



## M509T PM in the Lhcgr

## Genotyping protocol

**Kus6777 / IM00006777**

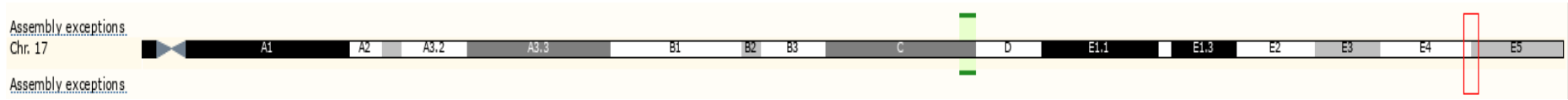
by Dr Marie-Christine Birling ([birlingm@igbmc.fr](mailto:birlingm@igbmc.fr))

# Strategy proposal

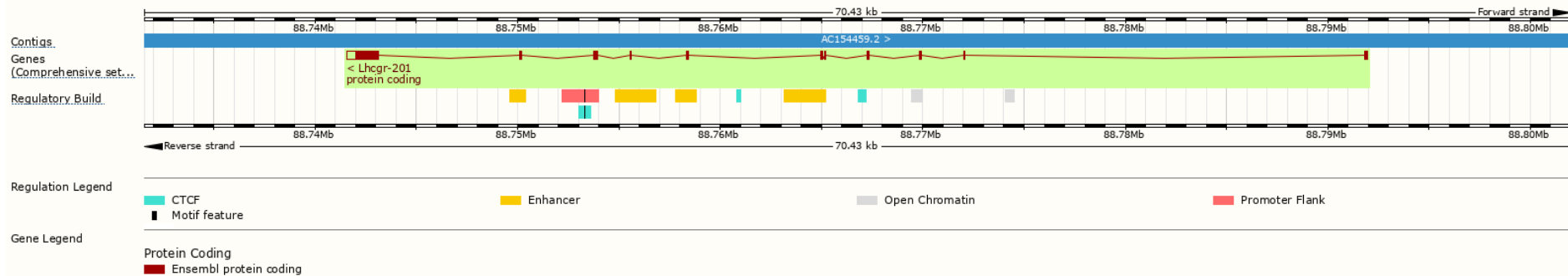
# Lhcgr Mouse Genomic locus



Chromosome 17: 88,741,549-88,791,976



Gene: Lhcgr ENSMUSG00000024107



# NOM mRNAs and proteins



Name	Transcript ID	bp	Protein	Biotype	CCDS	UniProt	RefSeq
Lhcgr-201	<a href="#">ENSMUST00000024916.5</a>	2553	<a href="#">700aa</a>	Protein coding	<a href="#">CCDS29025</a>	<a href="#">P30730</a>	<a href="#">NM_013582</a> <a href="#">NP_038610</a>

