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EXCELLENCE IN MOUSE PHENOGENOMICS

INVESTISSEMENTS D'AVENIR

R418W KI in Adcy5 by CRISPR-Cas9 Final report

Kus6795 / IM6795

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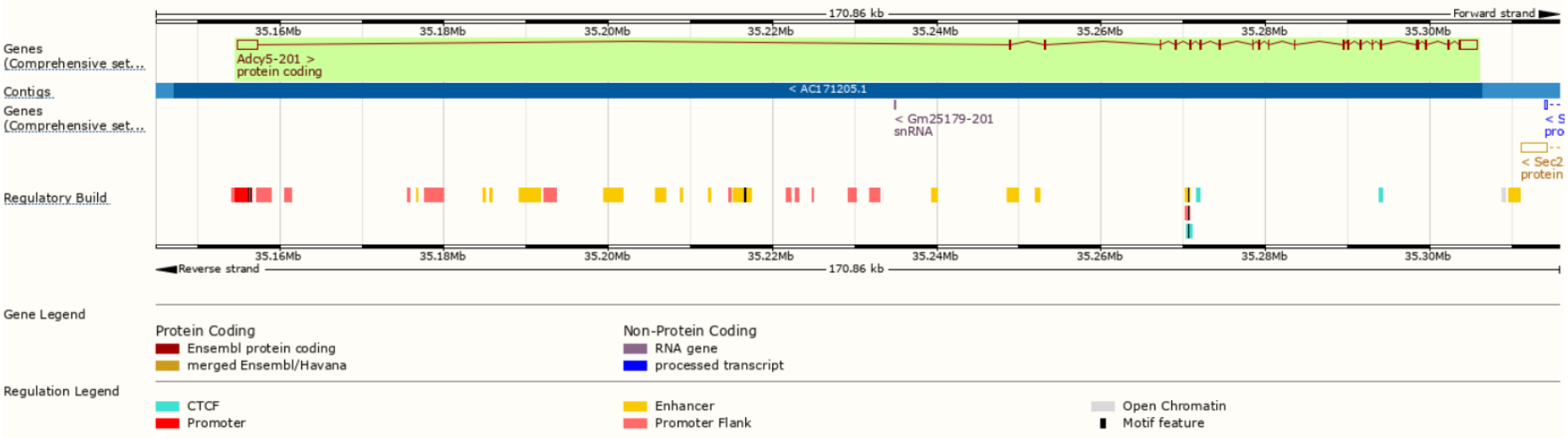
Adcy5 Mouse Genomic locus

Gene: Adcy5 ENSMUSG00000022840

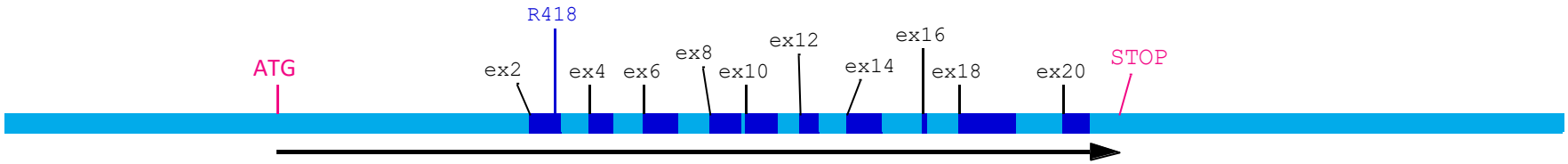


Chromosome 16: 35,154,877-35,305,737

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



sgRNA search

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	CCTGTTGCTGGTTCTCCCGC *TGG* Enzymes: *FauI, 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-S1pr3 show all...

