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EXCELLENCE IN MOUSE PHENOGENOMICS

Introduction of a causative point mutation in Nr2f1 by CRISPR/Cas9 Genotyping protocol

Kus7772 / IM7772

Done by Dr Marie-Christine Birling, birlingm@igbmc.fr

24/08/2021

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Strategy validated

cnrs

Université de Strasbourg

Instituts thématiques Inserm

Institut national de la santé et de la recherche médicale

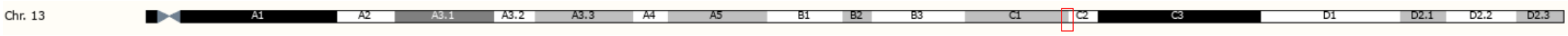
CERBM

centre européen de recherche en biologie et en médecine gie

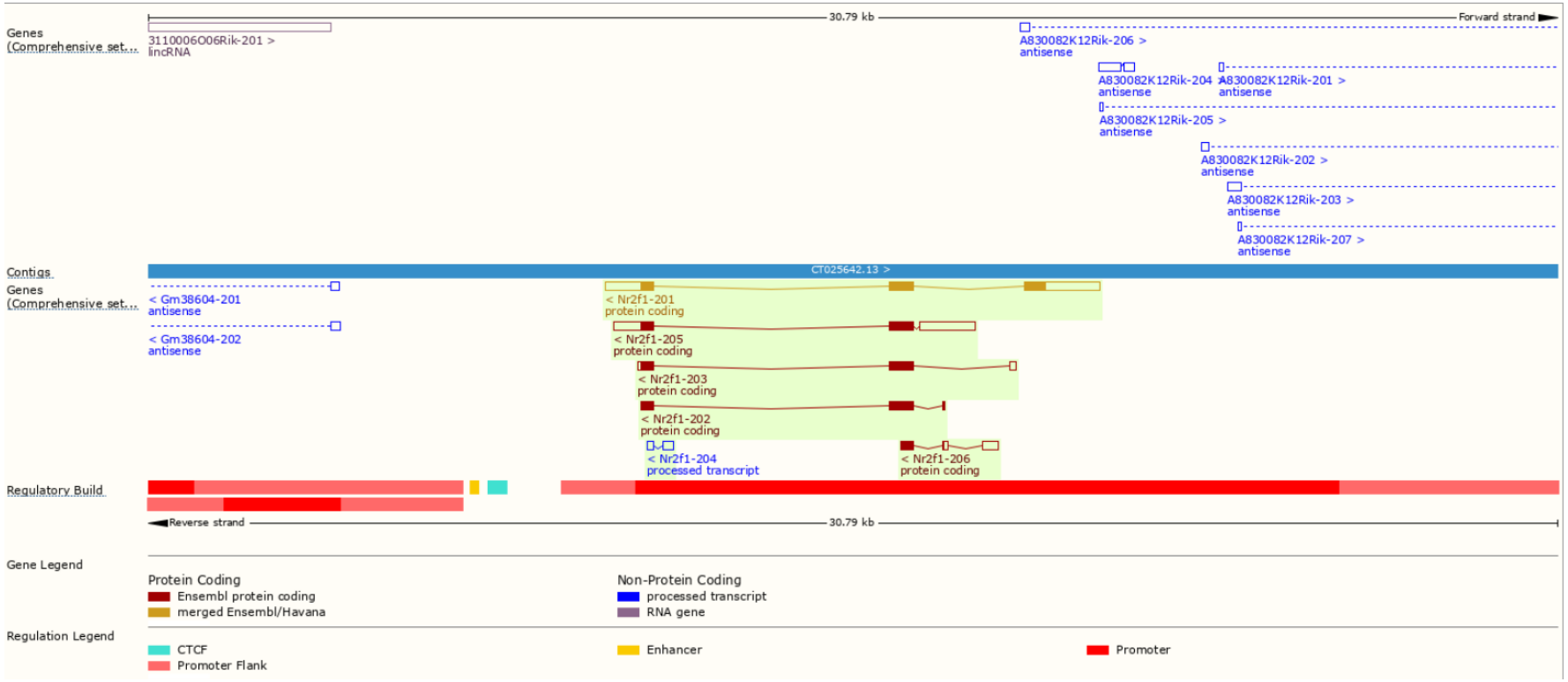
Aix*Marseille université

Nr2f1 Mouse Genomic locus

Location: Chromosome 13: 78,188,973-78,199,757



Gene: Nr2f1 ENSMUSG00000069171



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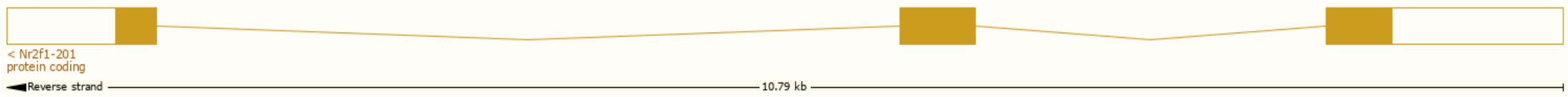
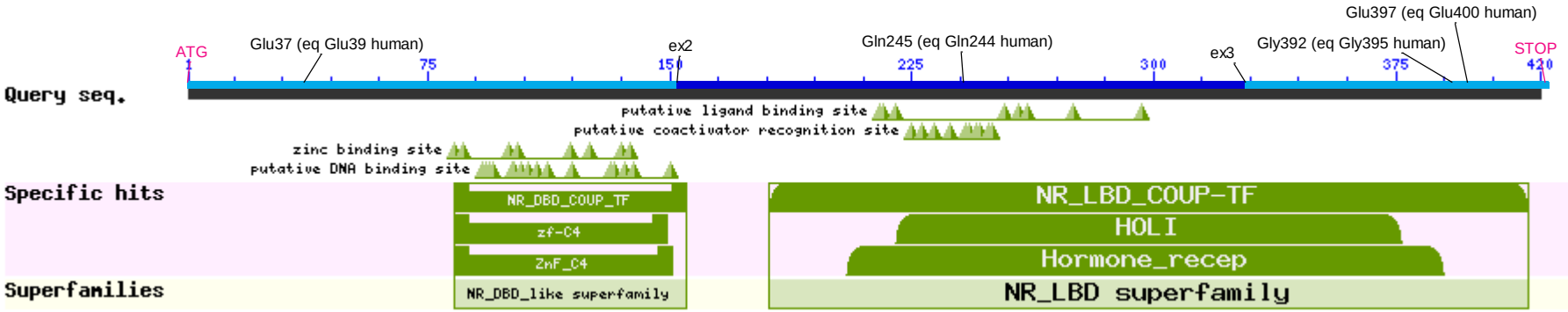
Nr2f1 mRNAs and proteins

Name	Transcript ID	bp	Protein	Biotype	CCDS	UniProt Match	Flags
Nr2f1-201	ENSMUST00000091458.12	3207	420aa	Protein coding	CCDS26658	Q32NY6	TSL:1GENCODE basicAPPRIS P1
Nr2f1-202	ENSMUST00000125176.2	841	273aa	Protein coding	CCDS84040	F7CII1	TSL:5GENCODE basic
Nr2f1-203	ENSMUST00000127137.2	991	263aa	Protein coding	CCDS84039	B8JJI9	TSL:5GENCODE basic
Nr2f1-204	ENSMUST00000135284.1	368	No protein	Processed transcript	-	-	TSL:3
Nr2f1-205	ENSMUST00000150498.7	2588	263aa	Protein coding	CCDS84039	B8JJI9	TSL:1GENCODE basic
Nr2f1-206	ENSMUST00000224798.1	721	95aa	Protein coding	-	A0A286YD96	CDS 3' incomplete

Transcript: Nr2f1-201 ENSMUST00000091458.12



Positions of the asked mutation in the Nr2f1 protein



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Alignement between human and mouse sequence

Query=human (NM_005654.6) vs Subject=Mouse (Nr2f1-201 ENSMUST00000091458.12)

