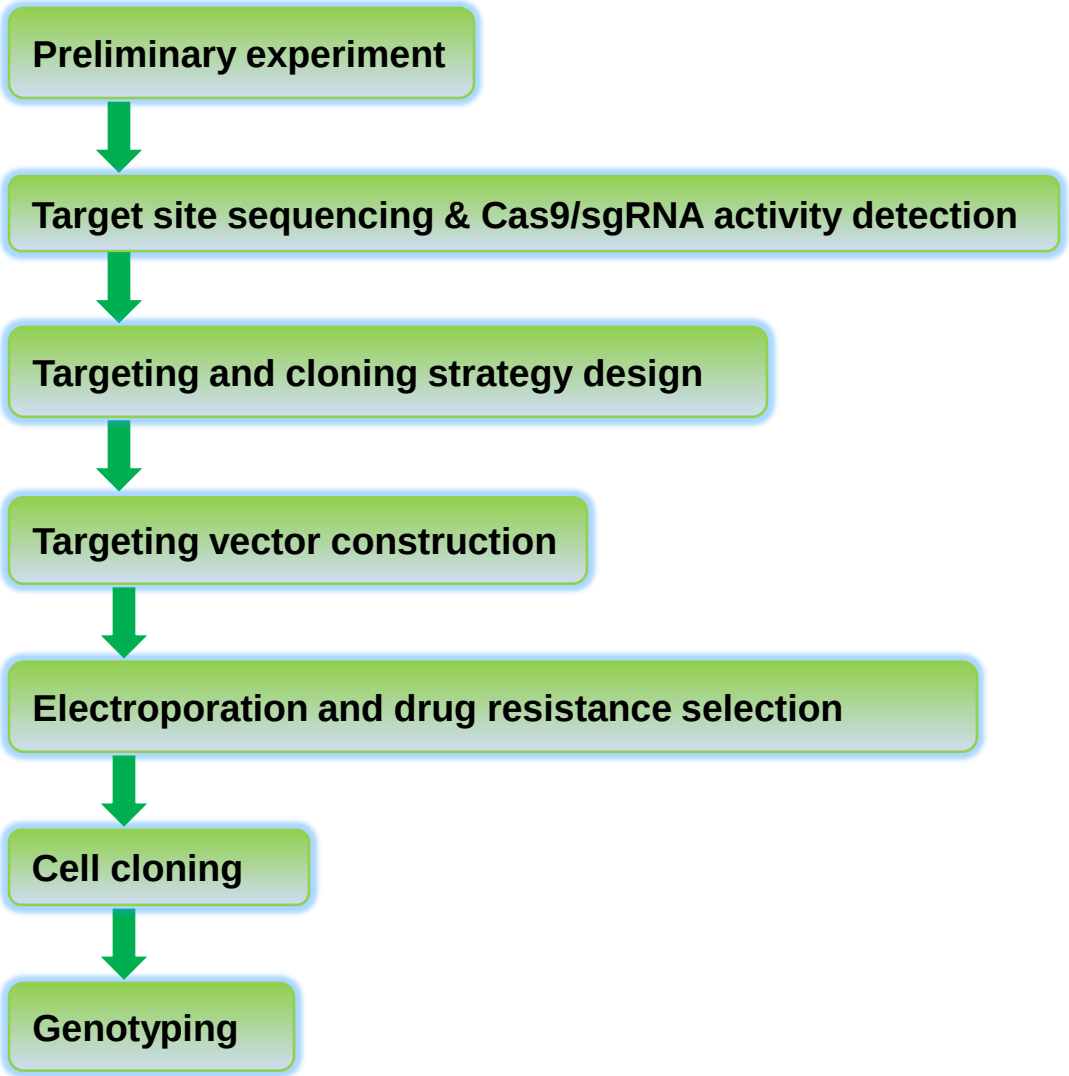
Project status report

2017.05.25~2018.08.15

Project: *CL-CYH-008* KI S18 cell line

Work Flow


Preliminary experiment
Target site sequencing & Cas9/sgRNA activity detection
Targeting and cloning strategy design
Targeting vector construction
Electroporation and drug resistance selection
Cell cloning
Genotyping

Part 1
Preliminary experiment

Titration of Antibiotic

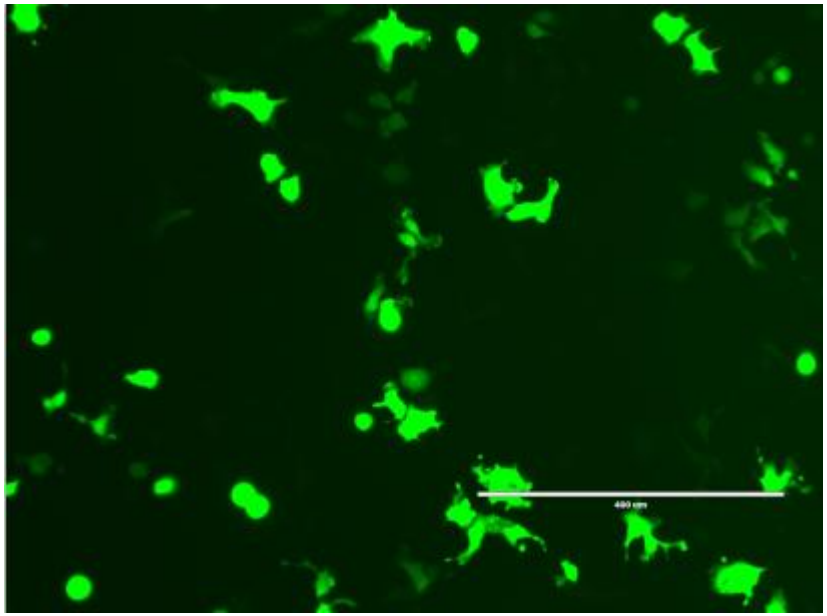
Antibiotic	Concentration (μg/mL)
Puromycin	0.5
G418	1200

Transfection Efficiency Optimisation

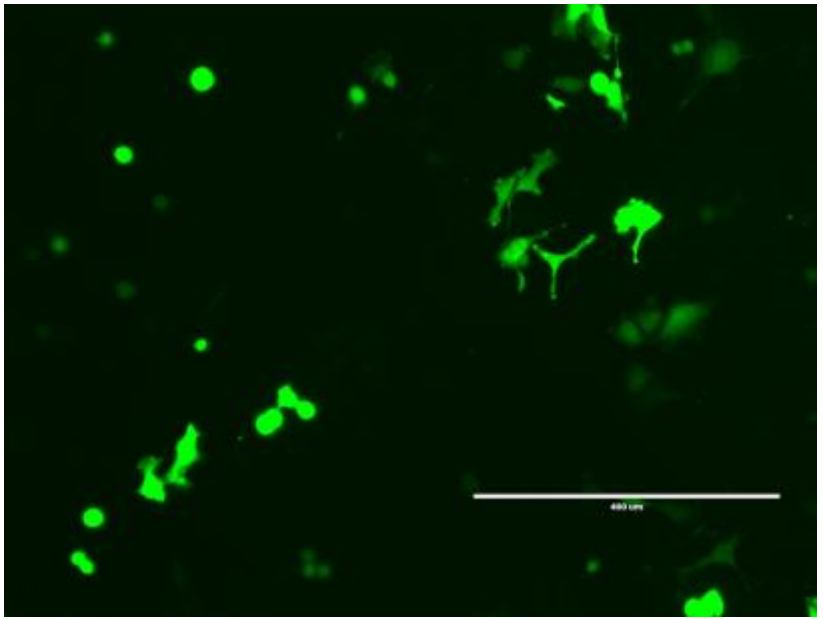
No.	Pulse Voltage	Pulse Width	Pulse no.	Transfection Efficiency	Cell Viability
1	1000	40	1	29.9%	70%
2	900	40	1	23.3%	73%