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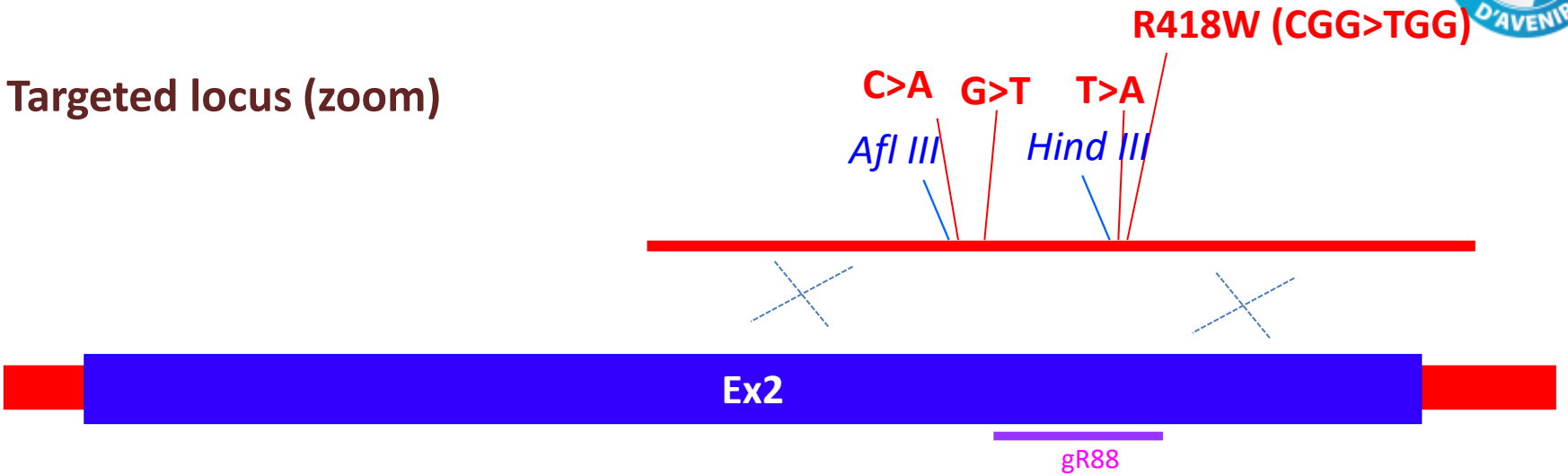
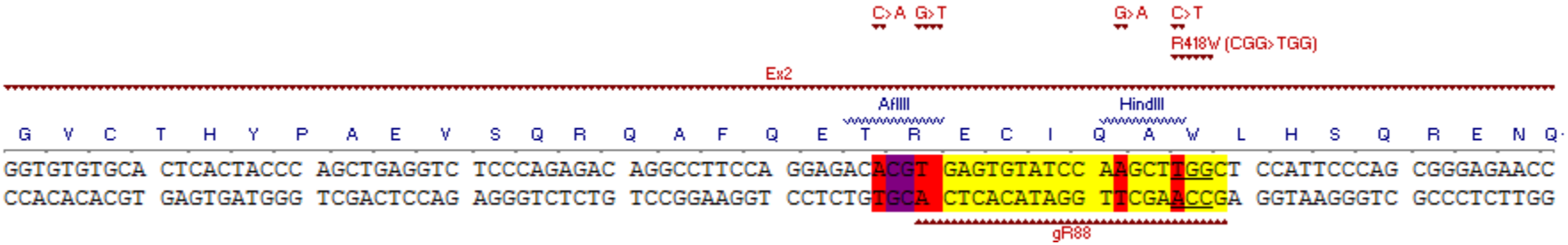
Strategy proposed: insertion of the R418W mutation

Targeted locus (zoom)

