Introduction of the C395Y point mutation in mouse Cckar (corresponding to C387Y in human) Genotyping protocol

Kus7497

CALL PHENOMIN-FMR LAUREATE PROJECT

Done by Marie-Christine Birling (PhD)

birlingm@igbmc.fr

phenomin
EXCELLENCE IN MOUSE PHENOGENOMICS

INVESTIR L'AVENIR

Approach 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

Introduction of the C395Y point mutation in mouse Cckar (corresponding to C387Y in human) via a CRISPR/Cas9 approach

Kus7497 / IM7497

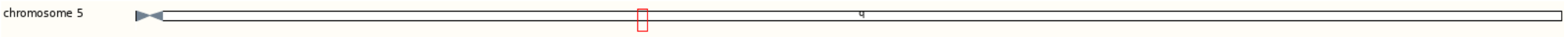
Strategy proposed by Marie-Christine Birling (PhD)

birlingm@igbmc.fr

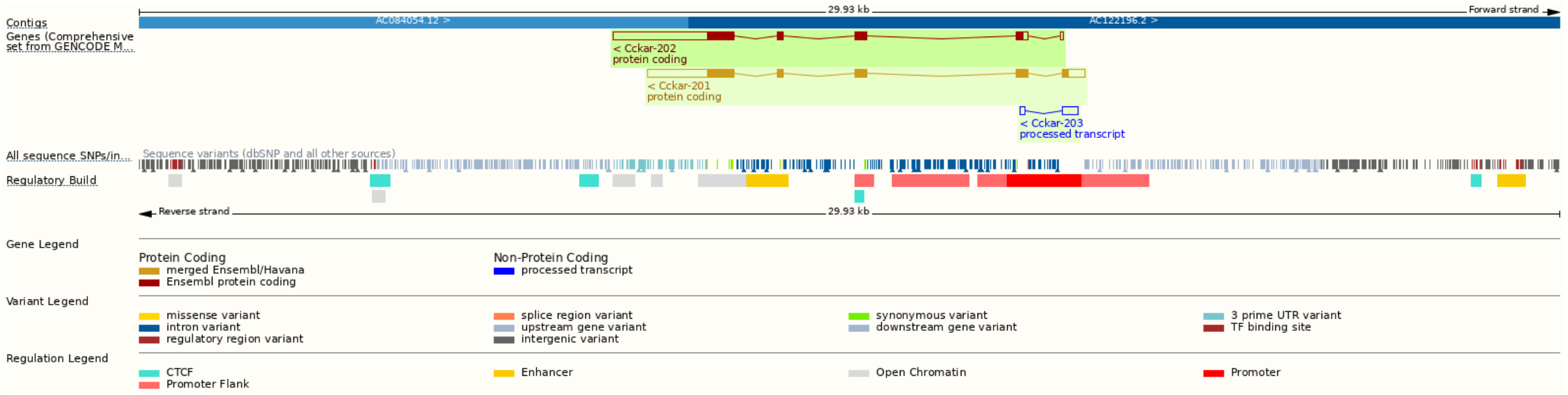
18/01/2022

Cckar Mouse Genomic locus

Location: Chromosome 5: 53,855,118-53,865,047



Gene: Cckar ENSMUSG00000029193



Cckar mRNAs and proteins

Transcript ID	Name	bp	Protein	Biotype	CCDS	UniProt Match
ENSMUST00000031093.5	Cckar-201	2930	436aa	Protein coding	CCDS19293	O08786
ENSMUST00000200691.4	Cckar-202	3230	365aa	Protein coding	CCDS84881	Q3TPL0
ENSMUST00000202946.2	Cckar-203	424	No protein	Processed transcript	-	-