FACS analysis

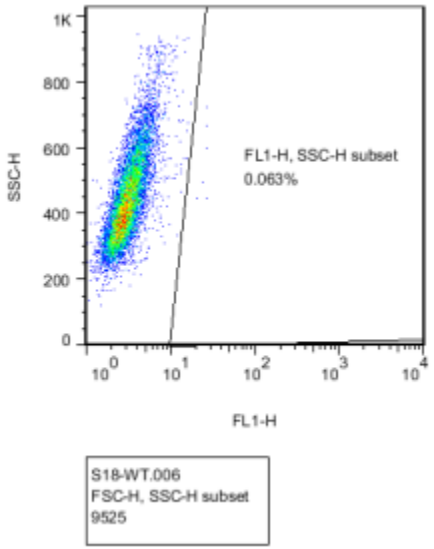
900v 40ms 1pulse

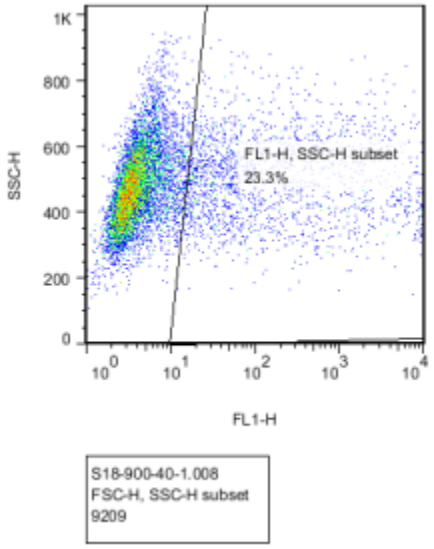


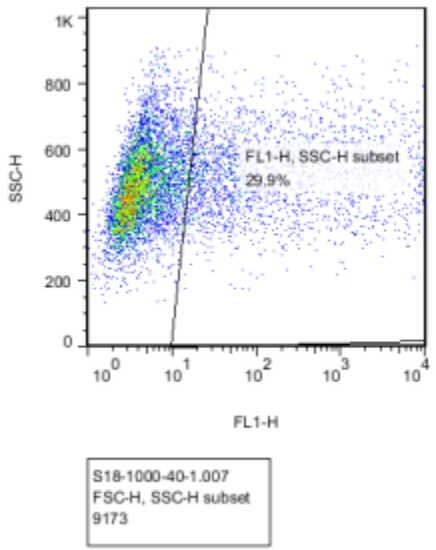
1000v 40ms 1pulse



FACS analysis







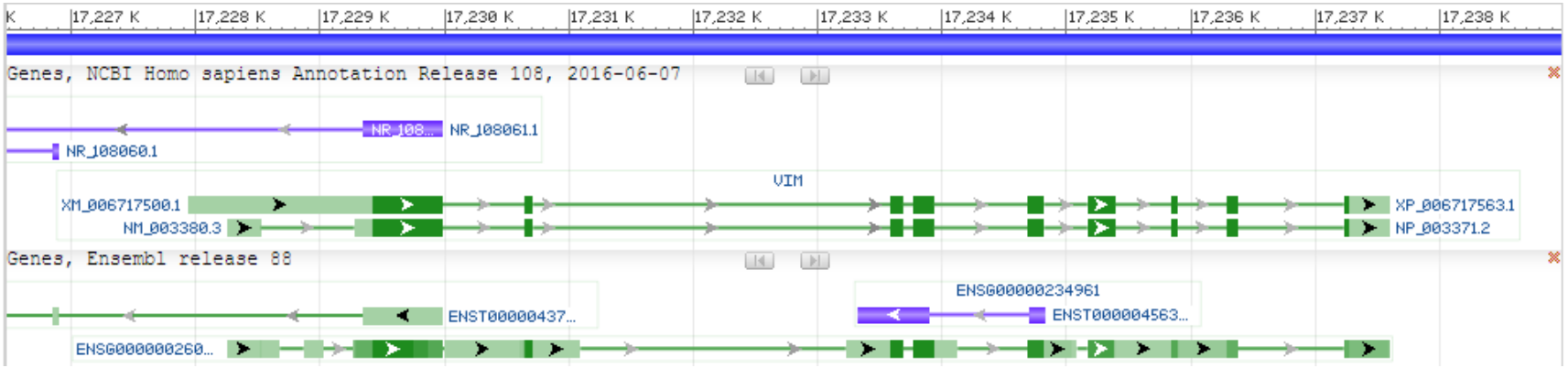
Conclusion

S18 cell line can be modified.

Part 2
Targeting and cloning strategy design

I. Background

1. Gene ID: 7431
2. Human *CL-CYH-008* gene spans about 9.7kb on chromosome 10 forward strand.



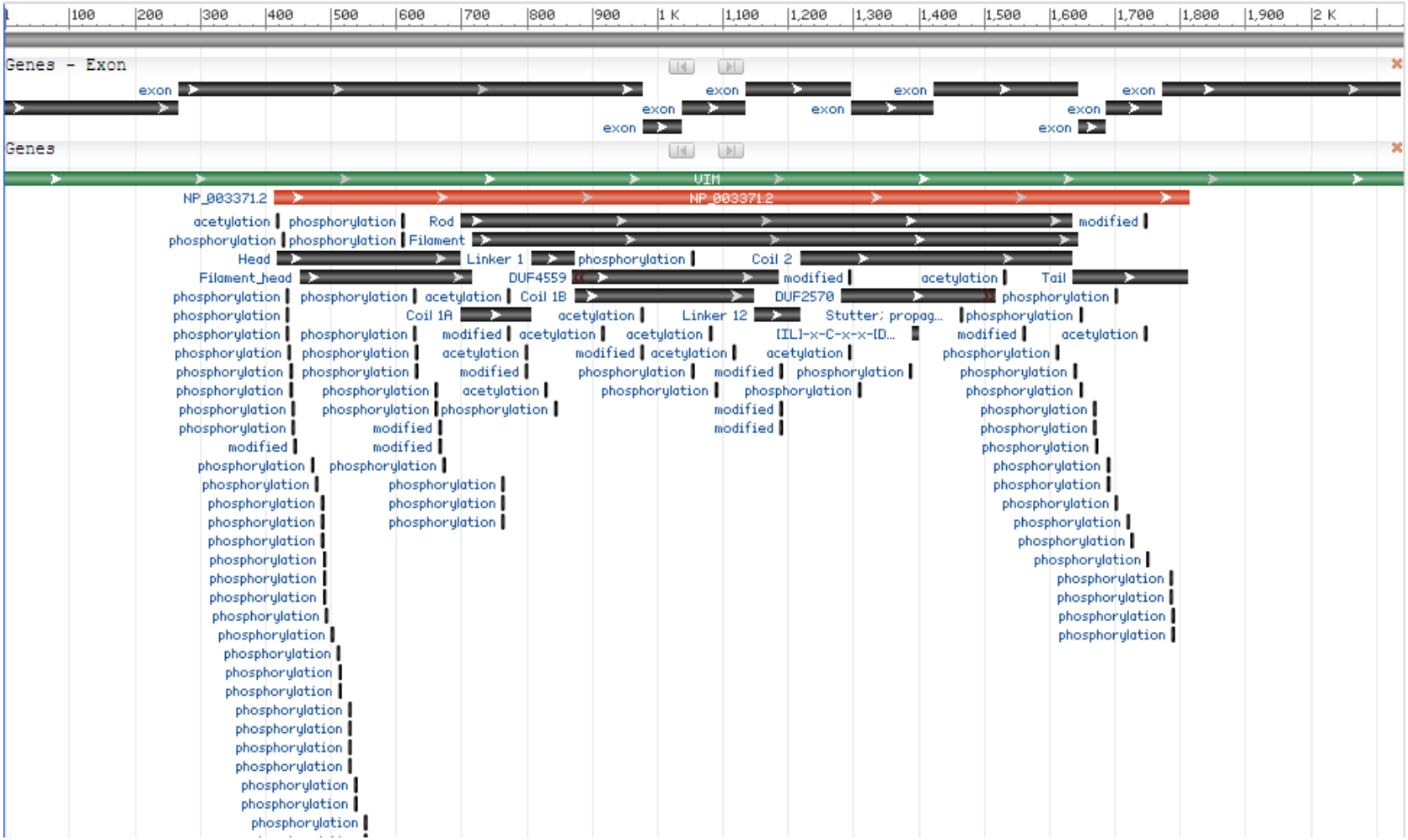
I. Background

3. There are 10 transcripts in this gene.

Name	Transcript ID	bp	Protein	Biotype	CCDS	UniProt	RefSeq	Flags
VIM-004	ENST00000544301.6	2135	466aa	Protein coding	CCDS7120	P08670 V9HWE1	NM_003380 NP_003371	TSL:1 APPRIS P1
VIM-001	ENST00000224237.9	1868	466aa	Protein coding	CCDS7120	P08670 V9HWE1	-	TSL:1 GENCODE basic APPRIS P1
VIM-005	ENST00000478746.1	757	149aa	Protein coding	-	A0A1B0GTT5	-	CDS 3' incomplete TSL:2
VIM-006	ENST00000497849.1	634	109aa	Protein coding	-	A0A1B0GVG8	-	CDS 3' incomplete TSL:2
VIM-003	ENST00000469543.5	2666	228aa	Nonsense mediated decay	-	B0YJC5	-	TSL:2
VIM-002	ENST00000487938.5	2272	431aa	Nonsense mediated decay	-	B0YJC4	-	TSL:5
VIM-007	ENST00000485947.1	1139	No protein	Processed transcript	-	-	-	TSL:2
VIM-010	ENST00000637053.1	508	No protein	Processed transcript	-	-	-	TSL:2
VIM-009	ENST00000495528.1	962	No protein	Retained intron	-	-	-	TSL:2
VIM-008	ENST00000421459.2	753	No protein	Retained intron	-	-	-	TSL:2