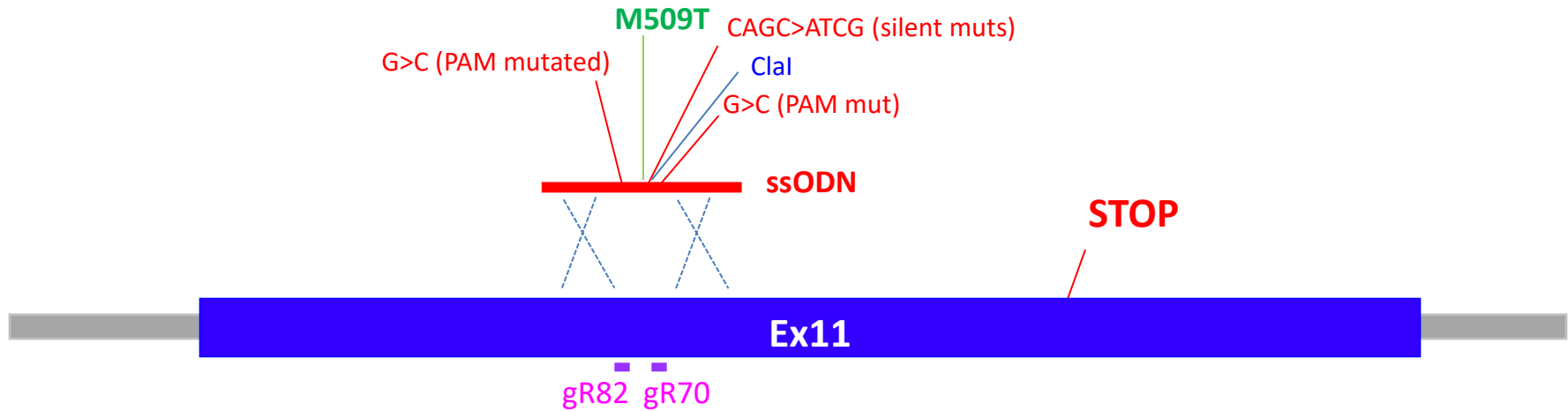
## Lhcgr-201 ENSMUST00000024916.5



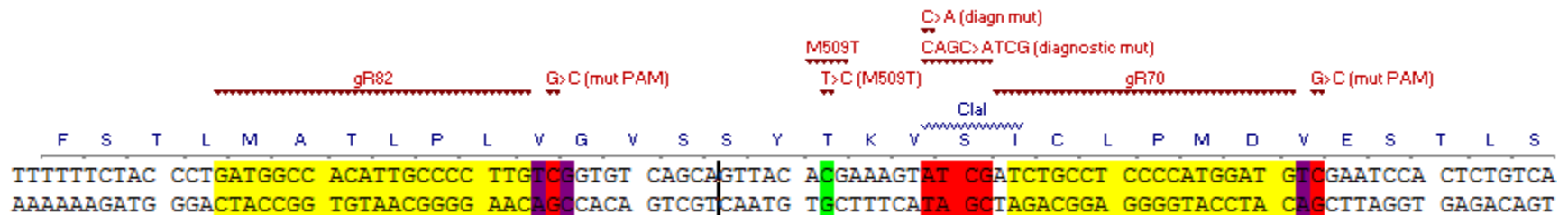
# Strategy proposed: M509T point mutation



## CRISPR/cas9 approach



## Sequence of the homology directed repair (HDR) resulting allele



# mRNA sequence after HDR



ATG

GGGCC ATG GGG CGG CGG GTC CCG GCT CTG AGA CAG CTG CTG GTG CTG GCA ATG CTG GTG CTG AAG CAG TCA CAG CTG CAC TCT CCA GAG TTG TCA GGG TCG CGC TGC CCT  
▶ M G R R V P A L R Q L L V L A M L V L K Q S Q L H S P E L S G S R C P

ex2

GAG CCC TGC GAC TGC GCG CCG GAT GGT GCC CTG CGC TGC CCT GGC CCT CGA GCT GGC CTC GCC CGA CTA TCT CTC ACC TAT CTC CCT GTC AAA GTA ATC CCA TCA CAA  
▶ E P C D C A P D G A L R C P G P R A G L A R L S L T Y L P V K V I P S Q

ex4

GCT TTC AGG GGA CTT AAT GAG GTC GTA AAA ATT GAA ATC TCT CAG AGT GAT TCC CTG GAA AGG ATA GAA GCT AAT GCC TTT GAC AAC CTC CTC AAT CTG TCT GAA ATA  
▶ A F R G L N E V V K I E I S Q S D S L E R I E A N A F D N L L N L S E I  
CTG ATC CAG AAC ACC AAA AAC CTG CTA TAC ATT GAA CCC GGT GCT TTT ACA AAC CTC CCT CGG TTA AAA TAC CTG AGC ATC TGT AAC ACA GGC ATC CGG ACC CTC CCA  
▶ L I Q N T K N L L Y I E P G A F T N L P R L K Y L S I C N T G I R T L P

ex6

GAT GTT TCG AAG ATC TCT TCC TCT GAA TTT AAT TTC ATT CTG GAA ATC TGT GAT AAC TTA TAC ATA ACC ACC ATA CCA GGG AAC GCT TTC CAA GGG ATG AAT AAT GAG  
▶ D V S K I S S S E F N F I L E I C D N L Y I T T I P G N A F Q G M N N E

ex8

TCC ATC ACG CTG AAA CTG TAT GGA AAT GGG TTT GAA GAA GTA CAA AGC CAT GCA TTC AAT GGG ACG ACG CTA ATC TCG CTG GAG TTA AAA GAA AAC ATC TAC CTG GAG  
▶ S I T L K L Y G N G F E E V Q S H A F N G T T L I S L E L K E N I Y L E  
AAG ATG CAC AGT GGC ACC TTC CAG GGG GCC ACG GGG CCC AGC ATC CTG GAT GTC TCT TCC ACC AAA TTG CAG GCC CTG CCG AGC CAC GGG CTG GAG TCC ATT CAG ACG  
▶ K M H S G T F Q G A T G P S I L D V S S T K L Q A L P S H G L E S I Q T  
CTC ATC GCC ACG TCA TCC TAC TCA CTG AAA ACT CTG CCC TCC AGA GAA AAA TTC ACC AGC CTA CTG GTT GCC ACG CTG ACC TAC CCT AGC CAC TGC TGT GCT TTC AGG  
▶ L I A T S S Y S L K T L P S R E K F T S L L V A T L T Y P S H C C A F R

ex10

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

CAGC>ATCG (silent plus diagnostic mut)