Amino acid alignment

Glu39

Score	Expect	Method	Identities	Positives	Gaps
867 bits(2239)	0.0	Compositional matrix adjust.	419/423(99%)	420/423(99%)	3/423(0%)

```
Query  1    MAMVVSSWRDPQDDVAGGNPGGPNPAAQAARGGGGGAGEQQQQAGSGAPHTPQTPGQPGA  60
            MAMVVSSWRDPQDDVAGGNPGGPNPAAQAARGGGGG  QQQQAGSGAPHTPQTPGQPGA
Sbjct  1    MAMVVSSWRDPQDDVAGGNPGGPNPAAQAARGGGGG---EQQQAGSGAPHTPQTPGQPGA  57

Query  61   PATPGTAGDKGQGPPGSGQSQQHIECVVCGDKSSGKHYGQFTCEGCKSFFKRSVRRNLTY  120
            PATPGTAGDKGQGPPGSGQSQQHIECVVCGDKSSGKHYGQFTCEGCKSFFKRSVRRNLTY
Sbjct  58   PATPGTAGDKGQGPPGSGQSQQHIECVVCGDKSSGKHYGQFTCEGCKSFFKRSVRRNLTY  117

Query  121  TCRANRNCPIDQHHRNQCQYCRLKKCLKVGMRREAVQRGRMPPTQPNPGQYALTNGDPLN  180
            TCRANRNCPIDQHHRNQCQYCRLKKCLKVGMRREAVQRGRMPPTQPNPGQYALTNGDPLN
Sbjct  118  TCRANRNCPIDQHHRNQCQYCRLKKCLKVGMRREAVQRGRMPPTQPNPGQYALTNGDPLN  177

Query  181  GHCYLSGYISLLLRAEPYPTSRYGSQCMQPNNIMGIENICELAARLLFSAVEWARNIPFF  240
            GHCYLSGYISLLLRAEPYPTSRYGSQCMQPNNIMGIENICELAARLLFSAVEWARNIPFF
Sbjct  178  GHCYLSGYISLLLRAEPYPTSRYGSQCMQPNNIMGIENICELAARLLFSAVEWARNIPFF  237

Query  241  PDLQITDQVSLLRLTWSELFVLNAAQCSMPLHVAPLLAAAGLHASPMSADRVVAFMDHIR  300
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Sbjct  238  PDLQITDQVSLLRLTWSELFVLNAAQCSMPLHVAPLLAAAGLHASPMSADRVVAFMDHIR  297

Query  301  IFQEQVEKLKALHVDSAEYSCLKAIVLFTSDACGLSDAAHIESLQEKSQCALEEYVRSQY  360
            IFQEQVEKLKALHVDSAEYSCLKAIVLFTSDACGLSDAAHIESLQEKSQCALEEYVRSQY
Sbjct  298  IFQEQVEKLKALHVDSAEYSCLKAIVLFTSDACGLSDAAHIESLQEKSQCALEEYVRSQY  357

Query  361  PNQPSRFGKLLLRLPSLRTVSSSVIEQLFFVRLVGKTPIETLIRDMLLSGSSFNWPYMSI  420
            PNQPSRFGKLLLRLPSLRTVSSSVIEQLFFVRLVGKTPIETLIRDMLLSGSSFNWPYMSI
Sbjct  358  PNQPSRFGKLLLRLPSLRTVSSSVIEQLFFVRLVGKTPIETLIRDMLLSGSSFNWPYMSI  417

Query  421  QCS  423
            QCS
Sbjct  418  QCS  420
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Gln244

Gly395

Glu400

Coding sequence alignment

Score	Expect	Identities	Gaps	Strand
1958 bits(1060)	0.0	1201/1269(95%)	9/1269(0%)	Plus/Plus

```
Query  1     ATGGCAATGGTAGTTAGCAGCTGGCGAGATCCGCAGGACGACGTggcggggggcaaccccc  60
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Sbjct  1     ATGGCAATGGTAGTTAGCAGCTGGCGAGATCCGCAGGACGACGTGGCCGGGGGCAACCCC  60

Query  61    ggcggccccaaccccgcagcgcaggcggcccgcggcggcggcggcggcgccggcgaggcag  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||  ||
Sbjct  61    GGCGGCCCCAACCCCGCAGCGCAGGCAGCCCGCGGCGGCGGCGGCGGCG---------AG  111

Query  121   cagcagcaggcgggctcgggcgcgccgcacacgccgcagACCCCGGGCCAGCCCGGAGCG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  112   CAGCAGCAGGCGGGCTCCGGCGCGCCGCACACGCCGCAGACCCCGGGCCAGCCCGGAGCG  171

Query  181   CCCGCCACCCCCGGCACGGCGGGGGACAAGGGCCAGGGCCCGCCCGGTTCGGGCCAGAGC  240
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Sbjct  172   CCCGCCACCCCCGGCACGGCAGGGGACAAGGGCCAGGGCCCGCCCGGTTCAGGCCAGAGC  231