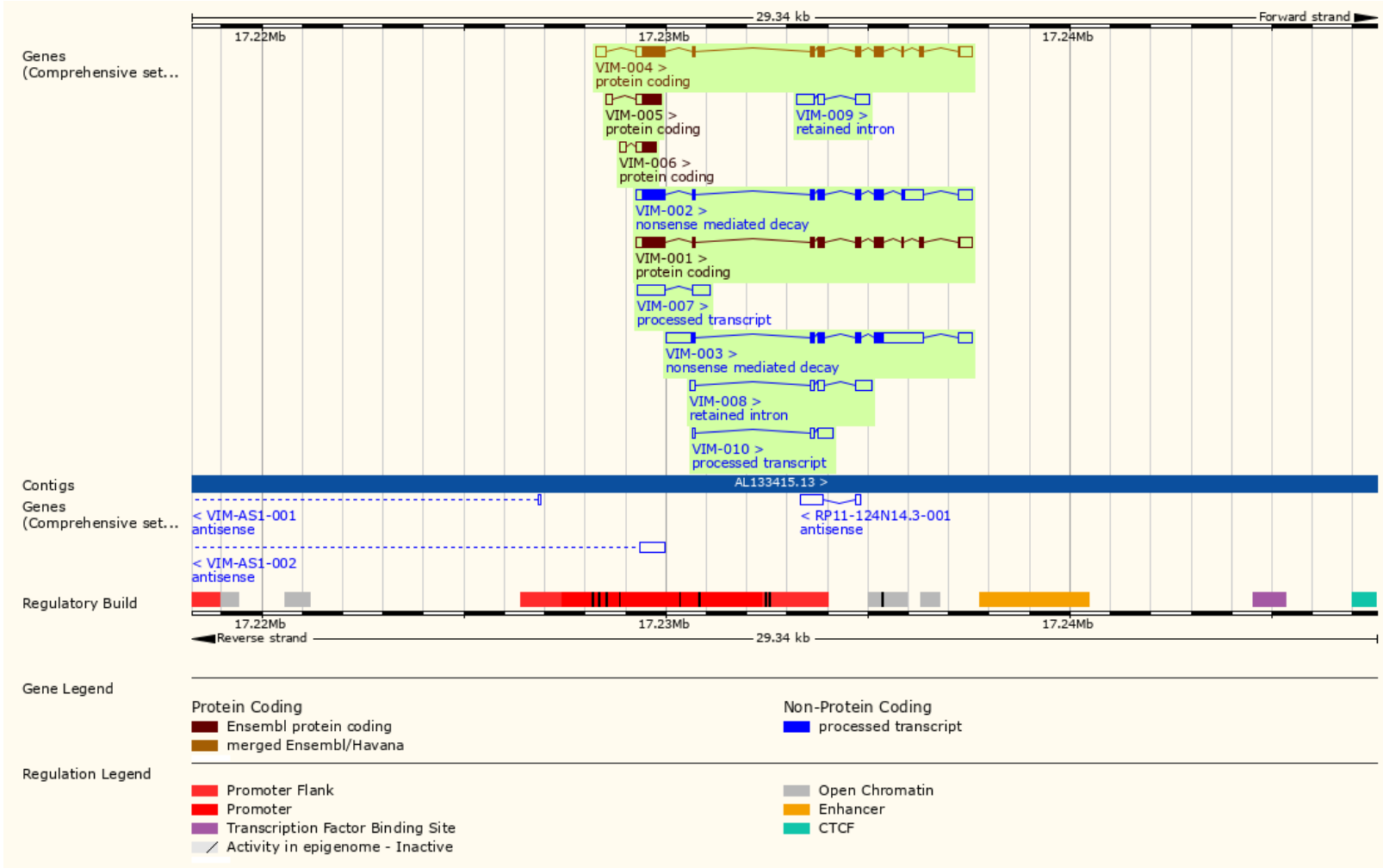
I. Background

4. Domains and features



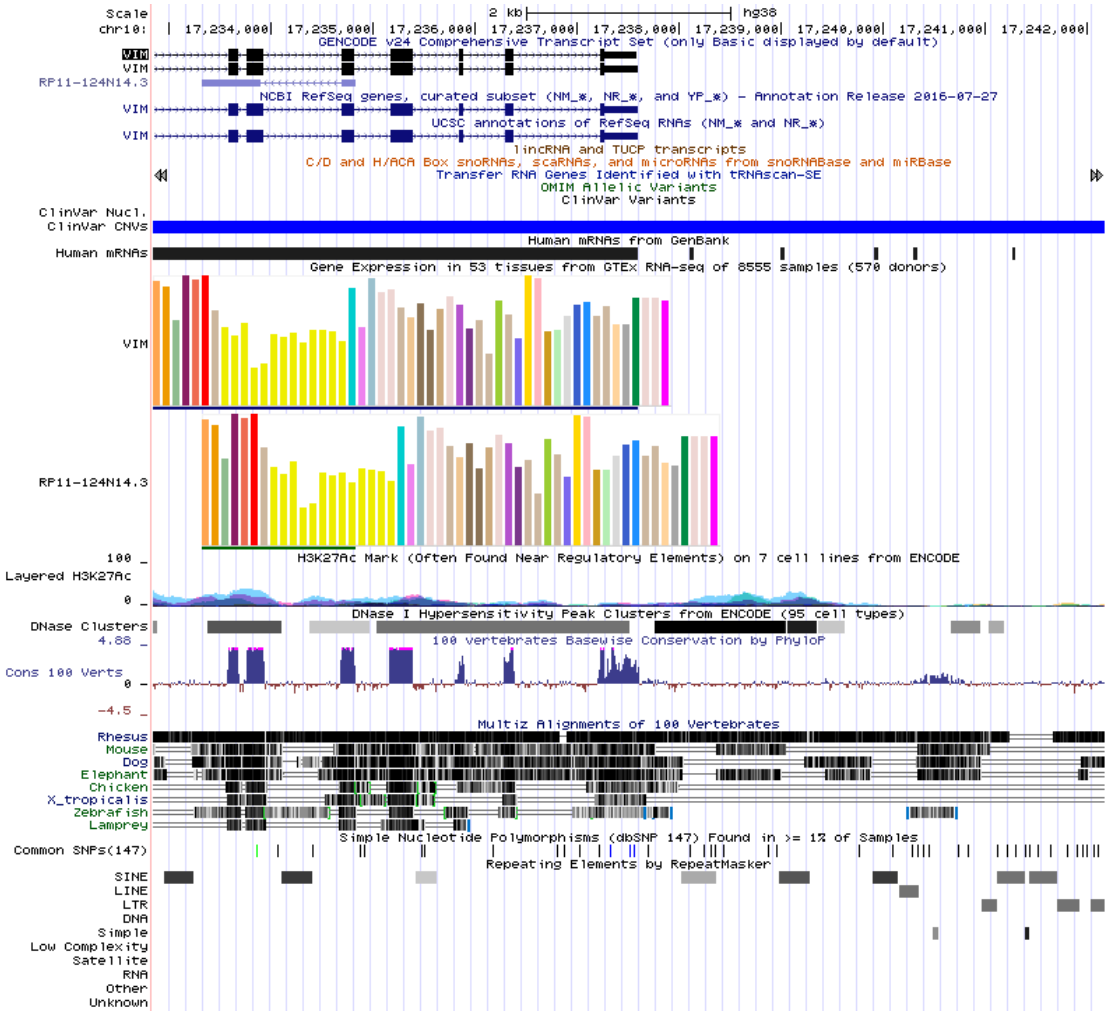
I. Background

5. Regulation



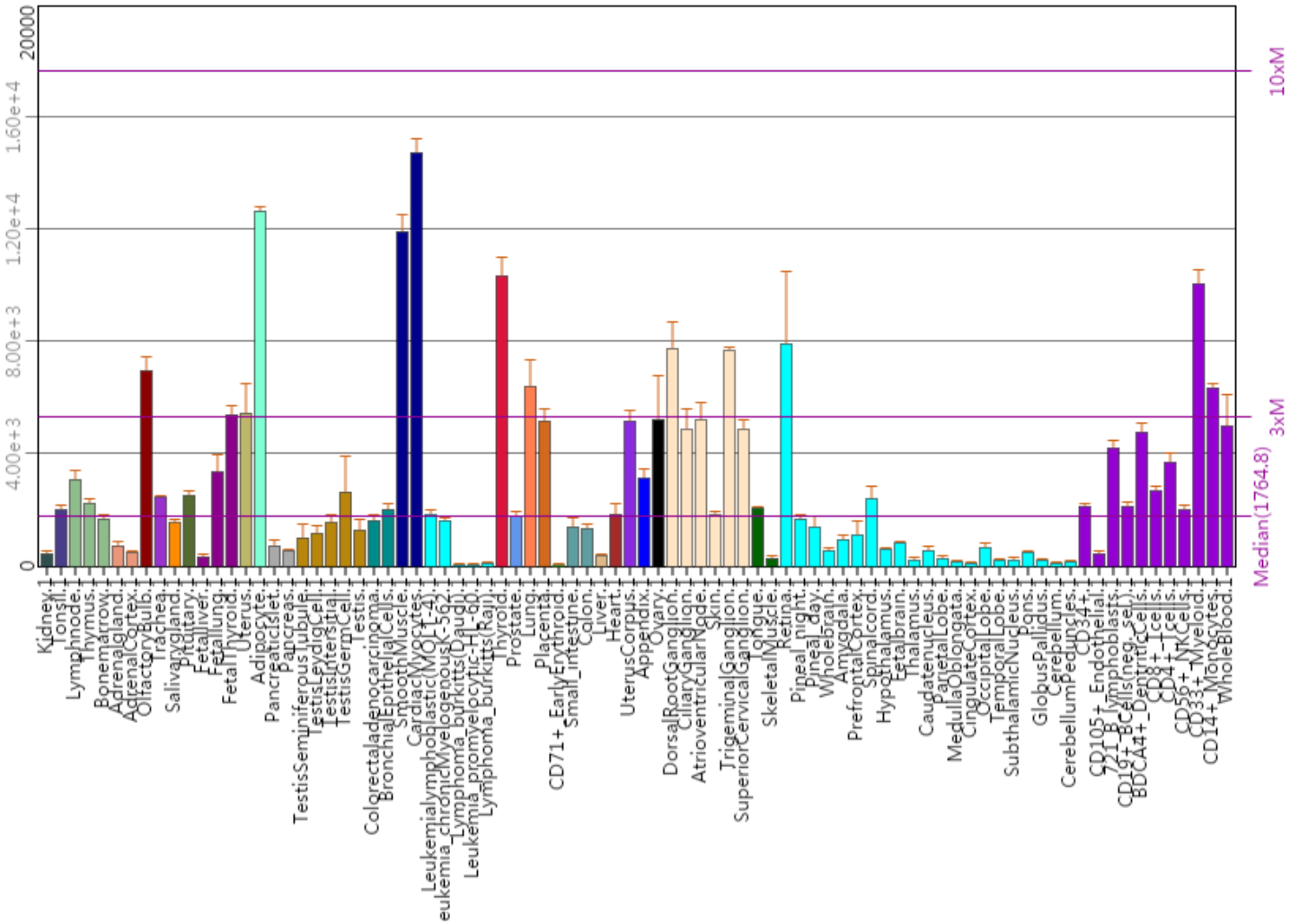
I. Background

6. Conservation



I. Background

7. Expression pattern (from BioGPS)



I. Background

8. Existing mouse models (from MGI)

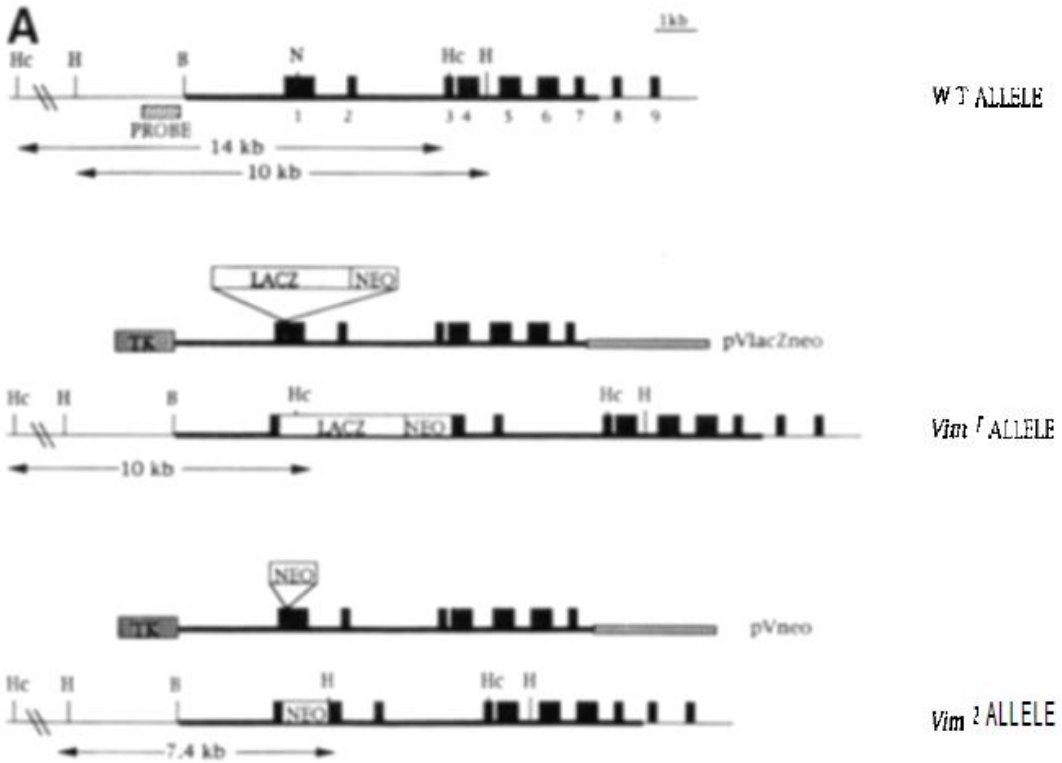
Allele Symbol Gene; Allele Name	Chr	Synonyms	Category	Abnormal Phenotypes Reported in these Systems	Human Disease Models
Vim^{tm1Cba} vimentin; targeted mutation 1, Charles Babinet	2	Vim-, Vim1	Targeted (Null/knockout, Reporter)	behavior, cardiovascular, cellular, hematopoietic, homeostasis, immune, mortality/aging, muscle, nervous system	
Vim^{tm2Cba} vimentin; targeted mutation 2, Charles Babinet	2	Vim-, Vim2	Targeted (Null/knockout)	cardiovascular, cellular	
Vim$^{tm1(KOMP)Vlcg}$ vimentin; targeted mutation 1, Velocigene	2		Targeted (Null/knockout, Reporter) **(Cell Line)**		
Vim$^{tm1a(EUCOMM)Wtsi}$ vimentin; targeted mutation 1a, Wellcome Trust Sanger Institute	2		Targeted (Conditional ready, Null/knockout, Reporter) **(Cell Line)**		
Vim$^{tm1e(EUCOMM)Wtsi}$ vimentin; targeted mutation 1e, Wellcome Trust Sanger Institute	2		Targeted (Null/knockout, Reporter) **(Cell Line)**		

http://www.informatics.jax.org/allele/summary?markerId=MGI:98932&alleleType=Targeted

