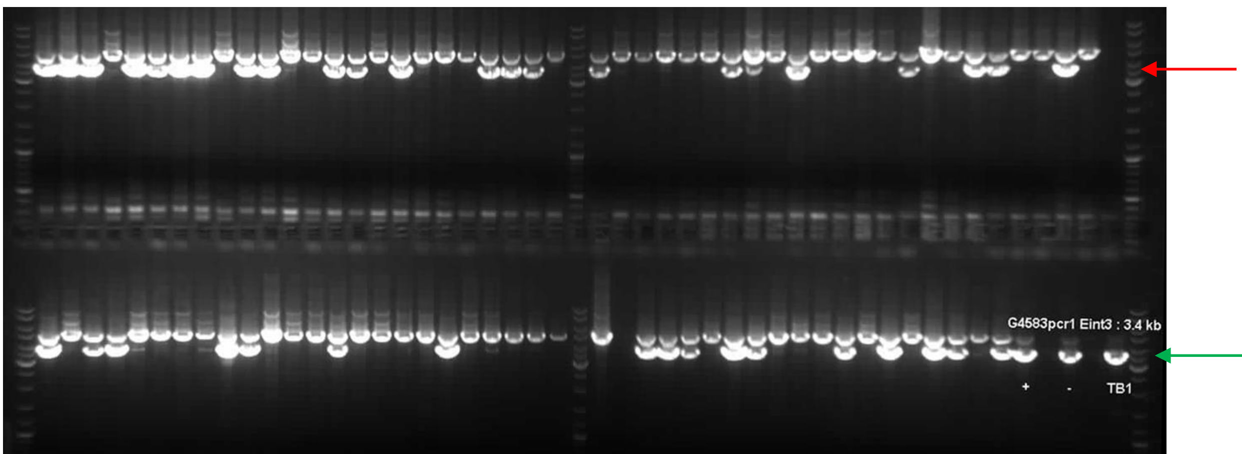
PCR	Primer Name	Primer sequences	PCR product size
5' PCR	Fext	GATGGGGGGTGGTACGCGCTCTCCC	3.59 kb
	Rlox	GTTATCTGCAGGTCGACCTTAAGCT	
5’ PCR	Fext	GATGGGGGGTGGTACGCGCTCTCCC	3.53 kb
	Rint	ACAGGGTTGGGAATCGCCTTTGTGG	
5’ PCR	Fext	GATGGGGGGTGGTACGCGCTCTCCC	5.1 kb
	Rneo	GCGGCCGGAGAACCTGCGTGCAATC	
3’ PCR	Fneo	AGGGGCTCGCGCCAGCCGAACTGTT	4.4 kb
	Rext	GAGCTATTTTATTATCCCAGTGGGC	

Long-Range 5' PCR screening – results



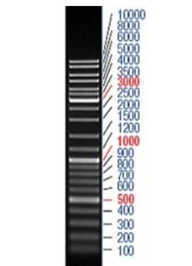


PR Fext – Rlox : 3.59 kb



+ / - / TB1 : Controls DNAs

PCR Fext – Rint : 3.53 kb



Ladder pattern

Six candidate clones out of the 40 positive clones were selected for 3' Long-Range PCR and Southern blot validation.

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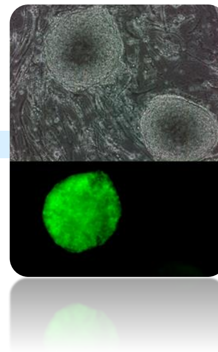
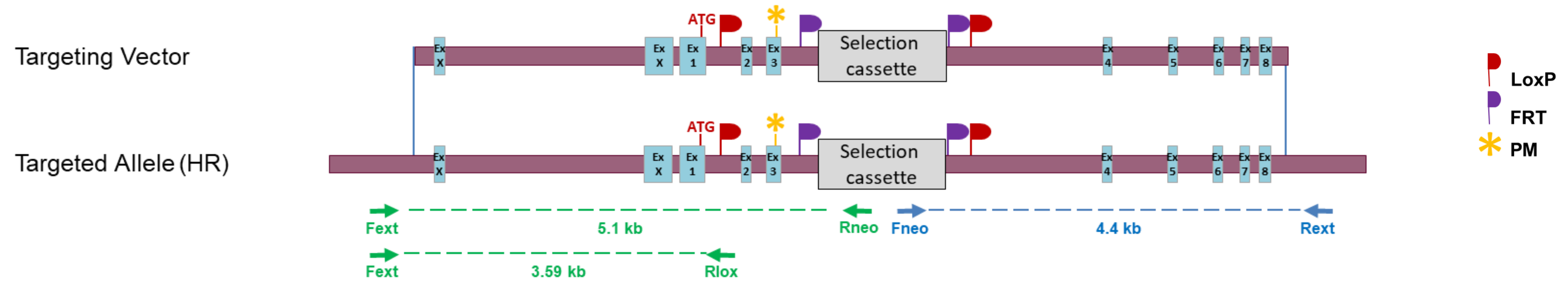
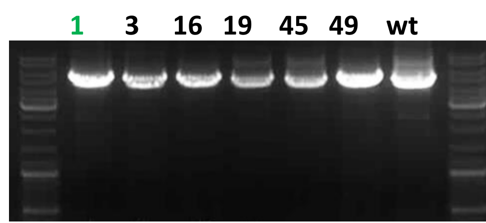
Recombinant ES validation by Long Range PCR