Query  241   CAGCAGCACATCGAGTGCGTGGTGTGCGGGGACAAGTCGAGCGGCAAGCACTACGGCCAA  300
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Sbjct  232   CAGCAGCACATCGAGTGCGTGGTGTGCGGGGACAAGTCGAGCGGCAAGCACTACGGCCAA  291

Query  301   TTCACCTGCGAGGGCTGCAAAAGTTTCTTCAAGAGGAGCGTCCGCAGGAACTTAACTTAC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  292   TTCACCTGCGAGGGCTGCAAAAGTTTCTTCAAGAGGAGCGTCCGCAGGAACTTAACTTAC  351

Query  361   ACATGCCGTGCCAACAGGAACTGTCCCATCGACCAGCACCACCGCAACCAGTGCCAATAC  420
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Sbjct  352   ACATGCCGTGCCAACAGGAACTGTCCCATCGACCAGCACCACCGCAACCAGTGCCAATAC  411

Query  421   TGCCGCCTCAAGAAGTGCCTCAAAGTGGGCATGAGGCGGGAAGCGGTTCAGCGAGGAAGA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  412   TGCCGCCTCAAGAAGTGCCTCAAAGTGGGCATGAGGCGGGAAGCGGTTCAGCGAGGAAGA  471

Query  481   ATGCCTCCAACCCAGCCCAATCCAGGCCAGTACGCACTCACCAACGGGGACCCCCTCAAC  540
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Sbjct  472   ATGCCTCCAACCCAGCCCAATCCAGGCCAGTATGCACTCACAAACGGGGATCCTCTCAAT  531

Query  541   GGCCACTGCTACCTGTCCGGCTACATCTCGCTGCTGCTGCGCGCCGAGCCCTACCCCACG  600
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Sbjct  532   GGCCACTGCTACCTGTCTGGCTACATTTCTCTGCTGCTGCGCGCAGAGCCCTACCCCACG  591

Query  601   TCGCGCTACGGCAGCCAGTGCATGCAGCCCAACAACATTATGGGCATCGAGAACATCTGC  660
             ||||| || |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  592   TCGCGTTATGGCAGCCAGTGCATGCAGCCCAACAACATCATGGGCATCGAGAACATCTGC  651

Query  661   GAGCTGGCCGCGCGCCTGCTCTTCAGCGCCGTCGAGTGGGCCCGCAACATCCCCTTCTTC  720
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Sbjct  652   GAGCTGGCAGCCCGCCTCCTCTTCAGCGCCGTCGAGTGGGCCCGCAACATCCCGTTCTTC  711

Query  721   CCGGATCTGCAGATCACCGACCAGGTGTCCCTGCTACGCCTCACCTGGAGCGAGCTGTTC  780
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Sbjct  712   CCGGATCTGCAGATCACGGACCAGGTGTCTCTGCTGCGCCTCACCTGGAGCGAGCTGTTC  771

Query  781   GTGCTCAACGCGGCCCAGTGCTCTATGCCGCTGCACGTGGCGCCGTTGCTGGCCGCCGCC  840
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Query  841   GGCCTGCATGCCTCGCCCATGTCTGCCGACCGCGTCGTGGCCTTCATGGACCACATCCGC  900
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Query  901   ATCTTCCAGGAGCAGGTGGAGAAGCTCAAGGCGCTACACGTCGACTCAGCCGAGTACAGC  960
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Sbjct  892   ATCTTTCAGGAACAGGTGGAGAAGCTCAAGGCGCTGCACGTCGACTCTGCCGAGTACAGC  951

Query  961   TGCCTCAAAGCCATCGTGCTGTTCACGTCAGACGCCTGTGGCCTGTCGGATGCGGCCCAC  1020
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Sbjct  952   TGCCTCAAAGCCATCGTGCTATTCACGTCAGATGCTTGTGGCCTGTCGGATGCTGCCCAC  1011

Query  1021  ATCGAGAGCCTGCAGGAGAAGTCGCAGTGCGCACTGGAGGAGTACGTGAGGAGCCAGTAC  1080
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Sbjct  1012  ATCGAAAGCCTGCAGGAGAAATCACAGTGTGCCCTGGAGGAGTATGTGAGAAGCCAGTAC  1071