Transcript: ENSMUST00000031093.5 Cckar-201

Strategy proposed: Selection of the best sgRNAs

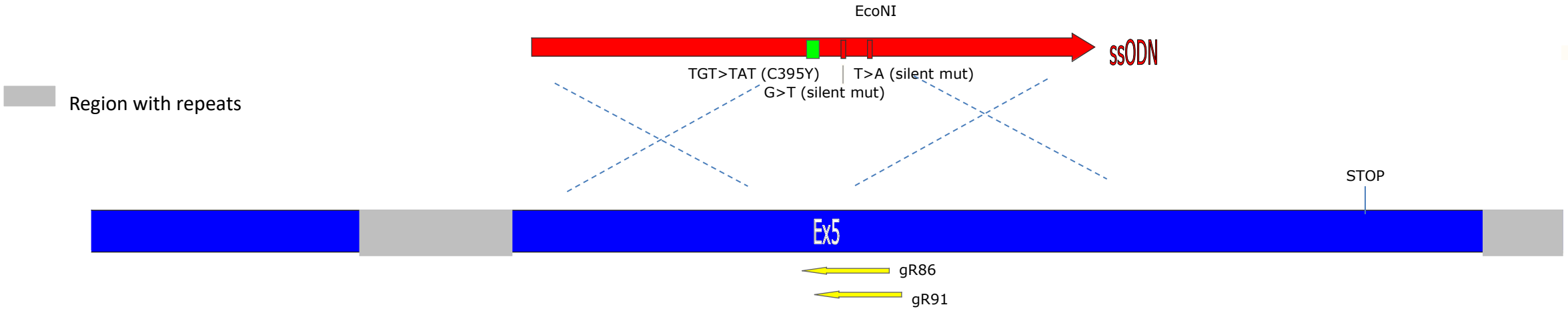
http://crispor.tefor.net/crispor.py?batchId=5KGHtECzxzICjylPvm1J

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 ☐ chr5 only
GGGACCAGGATTCGGGCAAC *AGG* . . . . **Cloning / PCR primers**	91	95	31	56	72	75	0 - 0 - 1 - 4 - 59 0 - 0 - 0 - 0 - 0 64 off-targets	4:intron:Stra6 4:intergenic:Tmem154-Fbxw7 4:intergenic:Mapk4/Gm9925-Ska1 show all...
GACCAGGATTCGGGCAACAG *GGG* . . . . **Cloning / PCR primers**	86	94	68	67	68	74	0 - 0 - 1 - 4 - 72 0 - 0 - 0 - 0 - 0 77 off-targets	4:intergenic:Gm22011-Mthfr-ps1 4:intron:Snx3 4:intergenic:Mir1971-Gm23652 show all...

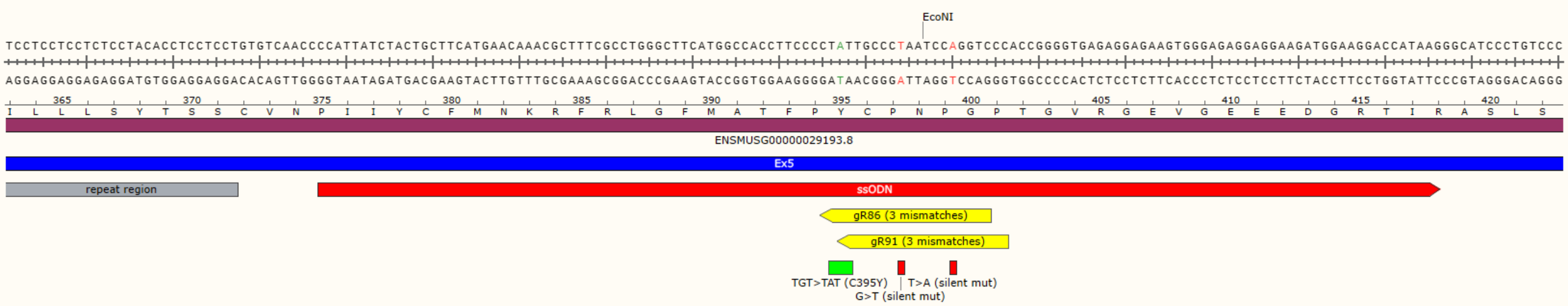
Haeussler, M. et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. **Genome Biol. 17, 148. doi:10.1186/s13059-016-1012-2**