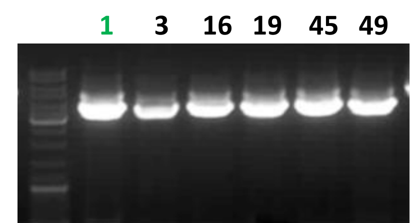


Confirmation and Validation of candidate recombinant ES clones by 5' and 3' PCRs

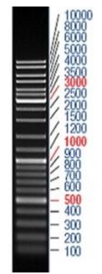




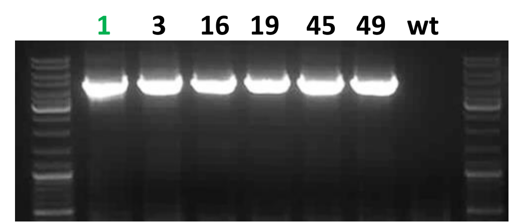
PCR Fext – Rneo : 5.1 kb



PCR Fext – Rlox : 3.59 kb



Ladder pattern



PCR Fneo – Rext : 4.4 kb

Six candidate clones identified by 5' PCR screening were further analysed by 3' PCR screening.
Six clones (clones #1, #3, #16, #19, #45 and #49) were confirmed.

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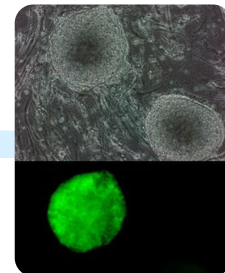
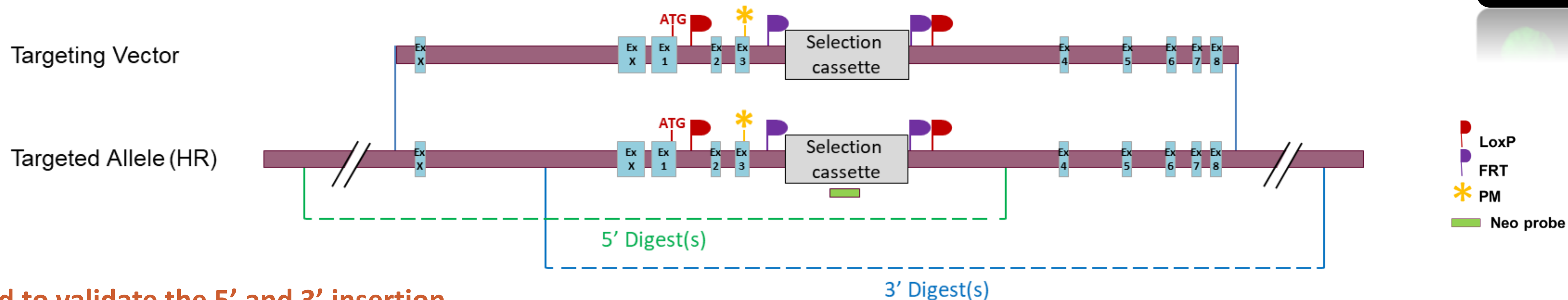
Recombinant ES clones validation by Southern Blot – Internal probe



Schematic Southern Blot validation strategy

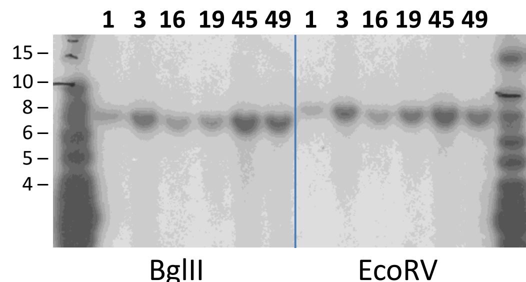
Digests on the scheme illustrate the position of the chosen restriction sites relative to the probe. They don't show the exact position of the restriction sites.