Query  1081  CCCAACCAGCCCAGCCGTTTTGGCAAACTGCTGCTGCGACTGCCCTCGCTGCGCACCGTG  1140
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Sbjct  1072  CCCAACCAGCCCAGCCGCTTTGGCAAACTGCTGCTGCGATTGCCCTCTCTTCGCACAGTG  1131

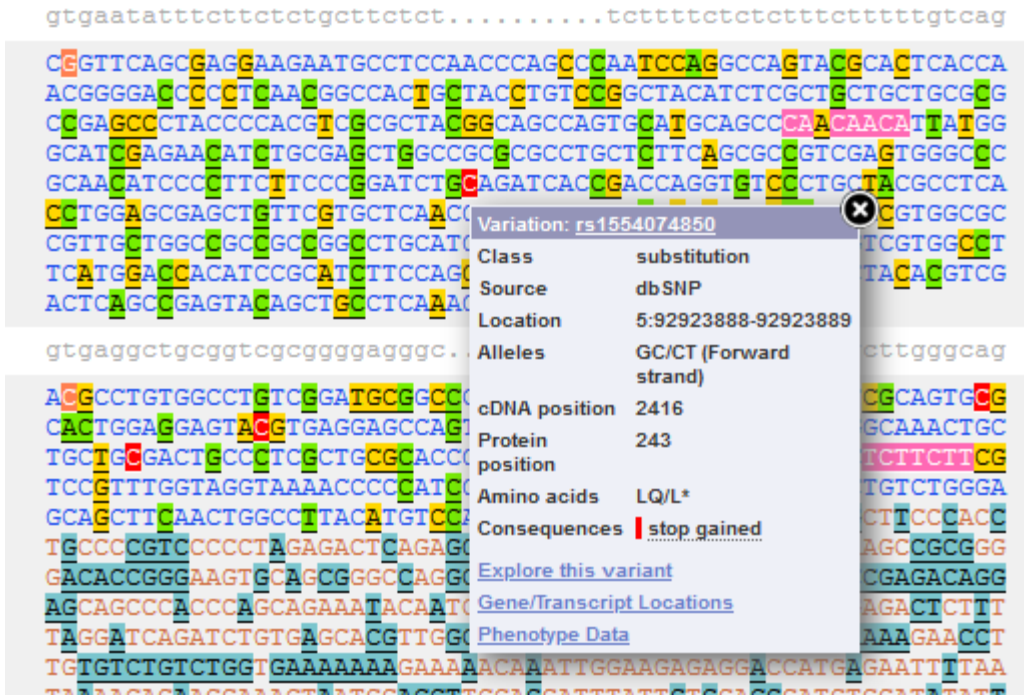
Query  1141  TCCTCCTCCGTCATCGAGCAGCTCTTCTTCGTCCGTTTGGTAGGTAAAACCCCCATCGAA  1200
             |||||||| |||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  1132  TCCTCCTCTGTCATCGAGCAACTCTTCTTCGTACGTTTGGTAGGTAAAACTCCCATCGAA  1191

