



R418W KI in Adcy5 by CRISPR-Cas9 Final report

Kus6795 / IM6795

by Marie-Christine Birling (PhD)

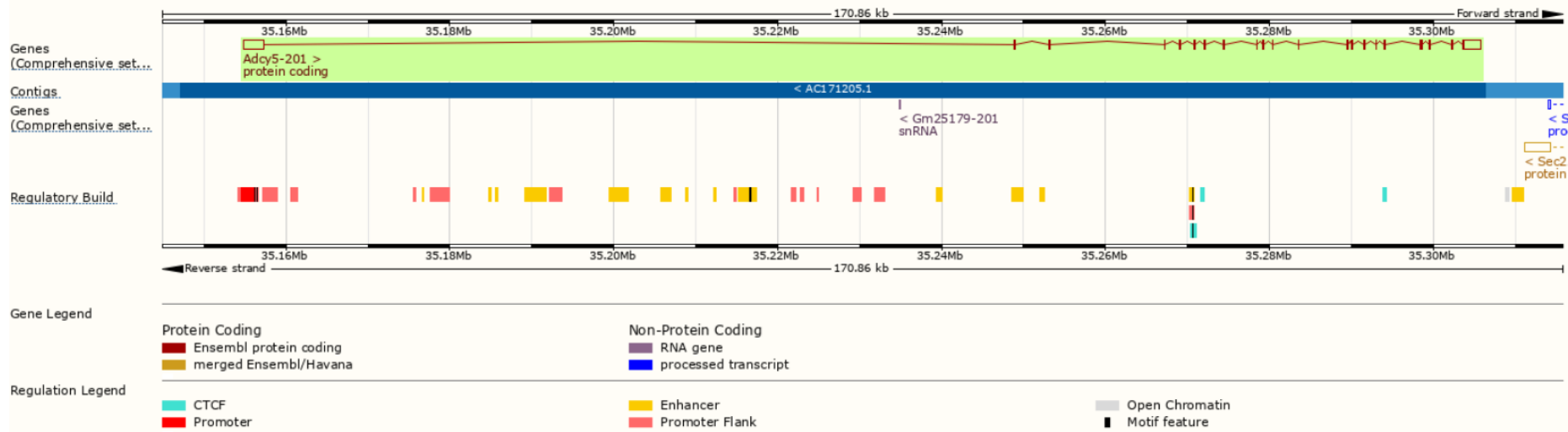
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08/01/2018

Adcy5 Mouse Genomic locus



Gene: Adcy5 ENSMUSG00000022840



Chromosome 16: 35,154,877-35,305,737

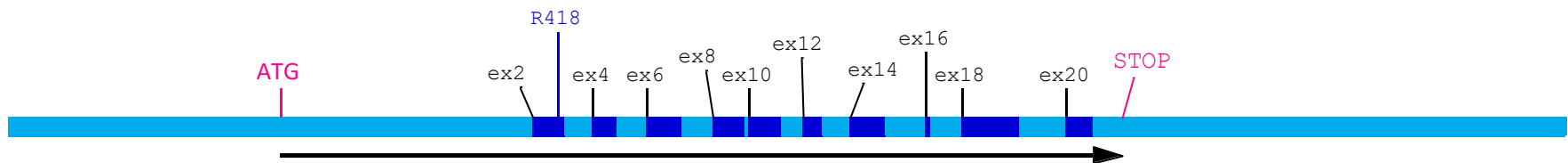


Adcy5 mRNA and protein



Name	Transcript ID	bp	Protein	Biotype	CCDS	UniProt	RefSeq	Flags
Adcy5-201	ENSMUST00000114913.1	7007	1262aa	Protein coding	CCDS37322	P84309	NM_001012765 NP_001012783	TSL:1GENCODE basicAPPRIS P