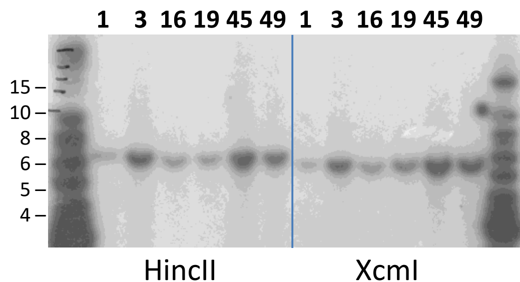
Digestions used to validate the 5' and 3' insertion

Probe		Genomic DNA digest	Targeted Allele (kb)
Neo	5' digest	BglII	7.5
		EcoRV	8.2
	3' digest	HincII	6.5
		XcmI	5.8

Southern blot - Neo 5'



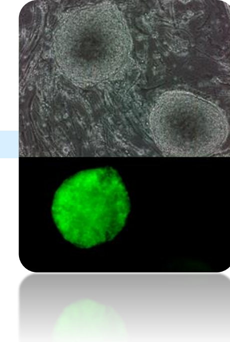
Southern blot - Neo 3'



Neo probe sequence

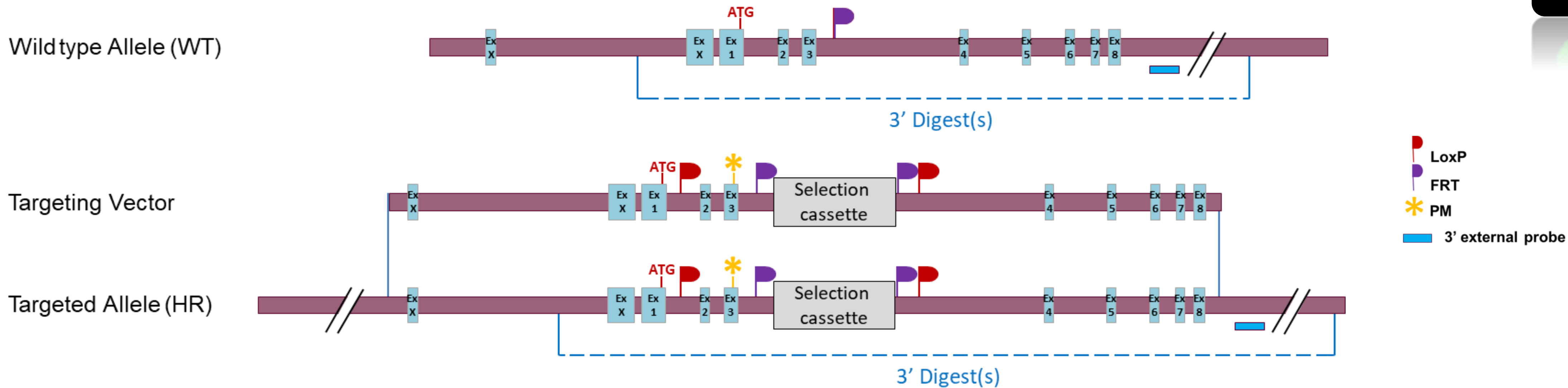
CTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTG
CTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGAT
CTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGG
CTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGA
GCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGG
CTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTC
GTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTC
ATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGAT
ATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCT
CCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTC

Recombinant ES clones validation by Southern Blot – External probe

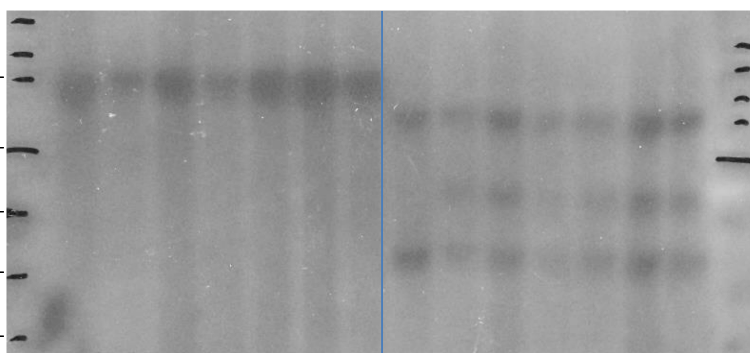


Schematic Southern Blot validation strategy

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 – 3' probe



BstEII 15.8 / 17.9 EcoRI 6.9 / 9

3' probe sequence

CCTTTGGCTCCTGCAGGAGCAGTGCCCACTGGGATAAT
AAAATAGCTCACTGCTCAAGGGCATGGGATTGGTGAGC
TGAGAGCTGTGATCCTGACCCCACGGCAGAGACTCAGC
CCAGACCTGATCTAAAGACAGTCCCCAGAGACCACGCC
ACCTGCAAGCTGATGGGGACAGAGTTGACTCTCTTCCT
CTGTCCATGCATCTCTGGGTTCCTCCCTGACTGATGAC
CAGGTCTCTGAACCCCGTTCTCTCAGGTGGCCAGTGTG
AGGAAGACAACAGTCCTGGATGTCATGAGGCGCCTGCT
ACAGCCCAAGAATGTGATGGTGTCCACAGGCCGGGATC
GTCAGACCAACCACTGCTACATCGCCATCCTCAACATC
ATCCAGG

Digestions used to validate the 5' and 3' insertion

Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
3' external probe	3' first digest	BstEII	15.8	17.9
	3' second digest	EcoRI	6.9	9

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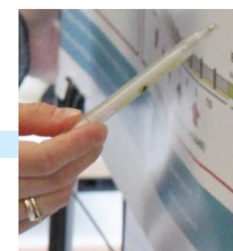
Recombinant ES clones validation –Sanger sequencing of the mutation



gcagatgatgagcactacatcccccgggctgtgctgctggacctggagccccgggtgatccattccatcctaaactcctcctatgctaagctctacaacccagagaacatctgcctgtcggagcatggcggaggagctggcaacaactgggccagcggattctc

cgtctactactcgtgatgtagggggcccgacacgacgacctggacctcggggcccactaggtaaggtaggatttgaggaggatacgattcgagatgttgggtctcttgtagacggacagcctcgtaccgcctcctcgaccgttgttgacccggtcgcctaagag

Floxed arm

Ex3

A>G (Y92C)

Move:

Aligned Sequences

Original Sequence

gcagatgatgagcactacatcccccgggctgtgctgctggacctggagccccgggtgatccattccatcctaaactcctcctatgctaagctctacaacccagagaacatctgcctgtcggagcatggcggaggagctggcaacaactgggccagcggattctc

1_G4583-sq8_G4583

GCAGATGATGAGCACTACATCCCCCGGGCTGTGCTGCTGGACCTGGAGCCCCGGGTGATCCATTCCATCCTAAACTCCTCCTATGCTAAGCTCTACAACCCAGAGAACATCTGCCTGTCGGAGCATGGCGGAGGAGCTGGCAACAACTGGGCCAGCGGATTCTC

1115 bases
vendredi 21 juin 2...

99 .. 1042 aligned
0 mismatches
4 gaps/insertions

110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260

Clone #1

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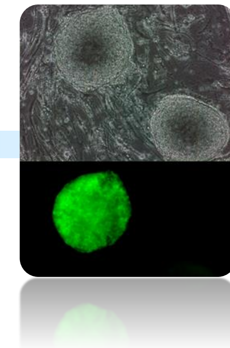
Aneuploidy screening in ES recombinant clones



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	qPCR	Giemsa
#1	**Pass**	**Pass**
#3	Pass	Pass
#16	Pass	Not done
#19	Pass	Not done
#45	Pass	Not done
#49	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/6N mouse strain (which have black coat colour). These cells were injected into blastocysts derived from an C57BL/6NTac ALBINO 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 #1 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
#1	**1**	**0**	**4**	**5**

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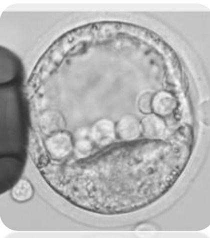
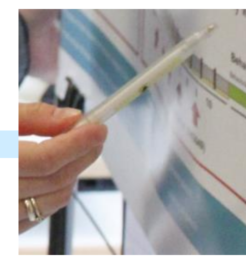
Breeding to F1 generation