Query  1201  ACTCTCATCCGCGATATGTTACTGTCTGGGAGCAGCTTCAACTGGCCTTACATGTCCATC  1260
             ||||||||| |||||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  1192  ACTCTCATCCGAGATATGTTGCTGTCAGGGAGCAGTTTCAACTGGCCTTACATGTCCATC  1251
```

Gln244, Gly395; Glu400 are located in very conserved region (aa or nucleotidique sequence)
Glu39* is located in a less conserved region

Detail concerning the potential Gln240*

délétion/insertion au niveau génomique 92923888_92923889 delinsCT; NM_005654.4: ADN codant c.729_730delinsCT; protéine Gln244*



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Selection of the best sgRNA for introduction of Gly395Ala and Glu400Ter

http://crispor.tefor.net/crispor.py?batchId=9fLgq8zPJacUC9vYfmvW

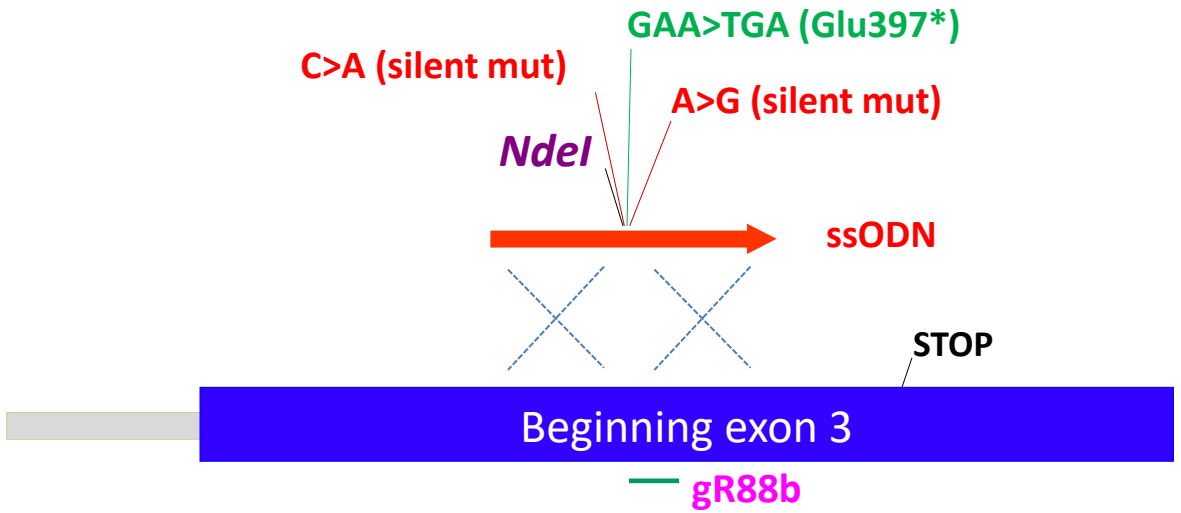
Guide Sequence + *PAM* + Restriction Enzymes + Variants ☐ Only G- ☐ Only GG- ☐ Only A-	MIT Specificity Score	CFD Spec. score	Predicted Efficiency Show all scores: Doench '16	Mor.-Mateos	Outcome: Out-of-Frame	Lindel	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score ☐ exons only ☐ chr13 only
GCAACTCTTCTTCGTACGTT *TGG* ⚠ Inefficient Enzymes: *MaeII, PspLI* **Cloning / PCR primers**	97	97	41	51	63	75	0 - 0 - 0 - 1 - 33 0 - 0 - 0 - 0 - 0 34 off-targets	4:intergenic:Lmo4-Hs2st1 4:intergenic:Gm12375-Gm12378 4:intergenic:Gm22459-Gm25041 show all...
ATCTCGGATGAGAGTTTCGA *TGG* Enzymes: *TaqI* **Cloning / PCR primers**	91	94	53	38	62	75	0 - 0 - 1 - 2 - 46 0 - 0 - 0 - 0 - 0 49 off-targets	2:exon:Nr2f2 4:intergenic:Gm22782-Rpl10l 4:intergenic:Avpr1a-mmu-mir-6412 show all...
CTCTTCTTCGTACGTTTGGT *AGG* **Cloning / PCR primers**	88	87	58	20	68	78	0 - 0 - 1 - 7 - 91 0 - 0 - 0 - 0 - 0 99 off-targets	4:intron:Slco3a1 4:intron:Axdnd1 4:intergenic:Gm8526-Edil3 show all...
TCTCGGATGAGAGTTTCGAT *GGG* Enzymes: *TaqI* **Cloning / PCR primers**	88 b	94	69	46	63	66	0 - 0 - 1 - 8 - 60 0 - 0 - 0 - 0 - 0 69 off-targets	4:intergenic:Gm5382-Gm14867 2:exon:Nr2f2 4:intergenic:9530052E02Rik-Gm15418 show all...