Adcy5-201 ENSMUST00000114913.1



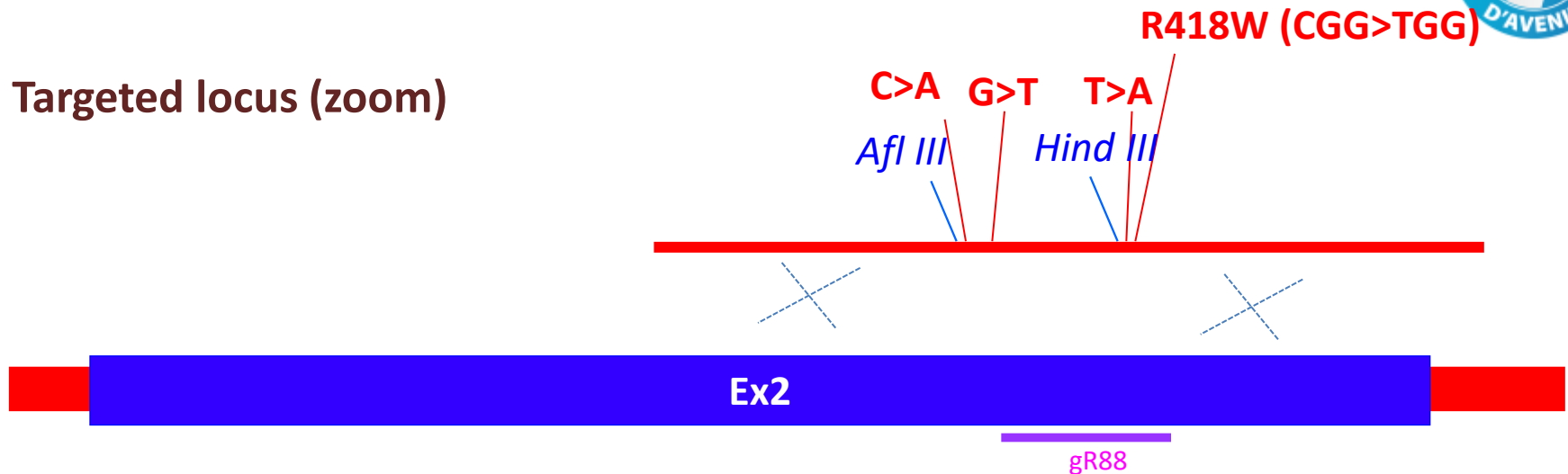
<http://crispor.tefor.net/crispor.py?batchId=JhHiZ9KWmTK04EqOxMnJ>

Position/ Strand ⓘ	Guide Sequence + PAM + Restriction Enzymes ⓘ + Variants ⓘ ☐ Only G- ☐ Only A- ⓘ	Specificity Score ⓘ	Predicted Efficiency ⓘ Show all scores Doench '16 Mor.-Mateos		Out-of- Frame score ⓘ Click on score to show micro- homology	Off-targets for 0-1-2-3-4 mismatches + next to PAM ⓘ	Genome Browser links to matches sorted by CFD off-target score ⓘ ☐ exons only ☐ chr16 only
27 / rev	GCCGAGCCTGGATACACTCC CGG Enzymes: BmeT110I, MspI, BcoDI, BseDI, NciI, BspTNI, StyD4I, Cfr9I Cloning / PCR primers	88	48	47	41	0-0-0-7-56 0-0-0-1-1 63 off-targets	4:intron:Rassf3 4:intron:Tbc1d14 4:intron:Lrrc16a show all...
57 / rev	CCTGTTGCTGGTTCCTCCGC TGG Enzymes: FaeI, MspA1I, SsiI Cloning / PCR primers	86	36	25	34	0-0-0-14- 114 0-0-0-0-0 128 off-targets	3:intron:Cacna2d2 4:intron:Spsb1 4:intron:Tbc1d22a show all...
41 / fw	GGAGACCCGGGAGTGTATCC AGG Enzymes: StyD4I, BstNI, BciVI Cloning / PCR primers	85	50	35	45	0-0-2-7-83 0-0-2-0-1 92 off-targets	4:intron:Tank 4:intergenic:4732491K20Rik-Plg 4:intergenic:Hist1h2al-51pr3 show all...

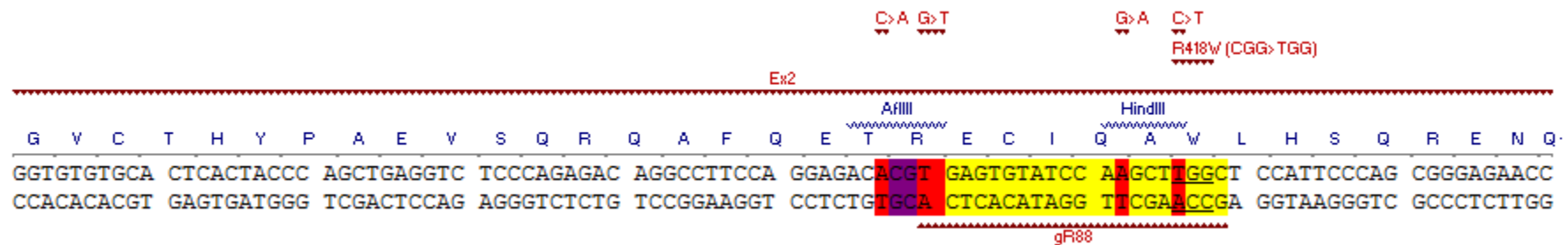
Strategy proposed: insertion of the R418W mutation