G>C (silent mut)

C>T (M509T)

G>C (silent mut)

TTG CCC CTT GTC GGT GTC AGC AGT TAC ACG AAA GTA TCG ATC TGC CTC CCC ATG GAT GTC GAA TCC ACT CTG TCA CAA GTC TAC ATA TTA TCC ATC TTG CTC CTC AAT  
▶ L P L V G V S S Y T K V S I C L P M D V E S T L T S Q V Y I L S I L L L N  
GCA GTG GCC TTT GTC GTC ATC TGT GCT TGC TAC GTT AGG ATA TAC TTT GCA GTT CAA AAT CCA GAG CTG ACG GCT CCT AAC AAG GAC ACA AAA ATT GCT AAG AAG ATG  
▶ A V A F V V I C A C Y V R I Y F A V Q N P E L T A A P N K D T K I A K K M  
GCC ATC CTC ATC TTC ACA GAC TTC ACA TGC ATG GCA CCC ATC TCA TTC TTT GCC ATC TCA GCT GCC TTC AAA GTA CCC CTT ATC ACT GTC ACC AAC TCA AAA GTT CTG  
▶ A I L I F T D F T C M A P I S F F A I S A A F K V P C L I T V T N S K V L  
CTG GTC CTT TTT TAT CCT GTC AAT TCT TGT GCC AAC CCA TTT CTG TAC GCA GTG TTC ACG AAG GCA TTT CAG AGA GAT TTC TTT CTC TTG CTG AGC AGA TTT GGT TGC  
▶ L V L F Y P V N S C A N P F L Y A V F T K A A F Q R D F F L L L S R F G C  
TGT AAG CAC CGG GCT GAA CTT TAC AGA AGG AAG GAA TTT TCT GCA TGT ACC TTC AAG TCC AAA AAC GGC TTT CCA AGA TCA AGT AAG CCT TCC CAG GCT GCC CTG AAG  
▶ C K H R A E L Y R R K E F S A C T F N S K N G F P R S S K P S Q A A L K  
TTA TCC ATA GTG CAC TGT CAA CAA CCT ACA CCT CCA AGA GTG TTA ATT CAG TAACTGCATTACTGAATTGTATCTAAATATGTTCCCCCCAAAAAGTCCACCCAAATTAGTCACTTTAATATAATGTG  
▶ L S I V H Q Q P T P R V L I Q  
TTTTGAAAAAATTTTATCTTTAAGCACCTTCAGGTGAATTAACCTGCTTCAAGGGGTGGCCCCAAGACACTTGGTGCACATAAAATTTTCAGAAGGGTTTGAAGAAATTTTTTATAATAATTTAGAAAGAAATGTTTTTGTGTGA  
ATCTAATATTAAGAAAATCTAAGTTGTGTGCAATTTTCCATGCTCTTGTATCTTTTTCACCTCAACTCTGTGATTTCATGTTGCCATCTCTAAATATATATTCATAACAGACTGGAAATTTAAAGTGGTCTTTGTCTCAGATAGTTTG  
ATAAATACATTCAAGAGATGCACTGTGCAGCGTATAGCTGTTAGCCTTACATGGTAAATAAAAAGTTTCTTAGCCATA

# Strategy proposed : HDR by CRISPR/Cas9

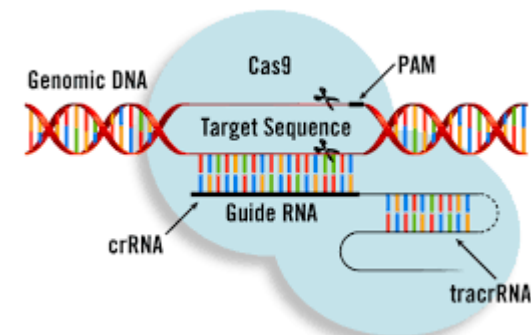


## ■ Pros

- As asked
- Two sgRNAs will be used in order to increase the chance of insertion of the asked mutation (double strand breaks occur 3 bps 5' of the PAM sequences, respectively 22 and 26 bps from the asked mutation)
- A diagnostic ClaI site will be created with changes in the codon (but not the amino acid sequence)
- Both Protospacer Adjacent Motif (PAM; NGG) will be mutated with silent mutation in order to avoid recuts after HDR.