- Six highly chimeric males generated in the previous phase by blastocyst injection of the ES clones were mated with WT C57BL/6N females to investigate whether the recombined ES cells have contributed to the germ layer.
- Germ line transmission was obtained the 28/05/2014.
- Allele nomenclature (following MGI guidelines) **Tubg1$^{\mathbf{tm1(Y92C)Ics}}$**
- The excision of the flipped selection marker was performed (by breeding with a Flp deleter line) for phenotyping purpose but the frozen allele is the one containing the flipped NeoR cassette.

6 SEQUENCE OF THE DELIVERED ALLELE



GAGAAGGGGCGGGGCGGGGCAAGTGCCGGAACGCCCACGCGCATGCGCAGACGCTCCGGTGCGCATTCATCAGACGGAAGAGAACACTGCGTCGAAATGCGTTCCCGGCGCTGA
GCGCGATTCGGAGCCGCGCACGCGCAACTCGCACGACTGAAGCCCGATGAAGGAGGAAATTTCATCTGCAGAACCCAGCTCCCATTGGAGCGGAGAACGATTAGGGGTGGAGCGCAT
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TCTCCCCCACAAAGGCGATTCCCAACCCTGTGGCGCGCCGCCCGGGCGGCCGGCCAAGCTTCTCGAGCTTAAGGTCGACCTGCAG**ATAACTTCGTATAATGTATGCTATACGAAGTTAT**T
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CTCTGAGTGGATGGATGGGAACAGAGTGGCTATCTTGAGGGAGAGCCTGCAGGAGCCAGAGTTGGGAGGCTTCAGGACTTGACCCATCCTGGCTTCCCTCTGACAGGCAGATGATGA
GCACTACATCCCCCGGGCTGTGCTGCTGGACCTGGAGCCCCGGGTGATCCATTCCATCCTAAACTCCTCCTATGCTAAGCTCTACAACCCAGAGAACATCT**G**CCTGTCGGAGCATGGCGG
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CCTTGGCTGTCCTGGAACTCACTTTGTAGACCAGGCTGGCCTCAAACAGAGATCCACCTGCCTCTGCCTCCTAAATGGCGAGGTTAAAGGTGTGCTCCGCCATTGCCCAGCTAATATGCA
ATTTATGAT

LoxP FRT Exons 2 & 3 A>G (Y92C)

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INVESTIR L'AVENIR

REPORT REDACTION & VALIDATION
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EXCELLENCE IN MOUSE PHENOGENOMICS

Genotyping protocol

Project Tubg1

(PHENOMIN-ICS reference IR00004583 / G4583)

This report has been **prepared** by: Amélie JEANBLANC

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Head of Genotyping Service

The first version of this report was finalized the: 25 Oct 2013
The last update of this report was done the: 29 Aug 2017