Targeted locus (zoom)



Sequence obtained



Introduction of diagnostic restriction sites at the site of the expected mutation (HindIII, will ease the genotyping) and a silent/diagnostic mutation (BsaAI and AflIII) to mutate the PAM sequence, avoid recutting after homologous directed repair)

Sequence of the mutated cDNA (partial sequence)



GCAAGGACACCTCTCCGTCCTCCGCTGCTCTCTCAACTCCAGCCTGAGCTCGACGCGAGCCAGGAGTCCGGACATCTCTGCTCCGGCGCAGCAGTCAGAGCTGTCCCACTGCCACCGCTCGGAGCCAACTCCGCTCTCGGACCTGGGACTCTGGACTCTAATTC
AGCTACTTCTCACTCCGCGGTGCGCTGTCTCCCGCTAGCCTTCCCGTTGTCTCTCCACGCTCAGGACGGGGTGCCACGATGTCCCGCTGCTGCGCAGGGCCCCGGGGCTCCCTCGACGTGTGACCTAGCCTGGTCCCCCTGCTTGGCTGTCCGCGCTCTCT

ATG

TGGAGACCCCCGGCCCCGGCCCCGGGGGAAGAGGAAGAAGACGACGAGGCCGAGGGGGG ATG TCC GGC TCC AAA AGC GTG AGC CCC CCG GGC TAC GCT GCA CAG ACA GCG GCG TCG CCA GCG CCC CGG GGA GGC CCG
▶ M S G S K S V S P P G Y A A Q T A A S P A P R G G P
GAG CAT CGC GCC GCT TGG GGA GAA GCC GAT TCC CGC GCC AAT GGC TAC CCC CAC GCC CCC GGG GGA TCA ACC CGC GGC TCC ACC AAG ACA TCT GGG GGA GCG GTG ACC CCA CAA CAG CAG CAG
▶ E H R A A W G E A D S R A N G Y P H A P G G S T R G G S T K R S G G A V T P Q Q Q Q
CGC CTG GCC AGC CGT TGG CGC GGT GGC GAT GAC GAG GAA GAC CCT CCA CTA AGC GGT GAT GAC GGT CTG GCT RGR GGC TTC CGC TCT AGC TTC CCG TCT TGG CAG CAG CCG GGT
▶ R L A S R W R G G D D D E D P P L S G D D P L A G G F G F S F R S K S A W Q E R G
GGC GAC GAC GGC GGT CGC GGC AGC AGG CGG CAG CGG CGG GGC GCG GCT GGA GGG GGC AGC ACC CGG GCG CCC CTT GCG GGC GGC AGC GGC AGT TCG GCG GCG GCC GCA GCG GCC GCA GGT GGC
▶ G D D G G R G S R R Q R R G A A G G G S T R A P A G G S S A A A A A A A G G
ACA GAG GTG CGC CCC CGC TCG GTG GAG CTG GGC CTG GAG GAG CGT CGA GGA AAA GGC CGA GCG GCC GAG GAG CTG GAG CCC GGG ACT GGC ATC GTC GAG GAT GGA GAC GGG TCG GAG GAT GGA
▶ T E V R P R S V E L G L E E R R G K G R A A E L E P G T G I V E D G D G S E D G
GGC AGT TCT GTG GCG TCA GGC TCT GGG ACC GGC GCG GTG CTG TCG TGT GGC GCC TGC TGC CTG GGC TTT CTG CAG ATA TTC CCG TCT AAG AAG TTC CCG TCG GAC AAA CTG GAG CAG CTG TAC
▶ G S S V A S G S G T G A V L S L G A C C L A L L Q I F R S K K F P S D K L E R L Y
CAG CGC TAC TTC TCC CGC CTG AAC CAG AGC AGT CTC ACC ATC CTC ATG GCC GTG CTG GTG CTT GTG TGC CTG GTC ATG CTG GCT TTC CAG GCG GCG CGC CCC CCG CTC CAG ATA GCC TAC CTG
▶ Q R Y F F R L N Q S S L T M L M A V L V L V C L V M L A F H A A R P L Q I A Y L
GCG GTG TTG GCA GCT GCT GTG GGC GTG ATC CTT ATC ATG GCC GTG CTC TGC AAC CGT GCA GCG TTC CAC CAG GAC CAC ATG GGC CTG GCC TGC TAT GCG CTC ATT GCA GTG GTG CTC GCC GTC
▶ A V L A A A V G V I L I M A V L C N R A A F H Q D H M G L A C Y A L I A V V L A V
CAG GTA GTG GGC CTG TTG CTG CCA CAG CCA GCG AGC GCC TCC GAG GGC ATC TGG TGG ACC GTG TTC TTC ATC TAT ACC ATC TAC ACC CTG CTG CCT GTG GCG ATG AGG GCT GCG GTG CTC AGC
▶ Q V V G L L L P Q P R S A S E G I W W T V F F I Y T I Y T L L P V R M R A A V L S