Haeussler, M. et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. **Genome Biol. 17, 148. doi:10.1186/s13059-016-1012-2**

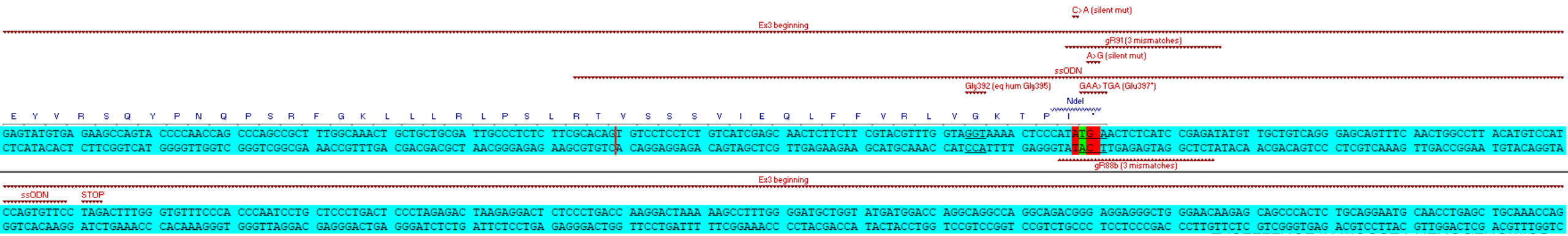
Strategy validated: introduction of Gln397* (corresponding to Glu400* in human)

Validated by the client

Approach proposed: Homology directed repair (HDR) via CRISPR/Cas9



Sequence of the allele after HDR



Strategy validated: introduction of Gln397* (corresponding to Glu400* in human)

PROS

- Two silent mutations (one in the STOP codon) will be added to the asked G>T mutation in order to avoid a recut after HDR. The 3 mutations will introduce a new NdeI diagnostic restriction site that will easy genotyping.

CONS

- Frequency of HDR is not predictable and can be low

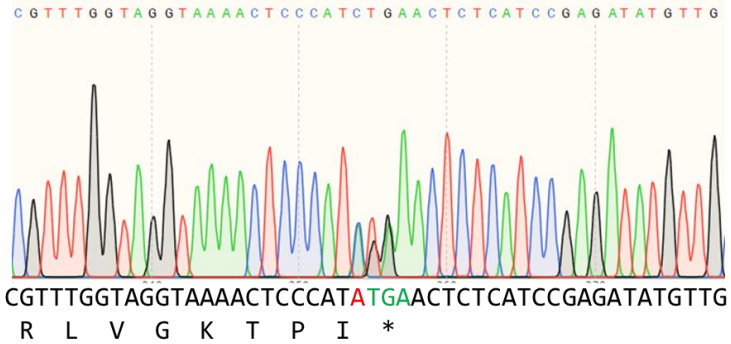
Line Kus7772-1-PM

F1 genotype

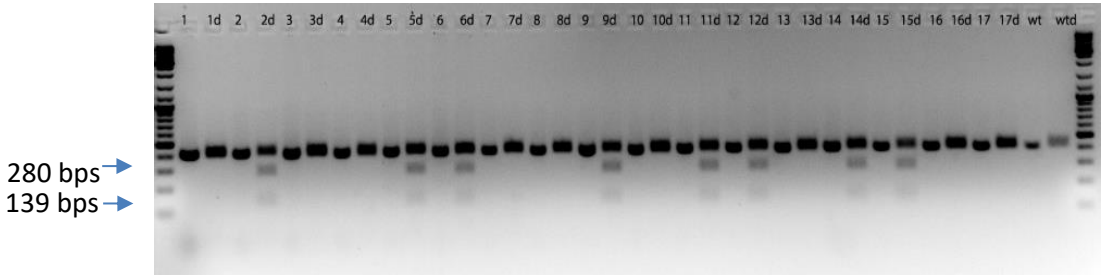
Sequence with PCR F1-R1
Primers in orange
Asked mutation in green
Silent mutation in red
NdeI site in italic and underlined

TGTATGCCTTTCCATCTCACTCACCTAACAATCTGTCCCCTTTCCCTGTCTCTCCCTCCTGTGGCTGCTCTGGCAGATGCTTGTGGCCTGTCGGATGCTGCCCACATCGAAAGCCTG
CAGGAGAAATCACAGTGTGCCCTGGAGGAGTATGTGAGAAGCCAGTACCCCAACCAGCCCAGCCGCTTTGGCAAACTGCTGCTGCGATTGCCCTCTCTTCGCACAGTGTCCTC
CTCTGTCATCGAGCAACTCTTCTTCGTACGTTTGGTAGGTAAAACTCC*CATATGA*ACTCTCATCCGAGATATGTTGCTGTCAGGGAGCAGTTTCAACTGGCCTTACATGTCCATCCA
GTGTTCCTAGACTTTGGGTGTTTCCCACCCAATCCTGCTCCCTGACTCCCTAGAGACTAAGAGGACTCTCCC

Sanger sequencing (F1 #2)



F1 genotyping



The line **Kus7772-1-PM wasestablished**. Four heterozygous males and 4 heterozygous females are available. Heterozygous pups with the premature STOP codon are viable at the heterozygous stage.
International nomenclature (according to MGI http://www.informatics.jax.org/mgihome/nomen/gene.shtml) : **Nr2f1^{em1Ics}**
MGI allele ID: 8253350