Strategy proposed: introduction of the Cys395Tyr point mutation via CRISPR/Cas9

Approach proposed: homology directed repair (HDR) after CRISPR directed double strand break in fertilized oocytes



Sequence detail of the locus <u>after</u> HDR



Strategy proposed: introduction of the Cys395Tyr point mutation via CRISPR/Cas9

PROS

- The double strand break obtained with CRISPR guide RNA gR86 or gR91 are ideally located close to the mutation to introduce
- Two additional silent mutations (C>T and T>A) will be introduced in order to avoid a new CRISPR guided double strand break after HDR. Three mismatches will be introduced in total in the donor ssODN.
- The silent mutations will also allow the introduction of a new diagnostic restriction site

CONS

- Frequency of HDR is not predictable and can be low

phenomin

EXCELLENCE IN MOUSE PHENOGENOMICS

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

Kus7497-13-PM

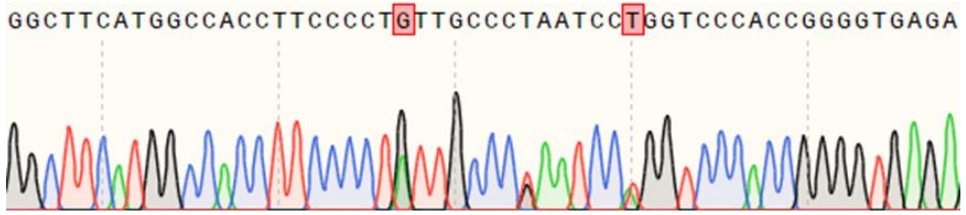
F1 genotyping

F1 and R1 are in orange
Silent mutation is in red
Asked mutation leading to the C395Y mutation is in green
EcoNI restriction sites are in italic and underlined

CATATGACACGGTTTCTGCCGAGAAACACCTCTCAGGGACCCCCATCTCCTTCATCCTCCTCCTCTCCTACACCTCCTCCTGTGTCAACCCCATTATCTACTGCTTCATGAACAAACGCTTTCGCCTGGGCTTCATGGCC
ACCTTCCCCTATTGCCCTAATCCAGGTCCCACCGGGGTGAGAGGAGAAGTGGGAGAGGAGGAAGATGGAAGGACCATAAGGGCATCCCTGTCCCGGTATTCCTACAGCCACATGAGCACCTCTGCCCCACCCCAC
TGAGCTGTACCTGGTTCGCTGCGGGCAGCAGAAAGGAGGGGACTGGGAGCAAGGAGGAAGAGGAAGTGGGAGGGAAGGAGAAGGAAGACCCATTTCTAACGAGGACTCTTCCGATGTCTGCTTTT