ex2

GGG GTG CTT CTG TCG GCT CTC CAC TTG GCC ATC TCT CTG CAC ACC AAC TCC CAG GAC CAG TTT CTG CTG AAA CAG CTT GTC TCC AAT GTC CTC ATC TTC TCC TGC ACC AAC ATT GTG GGT GTG
▶ G V L L S A L H L A I S L H T N S Q D D Q F L L A K Q L V S N V L I F S C T N I V G V

C>A G>T

G>A C>T

TGC ACT CAC TAC CCA GCT GAG GTC TCC CAG AGA CAG GCC TTC CAG GAG ACA CGT GAG TGT ATC CAA GCT TGG CTC CAT TCC CAG CGG GAG AAC CAG CAA CAG GAG CGT CTC CTG CTG TCT GTC
▶ C T H Y P A E V S Q R Q A F Q E T R E C I Q A W L H S Q R E N Q Q Q E R L L L S V

ex4

CTT CCC CGT CAT GTT GCC ATG GAG ATG AAA GCA GAC ATC AAC GCC AAG CAG GAG GAT ATG ATG TTC CAC AAG ATC TAC ATC CAG AAG CAT GAC AAT GTG AGC ATC CTG TTT GCT GAT ATC GAG
▶ L P R H V A M E M K A D I N A K Q E D M M F F H K I Y I Q K H D N V S I L F A A D I E
GGC TTC ACC AGC CTG GCT TCC CAG TGT ACT GCC CAA GAA CTG GTC ATG ACC CTC AAT GAG CTC TTC GCC CGC TTT GAC AAG TTG GCT GCG GAG AAT CAC TGT TTA CGG ATT AAG ATC CTC GGG
▶ G F T S L A S Q C T A Q D E L V M T L N E L F A R F D K L A A E N H C L R I K I L G

ex6

GAT TGT TAC TAC TGC GTC TCG GGG CTG CCT GAA GCC AGA GCC GAC CAT GCC CAC TGC TGC GTG GAG ATG GGA ATG GAC ATG ATC GAG GCC ATC TCG TTG GTC CGG GAG GTG ACA GGG GTG AAC
▶ D C Y Y C V S G L P E A R A D H A H C C V E M G M D M I E A I S L V R E V T G V N
GTG AAC ATG CGC GTG GGA ATT CAC AGC GGG AGA GTA CAC TGC GGT GCT CTT GGT CTC AGA AAG TGG CAA TTC GAC TGC TGT TCT AAC GAT GTC ACT CTG GCC AAC CAC ATG GAA GGT GGT GGC
▶ V N M R V G I H S G R V H C G V L G L R K W Q F D V W S N D V T L A N H M E A G G
AAG GCG GGC CGC ATC CAC ATC ACC AAG GCC ACA CTC AAC TAC CTG AAT GGG GAC TAT GAG GTG GAA CCA GGC TGT GGT GGC GAT CGC AAT GCC TAC CTC AAG GAG CAC AGC ATT GAG ACC TTC
▶ K A G R R I H I T K A T L N Y L N G D Y E V E C P G G G G D R N A Y L K E H S I E T F

ex8

CTC ATC CTG AGC TGT ACC CAG AAG CGG AAA GAA GAG AAG GCC ATG ATC GCC AAG ATG AAC CGC CAG AGA ACC AAC TCC ATC GGA CAC AAT CCG CCT CAC TGG GGA GCC GAG CGC CCC TTC TAC
▶ L I L S C T Q K R K E E K A M I A K M N R Q R T N S I G H N P P H W G A E R P F Y