I. Background

9. Targeting strategy of existing models

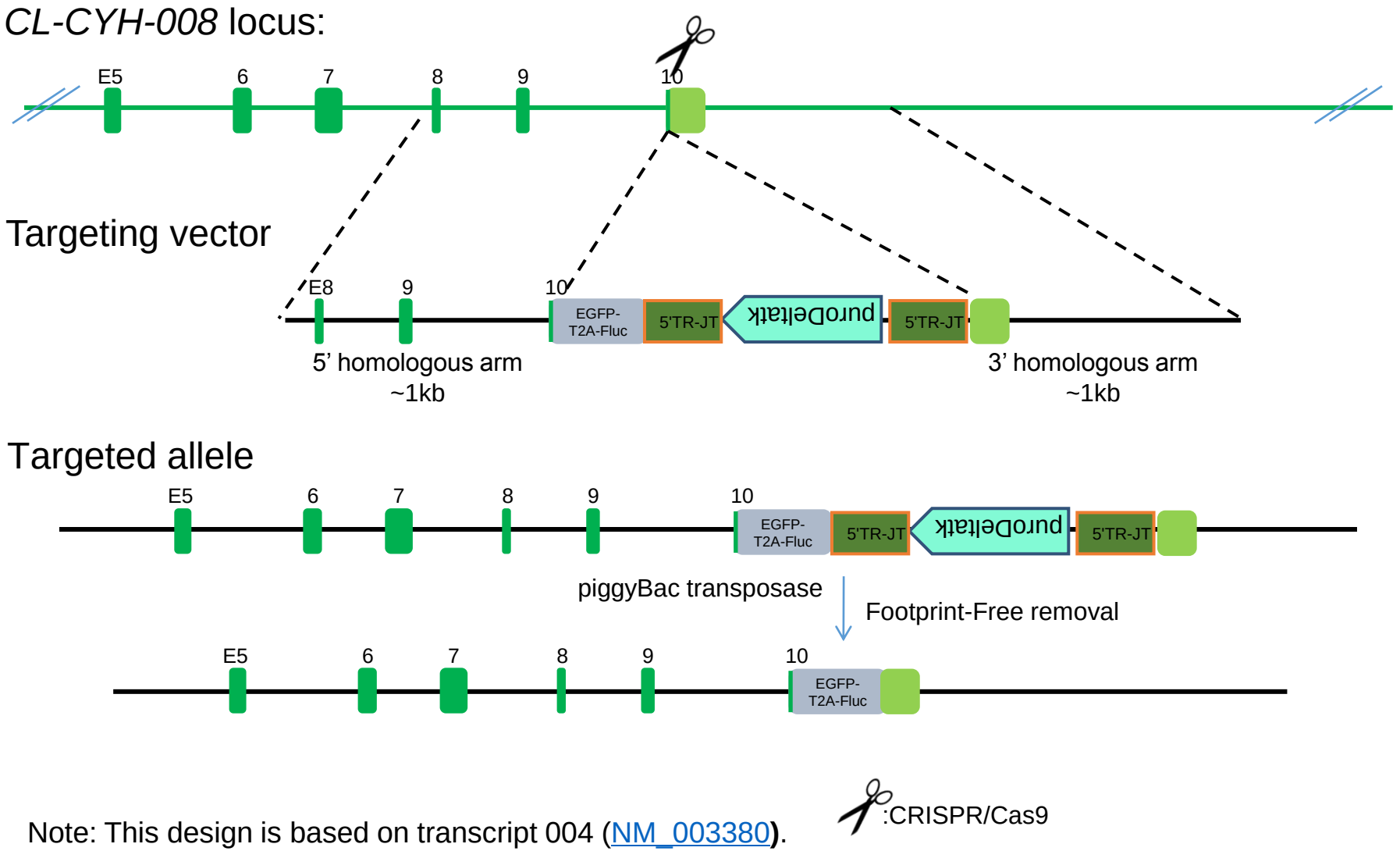
CL-CYH-008^{tm1Jjon}



Note:

The design for CL-CYH-008^{tm1Jjon} is based on Derksen PW, et al., Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. Cancer Cell. 2006 Nov;10(5):437-49.

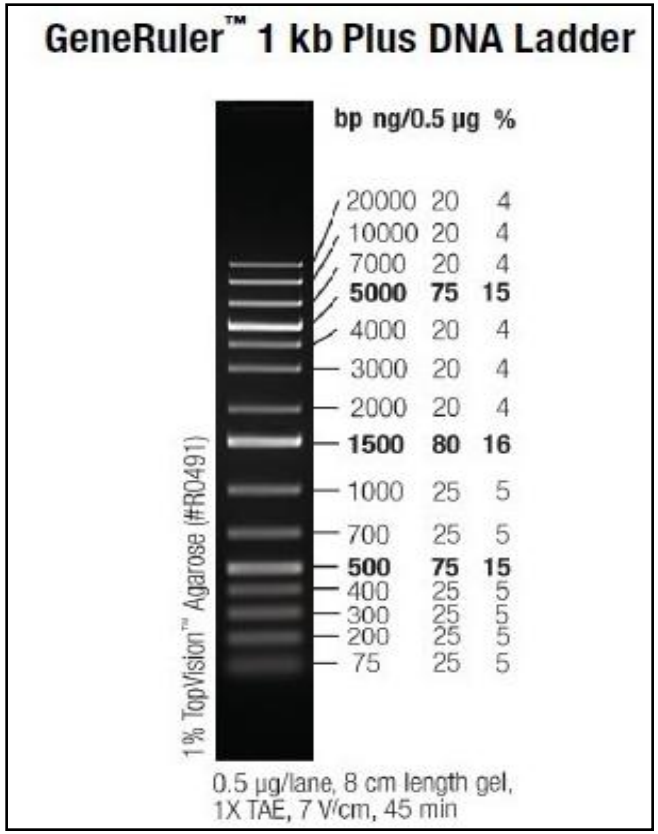
II. Targeting strategy (1) - EGE(CRISPR/Cas9) system



Note: This design is based on transcript 004 (NM_003380).

Marker used in the experiments

DNA electrophoresis

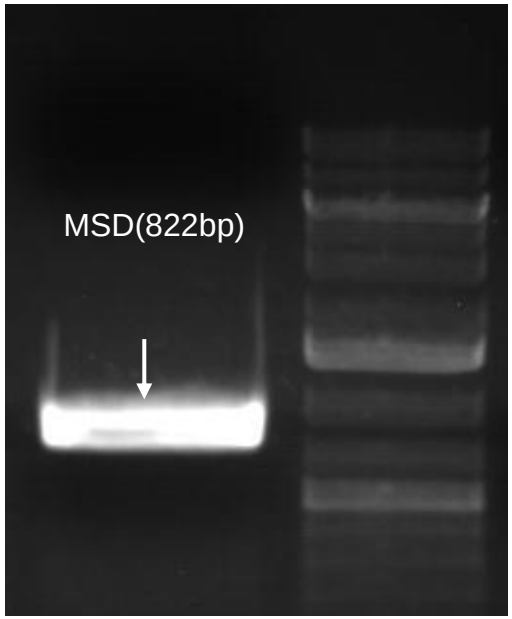


Part 3:
Cas9/sgRNA plasmid construction
&
UCA™ assay

I. Sequencing primer design of the target site

Primer	Sequence (5'-3')	Tm (℃)	Product size (bp)
CL-CYH-008-MSD-F	GCATCAAGCTTGGTACCGATTGCTTTTAAACT TGGCTGTATTGTGT	64	822
CL-CYH-008-MSD-R	ACTTAATCGTGGAGGATGATACTACATCTCAA GCATAAATAACCTGA	65	

PCR and sequencing of potential sgRNA target site:



Conclusion: The PCR products (S18 cell line) were sequenced and confirmed as same as those from the NCBI and Ensembl database.

II. sgRNA design

Guide	Score	Sequence (5'-3')
Guide #1	76	TCAGGAGCGCAAGATAGATT **TGG**
Guide #2	76	ACAAGACCCTCTTCCACTAC **AGG**
Guide #3	76	CAAGTTGGTTGGATACTTGC **TGG**
Guide #4	73	CAGCAAGTATCCAACCAACT **TGG**
Guide #5	68	GCGCAAGATAGATTTGGAAT **AGG**
Guide #6	67	CAGGCAATATAAGGATCCAG **TGG**
Guide #7	63	CTGCATGAGTGAAAACTAGA **AGG**

CRISPR design tool :http://crispr.mit.edu/

III. GuideRNA sequence

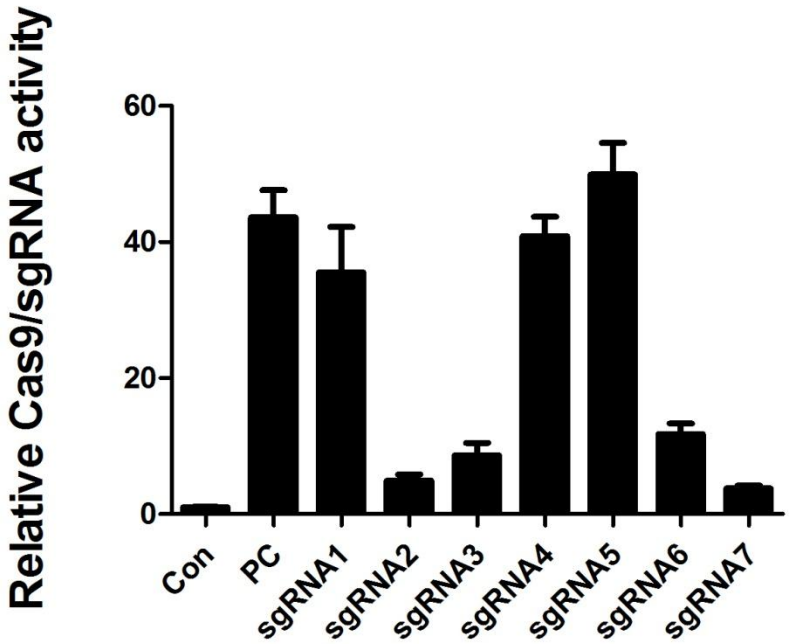
sgRNA	GuideRNA sequence
CL-CYH-008-sgRNA1	GGAGCGCAAGATAGATT
CL-CYH-008-sgRNA2	GGACAAGACCCTCTTCCACTAC
CL-CYH-008-sgRNA3	GGTTGGTTGGATACTTGC
CL-CYH-008-sgRNA4	GGCAAGTATCCAACCAACT
CL-CYH-008-sgRNA5	GGCGCAAGATAGATTTGGAAT
CL-CYH-008-sgRNA6	GGCAATATAAGGATCCAG
CL-CYH-008-sgRNA7	GGCATGAGTGAAAACTAGA

Cas9/sgRNA plasmid construction

Cas9/sgRNA plasmid construction was completed and the sequences were further confirmed by DNA sequencing.

UCA™ assay:

Note: UCA (Universal CRISPR Activity Assay), a sgRNA activity detection system developed by Biocytogen, is simpler and more sensitive than MSDase assay.



Conclusion: the sgRNA5 was used for next step.