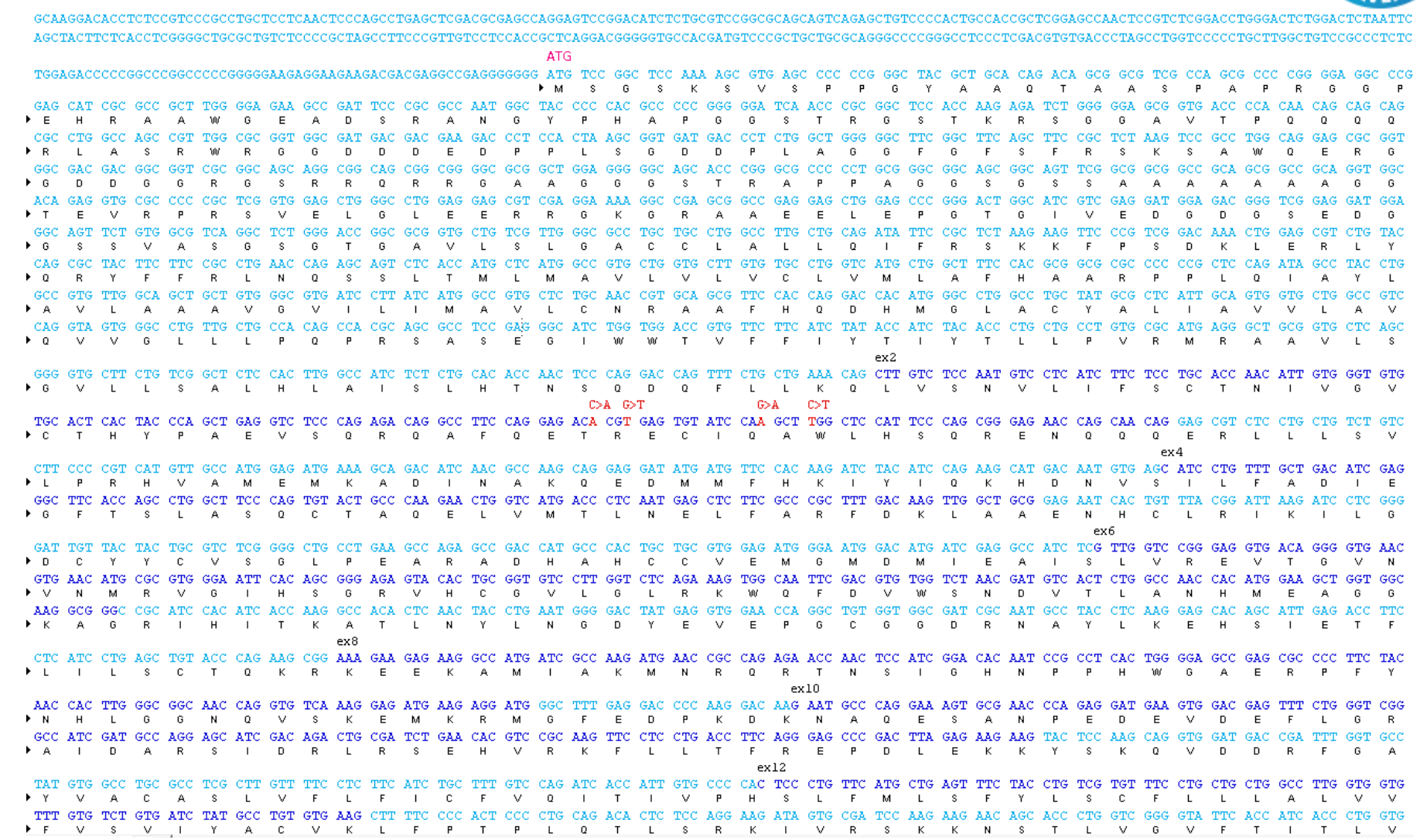


Sequence obtained



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)



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

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Results

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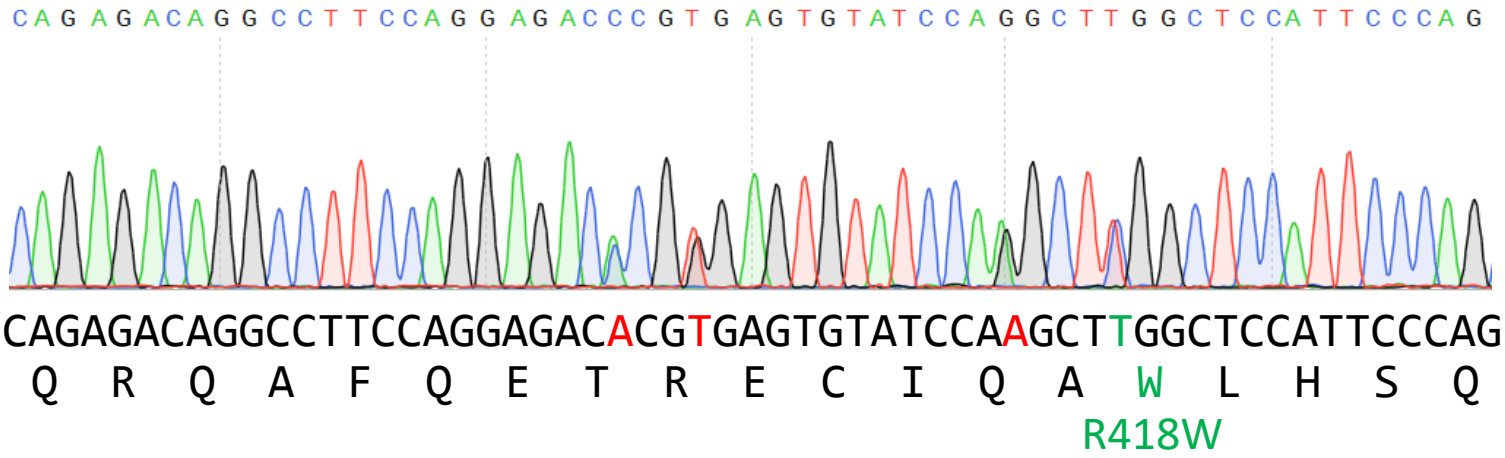
Microinjection