ex10

AAC CAC TTG GGC GGC AAC CAG GTG TCA AAG GAG ATG AAG AGG ATG GGC TTT GAG GAC CCC AAG GAC AAG AAT GCC CAG GAA AGT GCG AAC CCA GAG GAT GAA GTG GAC GAG TTT CTG GGT CGG
▶ N H L G G N Q V S K E M K R M G F E D P K D K N A Q E S A N P E D E V D E F L G R
GCC ATC ATG GCC AGG AGC ATC CAG AGA CTG CGA TCT GAA CAC GTC CGC AAG TTC CTC CTG ACC TTC AGG GAG CCC GAC TTA GAG AAG AAC TAC TCC AAG CAG GTG GAT GAC CGA TTT GGT GCC
▶ A I D A R S I D R L R S E H V R K F L L T F R E P D L E K K Y S K Q V D D R F G A

ex12

TAT GTG GCC TGC GGC TCG CTT GTT TTC CTC TTC ATC TGC TTT GTC CAG ATC ACC ATT GTG CCC CAC TCC CTG TTC ATG CTG AGT TTC TAC CTG TCG TGT TTC CTG CTG GGC TTC GTG GTG
▶ Y V A C A S L V F L F I C F V Q I T I V P H S L F M L S F Y L S C F L L L A L V V
TTT GTG TCT GTG ATC TAT GCC TGT GTG AAG CTT TTC CCC ACT CCC CTG CAG ACA CTC TCC AGG AAG ATA GTG CGA TCC AAG AAG AAC AGC ACC CTG GTC GGG GTA TTC ACC ATC ACC CTG GTG
▶ F V S V I Y A C V K L F P T P L Q T L S R K I V R S K K N S T L V G V F T I T L V

Strategy proposed: insertion of the R418W mutation



■ Pros

- As asked
- Insertion of silent diagnostic restriction sites (will ease genotyping and avoid recutting after HDR)

■ Cons

- The sgRNA efficiency of double strand break cannot be anticipated (even if the capacity to generate DSB is tested *in vitro*)
- Efficacy is highly variable from one target sequence to another



Results

Microinjection

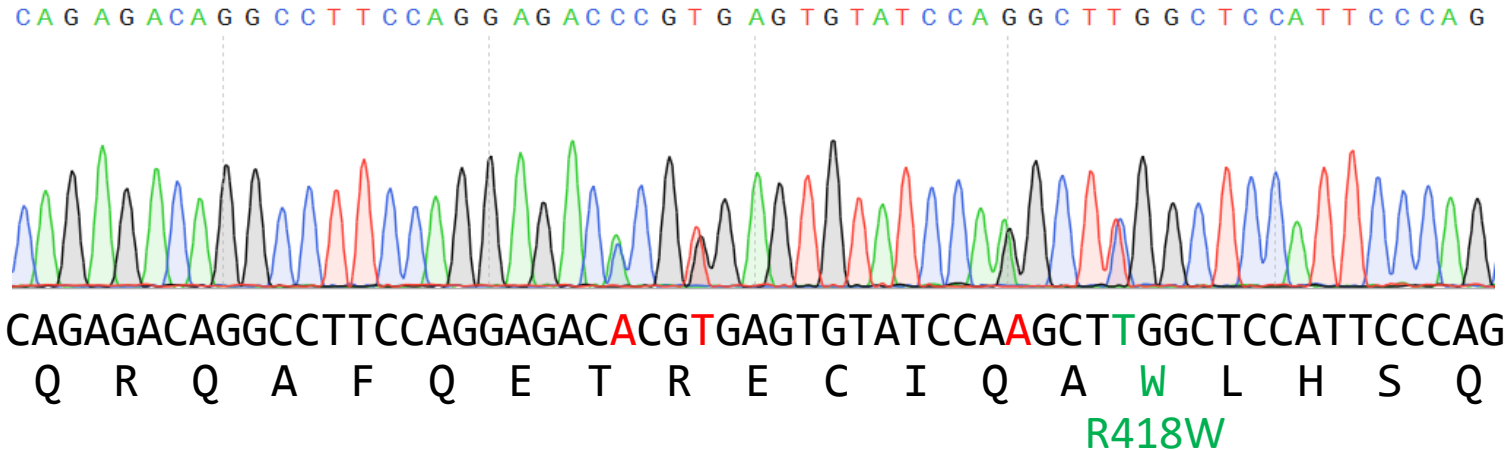


Injection session	Zygote background	# eggs reimplanted	# pups born	# Interesting founders	
21/11/2017	C57BL/6N	595	75	4 M	4 F