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Genotyping protocol

cnrs

Université de Strasbourg

Instituts thématiques Inserm

Institut national de la santé et de la recherche médicale

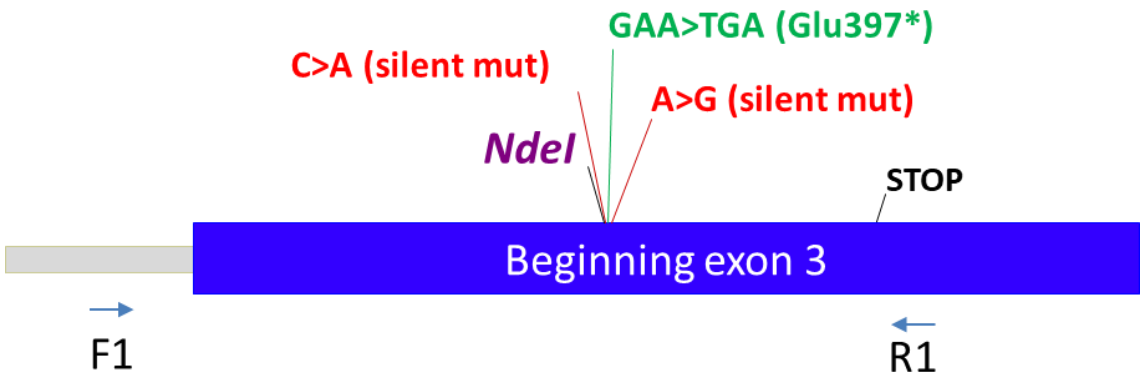
CERBM

centre européen de recherche en biologie et en médecine gie

Aix*Marseille université

GENOTYPING INSTRUCTIONS

Genotyping scheme



PCR genotyping primers and sequence

Primer ref.	Sequence	Amplification product size WT	Sizes observed if NdeI restriction present (associated with asked PM)
F1	TGTATGCCTTTCCATCTCACTCACC	419 bps	139 +280 bps
R1	GGGAGAGTCCTCTTAGTCTCTAGGG		

Sequence with PCR F1-R1
Primers in orange
Asked mutation in green
Silent mutation in red
NdeI site in italic and underlined

TGTATGCCTTTCCATCTCACTCACCTAACAATCTGTCCCCTTTCCCTGTCTCTCCCTCCTGTGGCTGCTCTGGCAGATGCTTGTGGCCTGTCGGATGCTGCCCACATCGAAAGCCTG
CAGGAGAAATCACAGTGTGCCCTGGAGGAGTATGTGAGAAGCCAGTACCCCAACCAGCCCAGCCGCTTTGGCAAACTGCTGCTGCGATTGCCCTCTCTTCGCACAGTGTCCTC
CTCTGTCATCGAGCAACTCTTCTTCGTACGTTTGGTAGGTAAAACTCC*CATATG*AACTCTCATCCGAGATATGTTGCTGTCAGGGAGCAGTTTCAACTGGCCTTACATGTCCATCCA
GTGTTCCTAGACTTTGGGTGTTTCCCACCCAATCCTGCTCCCTGACTCCCTAGAGACTAAGAGGACTCTCCC

GENOTYPING INSTRUCTIONS

PCR Protocol

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

Reagents:	Volume (per sample):
- Phusion HS (Thermo Scientific) 5X Buffer	4 µl
- 10mM dNTP	0.4 µl
- 5’ primer (100 µM)	0.1 µl
- 3’ primer (100 µM)	0.1 µl
- DNA (lysate 1/10)	2 µl
- Phusion Hot Start II	0.2 µl
- Sterile H2O	up to 20 µl

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 µl
Buffer 10X	2 µl
Restriction enzyme	0.2 µl
H20	7.8 µl

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

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REPORT REDACTION & VALIDATION

Protocol finalized on 2021/ 08 /24

By Marie-Christine Birling (PhD)
birlingm@igbmc.fr

CONTACT US

By email at mutagenesis@igbmc.fr
By phone at +33 (0)3 88 65 56 57

www.phenomin.fr