Part 4:
Targeting vector construction

I. Cloning primer design

Primer	Sequence (5'-3')	Restriction enzyme	Tm(℃)	Product size(bp)	Template
CL-CYH-008-LR-F	atcgCTCGAGTTGGCGAGTAGTACTTTACACAATT	XhoI	65	8164	Synthesis
CL-CYH-008-RR-R	cagtGCGGCCGCTAGCTATCACACACACACACAATAA	NotI	69		

Fragment LR-A-RR (Synthesis) will be ligated into LScKO-4G vector

Restriction analysis: CL-CYH-008 KI targeting vector

1. Restriction enzyme: XhoI+NotI

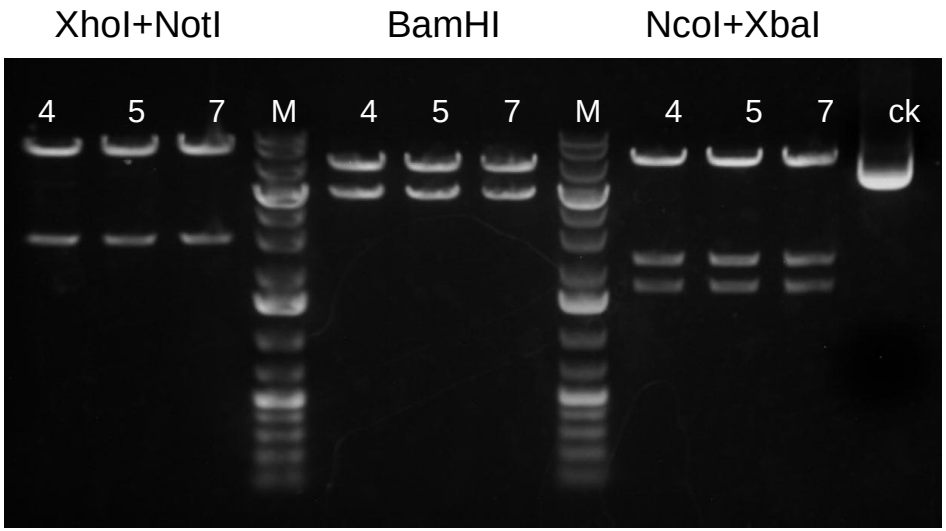
   Expected products: 8149bp+2773bp

2. Restriction enzyme: BamHI

   Expected products: 6419bp+4503bp

3. Restriction enzyme: NcoI+XbaI

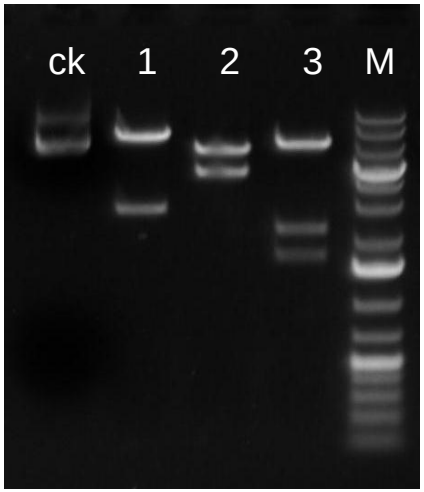
   Expected products: 7118bp+2198bp+1606bp



#4, #5 and #7 clones were correct. #5 and #7 were sent for DNA sequencing. Sequences of #5 and #7 were correct.

Restriction analysis: CL-CYH-008 KI targeting vector(#7) after maxi-prep

1. Restriction enzyme: XhoI+NotI
   Expected products: 8149bp+2773bp
2. Restriction enzyme: BamHI
   Expected products: 6419bp+4503bp
3. Restriction enzyme: NcoI+XbaI
   Expected products: 7118bp+2198bp+1606bp



Note: The number labeled in the pictures indicates the group number of restriction enzyme showed above.

Part 5
Electroporation & Cell cloning

Electroporation and clone screening

- sgRNA5 and CL-CYH-008-targeting vector were electroporated into S18 cell line.
- Drug resistance selection.
- Screening of mix colonies by PCR.
- Clonal growth of cells in semisolid media.
- 96 resistant clones were picked and expanded for further analysis.
- 96 colonies were ready for genotyping.

Electroporation and clone screening

- CL-CYH-008-piggyBac vector was electroporated into mix colonies.
- Drug resistance selection.
- Screening of mix colonies by PCR.
- Clonal growth of cells in semisolid media.
- 96 resistant clones were picked and expanded for further analysis.
- 96 colonies were ready for genotyping.

Electroporation and clone screening

- PCR screening was performed.
- Amplification and cryopreservation of posotive colonies.

Electroporation and clone screening(the 2nd)

- sgRNA5 and CL-CYH-008-targeting vector were electroporated into S18 cell line.
- Drug resistance selection.
- Screening of mix colonies by PCR.
- Clonal growth of cells in semisolid media.
- CL-CYH-008-piggyBac vector was electroporated into mix colonies.
- Drug resistance selection.

Electroporation and clone screening(the 2nd)

- 96 resistant clones were picked and expanded for further analysis.(CL-CYH-008 2#)
- 5 colonies were ready for genotyping. (CL-CYH-008 2#)
- 144 resistant clones were picked and expanded for further analysis. (CL-CYH-008-PB 2#)

Electroporation and clone screening

- 2 colonies were ready for genotyping. (CL-CYH-008 2#)
- PCR screening was performed. (CL-CYH-008 2#)
- Amplification and cryopreservation of posotive colonies. (CL-CYH-008 2#)
- 19 colonies were ready for genotyping. (CL-CYH-008-PB 2#)

Electroporation and clone screening

- PCR screening was performed. (CL-CYH-008-PB 2#)
- Amplification and cryopreservation of posotive colonies. (CL-CYH-008-PB 2#)
- Clonal growth of cells in semisolid media. (CL-CYH-008 2#)
- 96 resistant clones were picked and expanded for further analysis. (CL-CYH-008 2#)

Electroporation and clone screening

- 95 colonies were ready for genotyping.(CL-CYH-008 2#)
- PCR screening was performed. (CL-CYH-008 2#)
- Amplification and cryopreservation of posotive colonies.(CL-CYH-008 2#)
- CL-CYH-008-piggyBac vector was electroporated into 3-B5 . (CL-CYH-008-PB 3#)
- Drug resistance selection. (CL-CYH-008-PB 3#)

Electroporation and clone screening

- Drug resistance selection. (CL-CYH-008-PB 3#)
- Screening of mix colonies by PCR. (CL-CYH-008-PB 3#)
- Clonal growth of cells in semisolid media. (CL-CYH-008-PB 3#)

Electroporation and clone screening

- 96 resistant clones were picked and expanded for further analysis. (CL-CYH-008-PB 3#)
- 26 colonies were ready for genotyping. (CL-CYH-008-PBc 3#)
- PCR screening was performed. (CL-CYH-008-PB 3#)
- CL-CYH-008-piggyBac vector was electroporated into 1-F6. (CL-CYH-008-PB 4#)
- Drug resistance selection. (CL-CYH-008-PB 4#)

Electroporation and clone screening

- Amplification and cryopreservation of posotive colonies.(CL-CYH-008-PB 3#)
- Screening of mix colonies by PCR.(CL-CYH-008-PB 4#)
- Clonal growth of cells in semisolid media. (CL-CYH-008-PB 4#)

Electroporation and clone screening

- 96 resistant clones were picked and expanded for further analysis.(CL-CYH-008-PB 4#)
- 96 colonies were ready for genotyping.(CL-CYH-008-PB 4#)

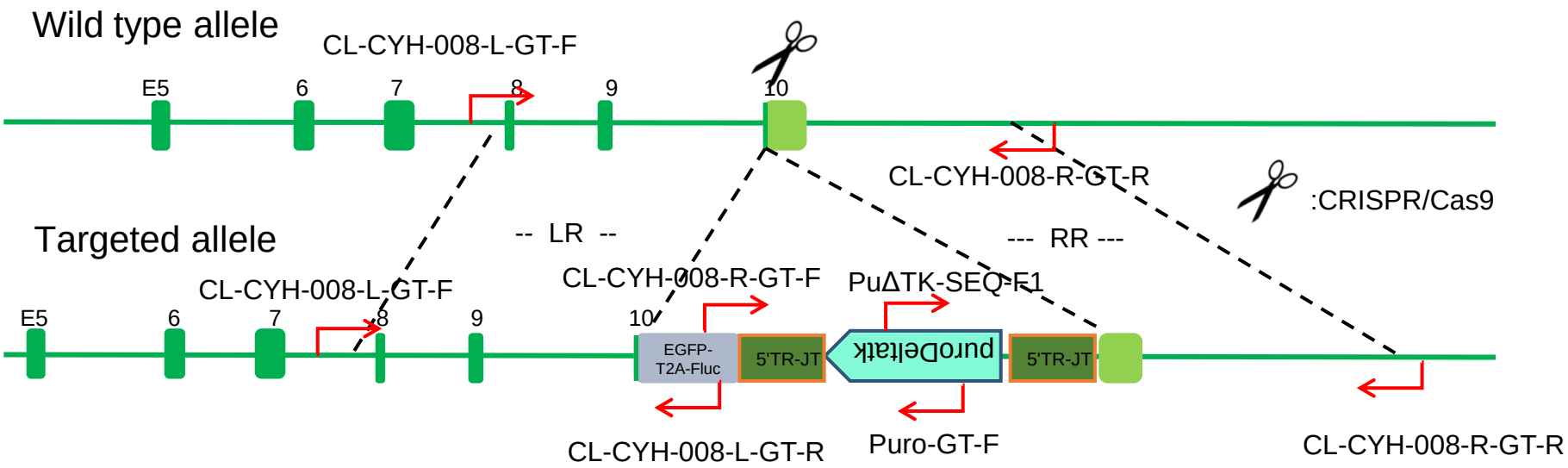
Electroporation and clone screening

- PCR screening was performed.(CL-CYH-008-PB 4#)