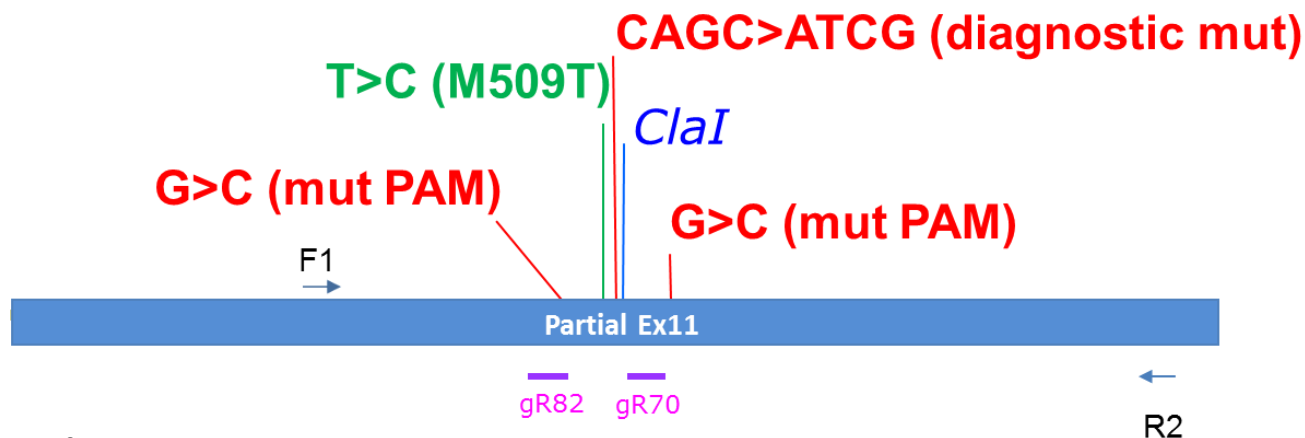
## ■ Cons

- Silent mutations will be introduced and may change the genomic environment (ie splicing could be affected, other?)
- 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



# Genotyping protocol

# GENOTYPING INSTRUCTIONS



## PCR genotyping strategy

Primer ref.	Sequence	Amplification product size WT	Sizes observed if <i>ClaI</i> restriction present (associated with asked PM)
F1	AAAGGTGGCACACCATCACCTATGC	436 bps	288 bps + 148 bps
R2	AGCAGAACTTTTGAGTTGGTGACAG		

## Sequence of the PCR product

AAAGGTGGCACACCATCACCTATGCTGTTTCAGCTGGACCAAAGCTGAGGCTGAGACATGCCATCCCAATTATGCTCGGAGGATGGATTT  
 TTTCTACCCTGATGGCCACATTGCCCTTGT<sup>*CGGTGTCAGCAGTTACACGAAAGTATCG*</sup>A7CTGCCTCCCCATGGATGT<sup>*CGAATCCACTCTG*</sup>  
 TCACAAGTCTACATATTATCCATCTTGCTCCTCAATGCAGTGGCCTTTGTGTCATCTGTGCTTGTACGTTAGGATATACTTTGCAGTTCAA  
 ATCCAGAGCTGACGGCTCCTAACAAGGACACAAAATTGCTAAGAAGATGGCCATCCTCATCTTCACAGACTTCACATGCATGGCACCCAT  
 CTCATTCTTTGCCATCTCAGCTGCCTTCAAAGTACCCTTATCA<sup>*CTGTCACCAACTCAAAGTTCTGCT*</sup>

Primers in orange

*ClaI* site in italic

## PCR Protocol

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

Reagents:	Volume (per sample):
- Phusion HS (Thermo Scientific) 5X Buffer	4 $\mu$ l
- 10mM dNTP	0,4 $\mu$ l
- 5' primer (100 $\mu$ M)	0.1 $\mu$ l
- 3' primer (100 $\mu$ M)	0.1 $\mu$ l
- DNA (lysate 1/10)	2 $\mu$ l
- Phusion Hot Start II	0,2 $\mu$ l
- Sterile H2O	up to 20 $\mu$ 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 $\mu$ l
Buffer 10X	2 $\mu$ l
Clal Restriction enzyme	0,2 $\mu$ l
H2O	7,8 $\mu$ l

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