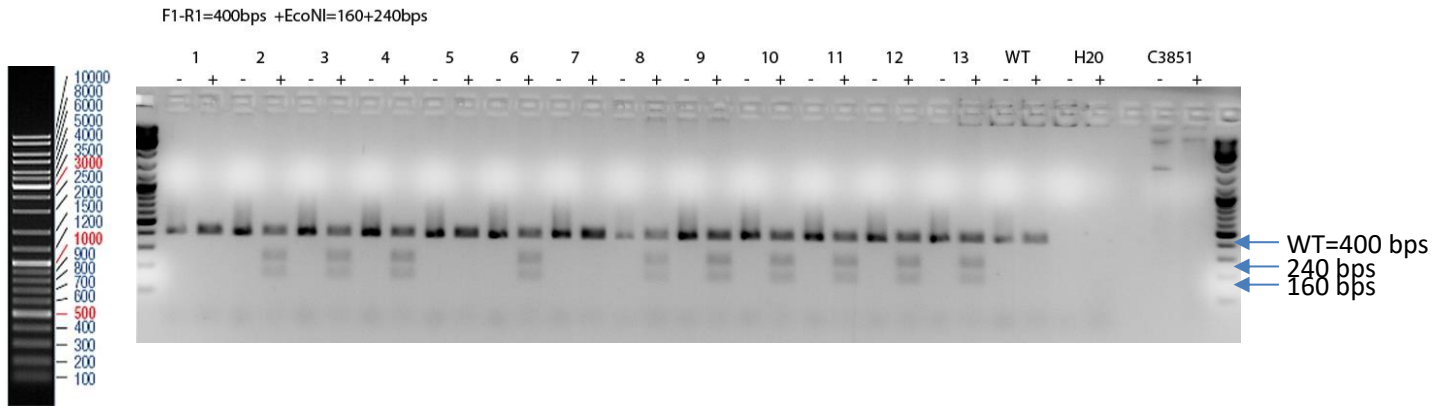
Sanger sequencing

F1#2



GGCTTCATGGCCACCTTCCCCTATTGCCCTAATCCAGGTCCCACCGGGGTGAGA
G F M A T F P Y C P N P G P T G V R

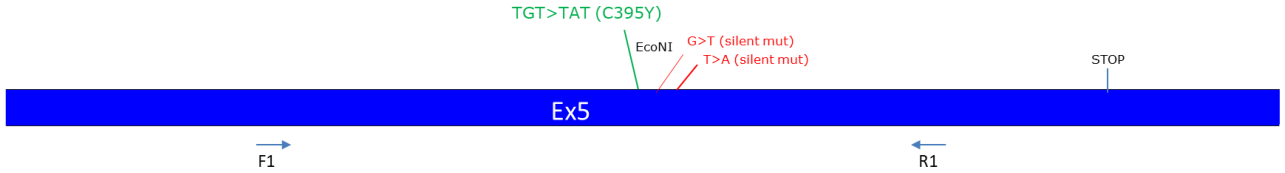
F1 genotyping



The line Kus7497-13-PM is established and cryopreserved

GENOTYPING INSTRUCTIONS

Genotyping Scheme



PCR genotyping primers and sequence

Primer ref.	Sequence	Amplification product size WT	Sizes observed if new EcoNI restriction present (associated with asked PM)
F1	CATATGACACGGTTTCTGCCGAGAA	400 bps	240 bps + 160 bps
R1	AAAAGCAGACATCGGAAGAGTCCTC		

CATATGACACGGTTTCTGCCGAGAAACACCTCTCAGGGACCCCCATCTCCTTCATCCTCCTCCTCTCCTACACCTCCTCCTGTGTCAACCCCATTATCTACTGCTT
CATGAACAAACGCTTTCGCCTGGGCTTCATGGCCACCTTCCCCTATTGC*CCTAATCCAGG*TCCCACCGGGGTGAGAGGAGAAGTGGGAGAGGAGGAAGATG
GAAGGACCATAAGGGCATCCCTGTCCCGGTATTCCTACAGCCACATGAGCACCTCTGCCCCACCCCACTGAGCTGTACCTGGTTCGCTGCGGGCAGCAGAAA
GGAGGGGACTGGGAGCAAGGAGGAAGAGGAAGTGGGAGGGAAGGAGAAGGAAGACCCATTTCTAACGAGGACTCTTCCGATGTCTGCTTTT

F1 and R1 are in orange
Silent mutation is in red
Asked mutation leading to the C395Y mutation is in green
EcoNI restriction sites are in italic and underlined

phenomin
EXCELLENCE IN MOUSE PHENOGENOMICS

GENOTYPING INSTRUCTIONS

PCR Protocol

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

Lysis buffer: 50 µl DNA Extract All Reagents – appliedbiosystems, Ref 4402616 (25 µl Lysis buffer +25 µl stabilising buffer)

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	5 min	1
96°C	8s	30
62°C	10s	
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

phenomin

EXCELLENCE IN MOUSE PHENOGENOMICS

REPORT REDACTION

Report performed on 2022/12/19

by Marie-Christine BIRLING, PhD

birlingm@igbmc.fr

CONTACT US

By email at mutagenesis@igbmc.fr

By phone at +33 (0)3 88 65 57 43

www.phenomin.fr