Electroporation and clone screening

- Amplification and cryopreservation of posotive colonies.(CL-CYH-008-PB 4#)

Screening of mix colonies-Primer design



Screening of mix colonies-Primer design

Primer	Sequence (5'-3')	Tm(℃)	Product size
CL-CYH-008-L-GT-F	TCCTGCTGCAAGTACTATCTCATCC	59	Mut: 2901bp
CL-CYH-008-L-GT-R	GTTGCTTAGGTCGTACTTGTCGATG	59	
CL-CYH-008-R-GT-F	ATGGATAGCAAGACCGACTACCAGG	61	Mut: 3301bp
Puro-GT-F	GCAACAGATGGAAGGCCTCCTGGCG	67	
PuΔTK-SEQ-F1	AGTAGCGTGGGCATGGATCC	61	Mut: 3113bp
CL-CYH-008-R-GT-R	CGAAGTTTTCACTCCAGAACACACA	59	

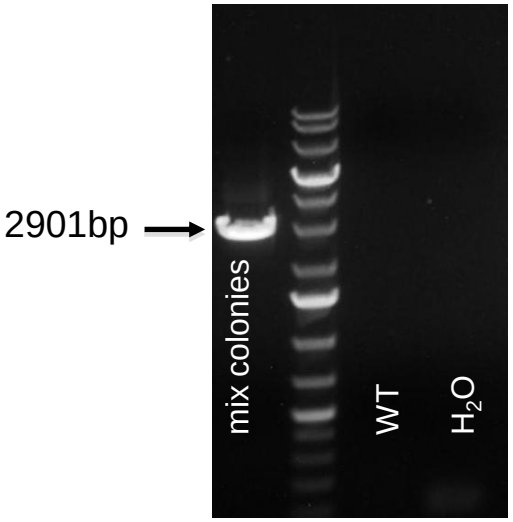
Enzyme: KOD-FX

Program: Touchdown PCR

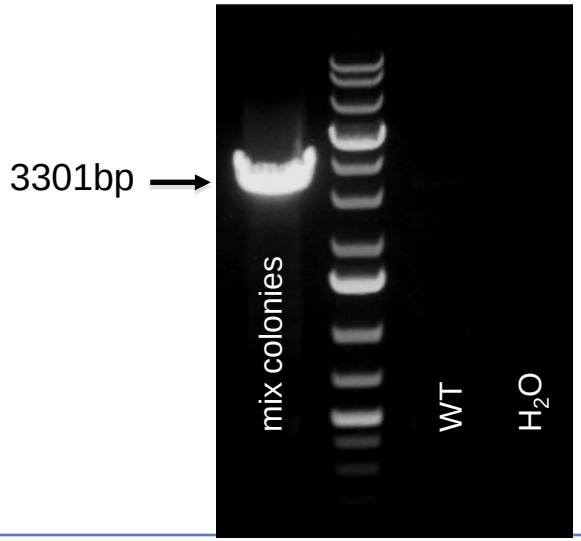
94 ℃	2 min	
98 ℃	10 sec	15 cycles
67 ℃	30 sec（- 0.7℃/cycle）	
68 ℃	1 kb / min	
98 ℃	10 sec	25 cycles
57 ℃	30 sec	
68 ℃	1 kb / min	
68 ℃	10 min	
4 ℃	forever	

Screening of mix colonies-Genotyping

Primers: CL-CYH-008-L-GT-F/CL-CYH-008-L-GT-R

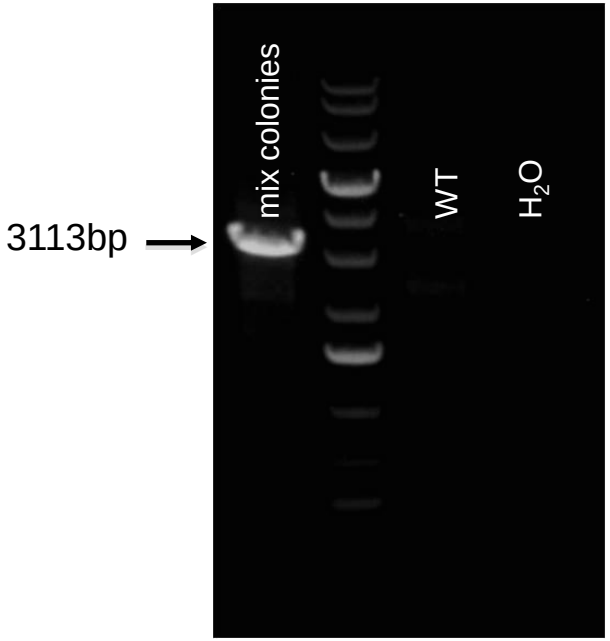


Primers: CL-CYH-008-R-GT-F/Puro-GT-F



Screening of mix colonies-Genotyping

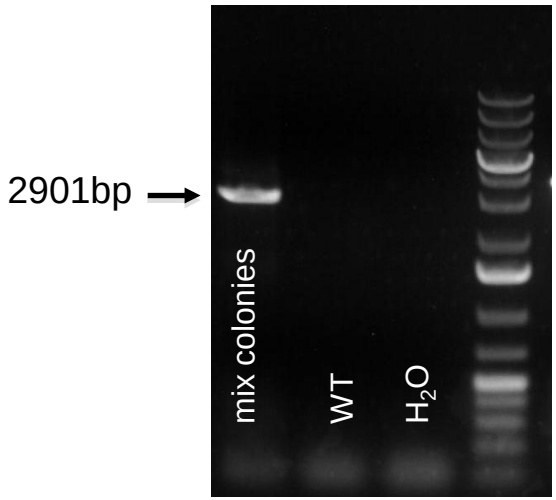
Primers: PuΔTK-SEQ-F1/CL-CYH-008-R-GT-R



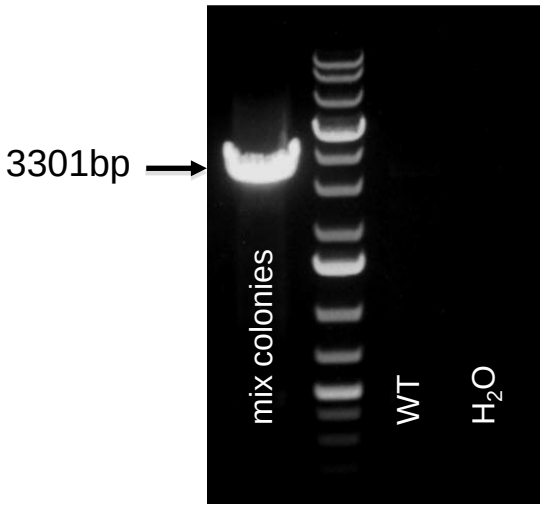
Positive colonies will be picked and expanded for further analysis.

Screening of mix colonies-Genotyping(the 2nd time)