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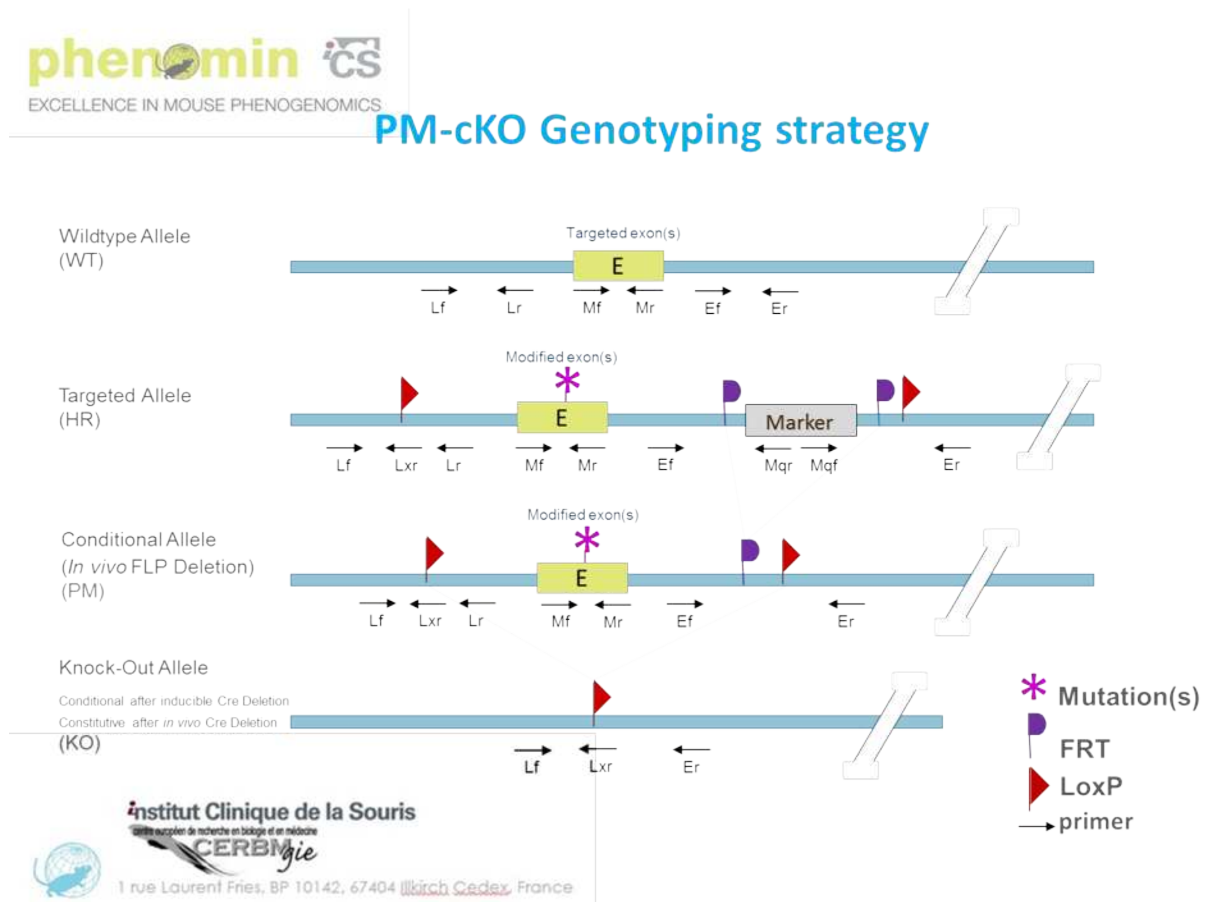
1. Genotyping protocol and data

This section describes the condition used at the Mouse Clinical Institute (ICS) to genotype your **Tubg1** Conditional Point mutation / Knockout (PM-cKO) 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[2]	7298	GGTCAGAGATGTTCGAGTGCTGAG
Ef	7297	GGAACGCTAAGGACGCAGTCTAG
Er[2]	7300	GAGAGGCCTGAGTGCTGACAATAAG
Er	7299	GCCTTCCTTCTCCATTGCGCATTG
Lf[2]	7293	CAGTGCGGCAATCAGAGTGAGC
Lf	7292	CCTCGCAATAAGGTCATAGCTTCGG
Lr	7294	GGTAGGTTCCAAATAGCCAGTGGG
Lxr	5049	CATACATTATACGAAGTTATCTGCAG
Mf	7295	GTGATCCATTCCATCCTAAACTCCTC
Mq1f	6	GAAGAACGAGATCAGCAGCCTCTGTTCC
Mq1r	265	TGCTAAAGCGCATGCTCCAGACTGC
Mr	7296	CTGTGCCCACCCACAGAGTTGAG

[2]: for a selected position, a second primer was designed

PCR fragments expected size (bp):

Region analyzed	Primers used	Position on the primer *(see the map above)*	Targeted allele (HR)	PM allele	KO allele	WildType allele (WT)
WildType / Mutated alleles	7295-7296	Mf / Mr	163	163	---	163
Presence of the distal loxP	7293-7294	Lf[2] / Lr	370	370	---	274
Excision of the selection marker	7298-7300	Ef[2] / Er[2]	2268*	414	---	310
5' part of the selection marker	7297-265	Ef / Mq1r	192	---	---	---
3' part of the selection marker	6-7299	Mq1f / Er	318	---	---	---
LoxP specific PCR	7293-5049	Lf[2] / Lxr	219	219	219	---
Excision of the floxed exon(s), i.e. knock out	7292-7299	Lf / Er	3099*	1245*	311**	1045*

*: 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

You will find the genotyping protocol in the publication:

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. Epub 2012 Mar 20.