Line Kus6795-12-PM



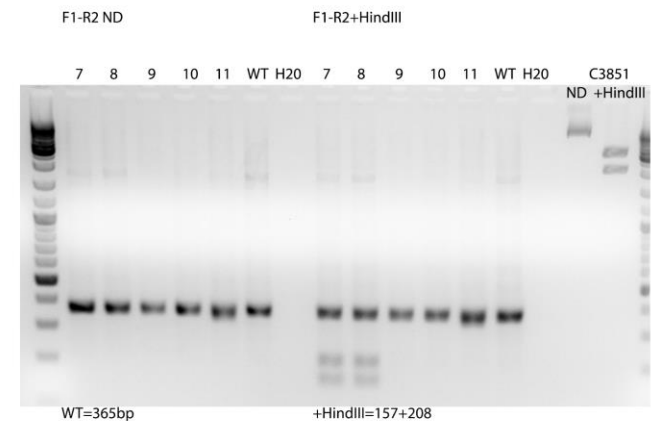
Sanger sequencing



FO	Nb F1 born	F1 heterozygote for the PM	
		M	F
Kus6795-12	30	3	2

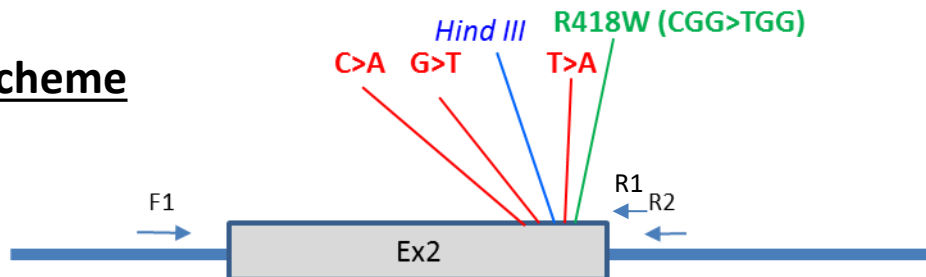
This line was created, cryopreserved and phenotyped

F1 genotyping



Genotyping protocol

Genotyping Scheme



Sequence PCR fragment F1-R2 (F1-R1)

CAGGCCCGCTGTGTTCTGAGTGTCTTGTCTTCTCCCTCTGCAGCTTGTCTCCAATGTCCTCATCTTCTCCTGCACCAACATTGTG
GGTGTGTGCACTCACTACCCAGCTGAGGTCTCCAGAGACAGGCCTTCCAGGAGACACGTGAGTGTATCCAAGCTGGCTCCATTC
CCAGCGGGAGAACCAGCAACAGGTGAGTGAAGAGGAGCCAGGAGGCCTGGATGTCAGCCAAGGCCTGCTGCAGACACACAAGCAAA
CGGCTGGCTTGCATCTGCCTGCCTCTCCCCCAGGCCATGCTCATCCCTCTGCCAGTCCCTCCTCCTAGCAGTATTTCCTGCAG
GGAGCCCGACTCTGTGGTTCC

PCR genotyping strategy

Primer ref.	Sequence	Amplification product size WT	Sizes observed if AclI restriction present (associated with asked PM)
F1	CAGGCCCGCTGTGTTCTGAGTGTCT	319 bps	159 bps + 160bps
R1	GGACTGGGCAGAGGGATGAGCATGG		
F1	CAGGCCCGCTGTGTTCTGAGTGTCT	365 bps	159 bps + 206 bps
R2	GGAACCACAGAGTCGGGCTCCCTGC		

PCR Protocol

This section describes the composition of the mix and the cycling conditions used for genotyping F0 and F1 genotyping.

Reagents:

- Phusion HS (Thermo Scientific) 5X Buffer
- 10mM dNTP
- 5' primer (100 μ M)
- 3' primer (100 μ M)
- DNA (lysate 1/10)
- Phusion Hot Start II
- Sterile H₂O

Volume (per sample):

4 μ l
0,4 μ l
0.1 μ l
0.1 μ l
2 μ l
0,2 μ l
up to 20 μ l

Cycling conditions

Temp	Time	#Cycles
96°C	5min	1
96°C	8s	
62°C	10s	30
68°C	45s	
68°C	5min	1
12°C	5min	1

Digestion protocol

Volume / sample

PCR product	10 μ l
Buffer 10X	2 μ l
Restriction enzyme	0.2 μ l
H ₂ O	7.8 μ l

This reaction is incubated 15 mins at 37°C then leaded on a 3% agarose. The 10 μ l left over PCR reaction serves as negative control