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

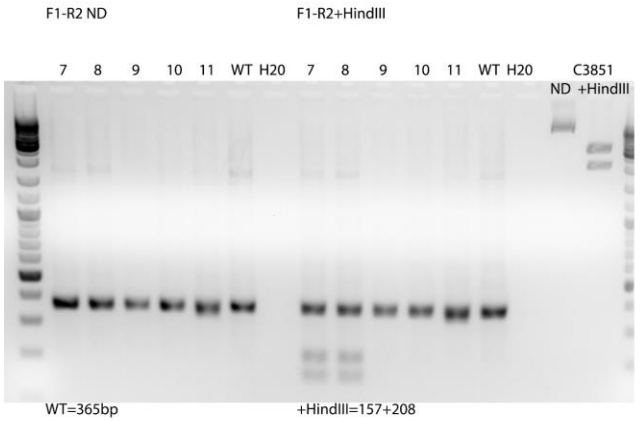
Line Kus6795-12-PM

Sanger sequencing



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

F1 genotyping



This line was created, cryopreserved and phenotyped

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Genotyping protocol

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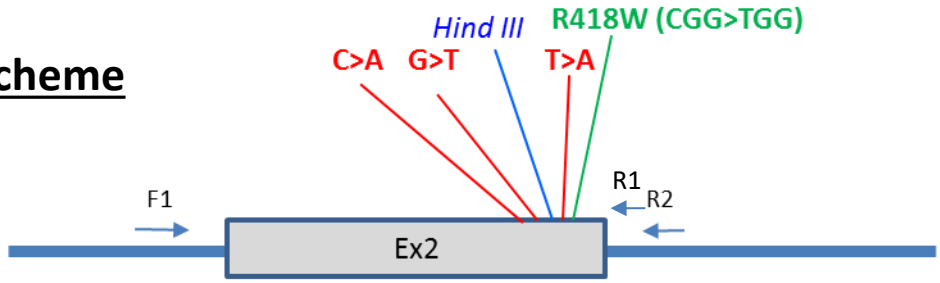
GENOTYPING INSTRUCTIONS

Genotyping Scheme



Sequence PCR fragment F1-R2 (F1-R1)

CAGGCCCGCTGTGTTCCTGAGTGTCTTGTTTTCTCCCTCTGCAGCTTGTCTCCAATGTCCTCATCTTCTCCTGCACCAACATTGTG
GGTGTGTGCACTCACTACCCAGCTGAGGTCTCCCAGAGACAGGCCTTCCAGGAGACACGTGAGTGTATCCAAGCTTGGCTCCATTC
CCAGCGGGAGAACCAGCAACAGGTGAGTGAAGAGGAGCCAGGAGGCCTGGATGTCAGCCAAGGCCTGCTGCAGACACACAAGCAAA
CGGCTGGCTTGCGATCTGCCTGCCTCTCCCCCCAGGCCATGCTCATCCCTCTGCCCAGTCCCTCCTCCTAGCAGTATTTCCTGCAG
GGAGCCCGACTCTGTGGTTCC

PCR genotyping strategy

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

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	0,4µl
- 5' primer (100 µM)	0.1µl
- 3' primer (100 µM)	0.1µl
- DNA (lysate 1/10)	2µl
- Phusion Hot Start II	0,2µl
- 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