Primers: CL-CYH-008-L-GT-F/CL-CYH-008-L-GT-R

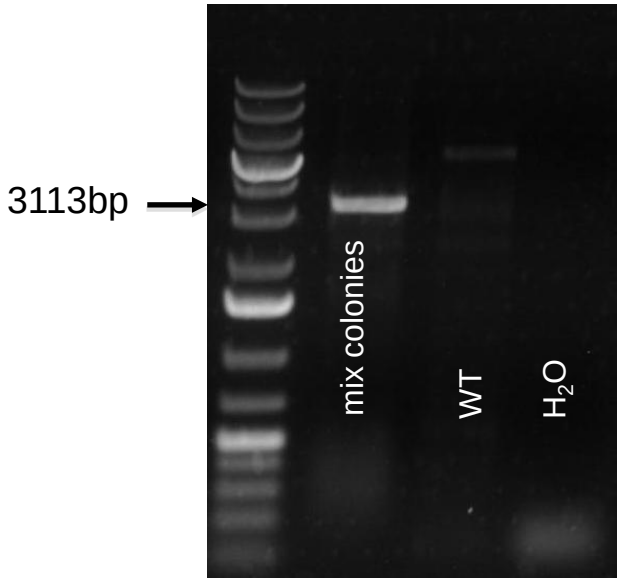


Primers: CL-CYH-008-R-GT-F/Puro-GT-F



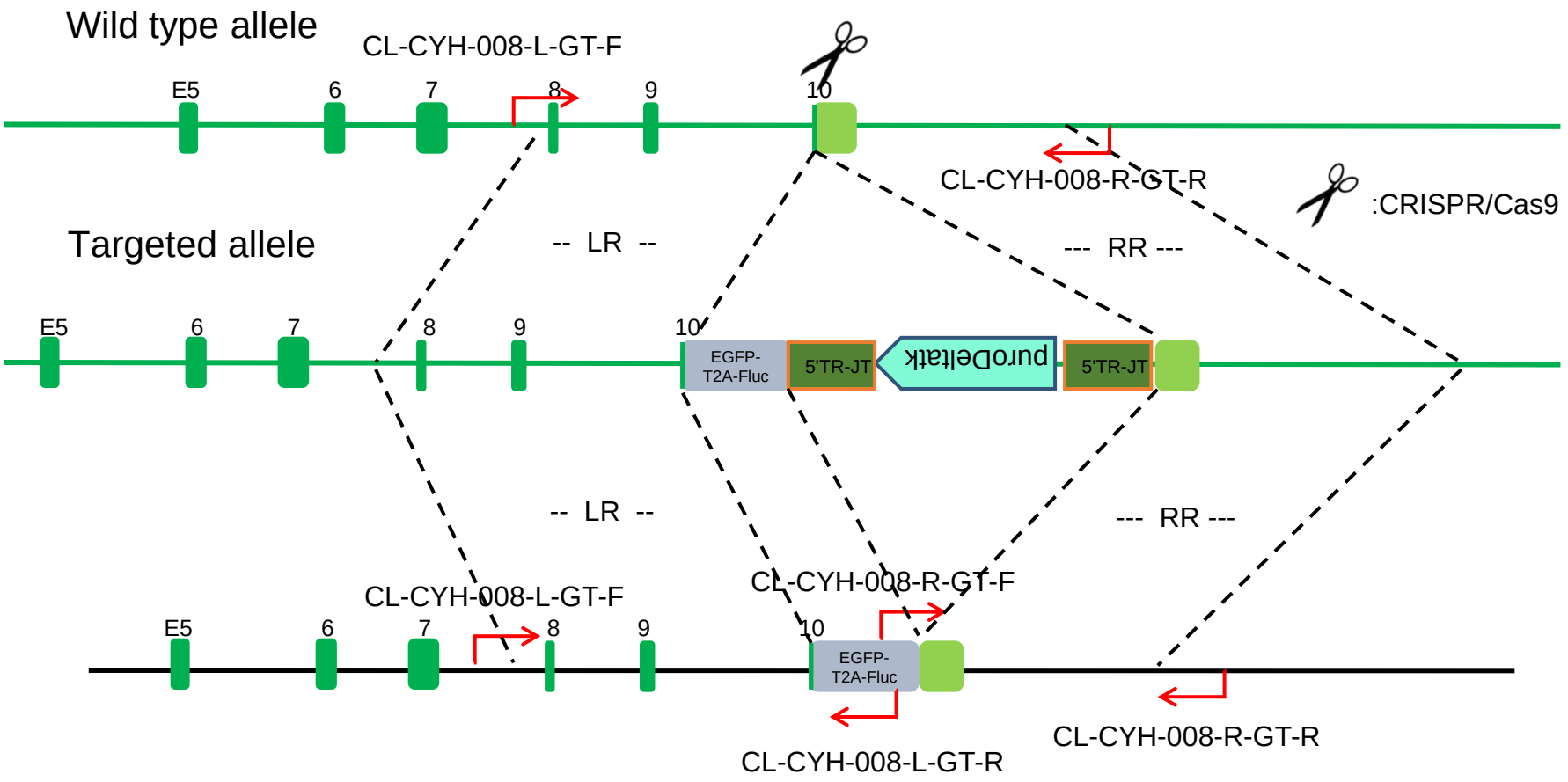
Screening of mix colonies-Genotyping(the 2nd time)

Primers: PuΔTK-SEQ-F1/CL-CYH-008-R-GT-R



Positive colonies will be picked and expanded for further analysis.

Screening of mix colonies-piggyBac-Primer design



Screening of mix colonies-piggyBac-Primer design

Primer	Sequence (5'-3')	Tm(℃)	Product size
CL-CYH-008-L-GT-F	TCCTGCTGCAAGTACTATCTCATCC	59	Mut: 2901bp
CL-CYH-008-L-GT-R	GTTGCTTAGGTCGTACTTGTCGATG	59	
CL-CYH-008-R-GT-F	ATGGATAGCAAGACCGACTACCAGG	61	Mut: 2680bp
CL-CYH-008-R-GT-R	CGAAGTTTTCACTCCAGAACACACA	60	

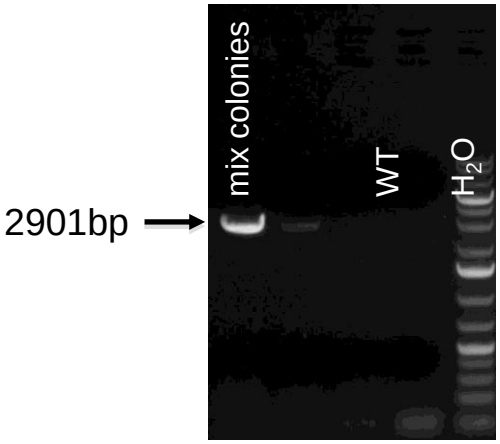
Enzyme: KOD-FX

Program: Touchdown PCR

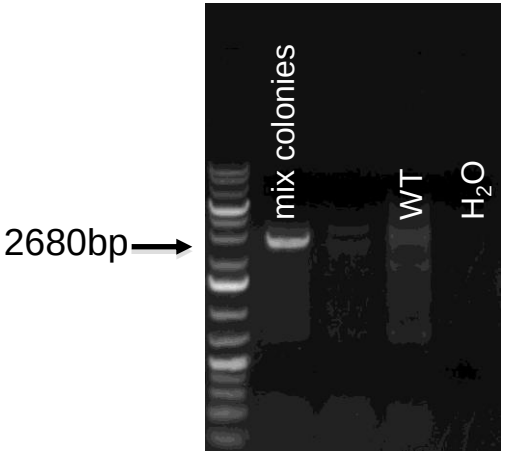
94 ℃	2 min	
98 ℃	10 sec	15 cycles
67 ℃	30 sec（- 0.7℃/cycle）	
68 ℃	1 kb / min	
98 ℃	10 sec	25 cycles
57℃	30 sec	
68 ℃	1 kb / min	
68 ℃	10 min	
4 ℃	forever	

Screening of mix colonies-Genotyping-piggyBac

Primers: CL-CYH-008-L-GT-F/CL-CYH-008-L-GT-R



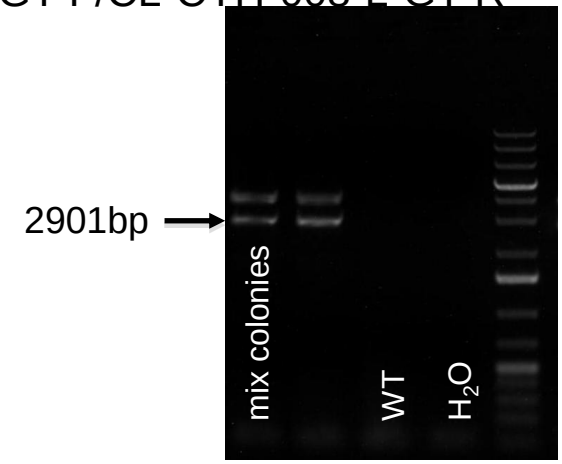
Primers: CL-CYH-008-R-GT-F/ CL-CYH-008-R-GT-R



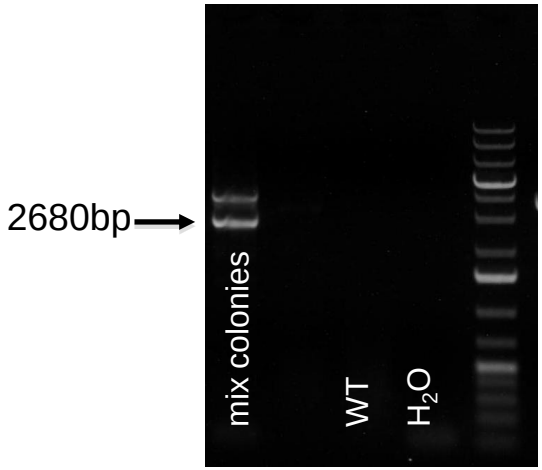
Positive colonies will be picked and expanded for further analysis.

Screening of mix colonies-Genotyping-piggyBac(the 2nd time)

Primers: CL-CYH-008-L-GT-F/CL-CYH-008-L-GT-R



Primers: CL-CYH-008-R-GT-F/ CL-CYH-008-R-GT-R

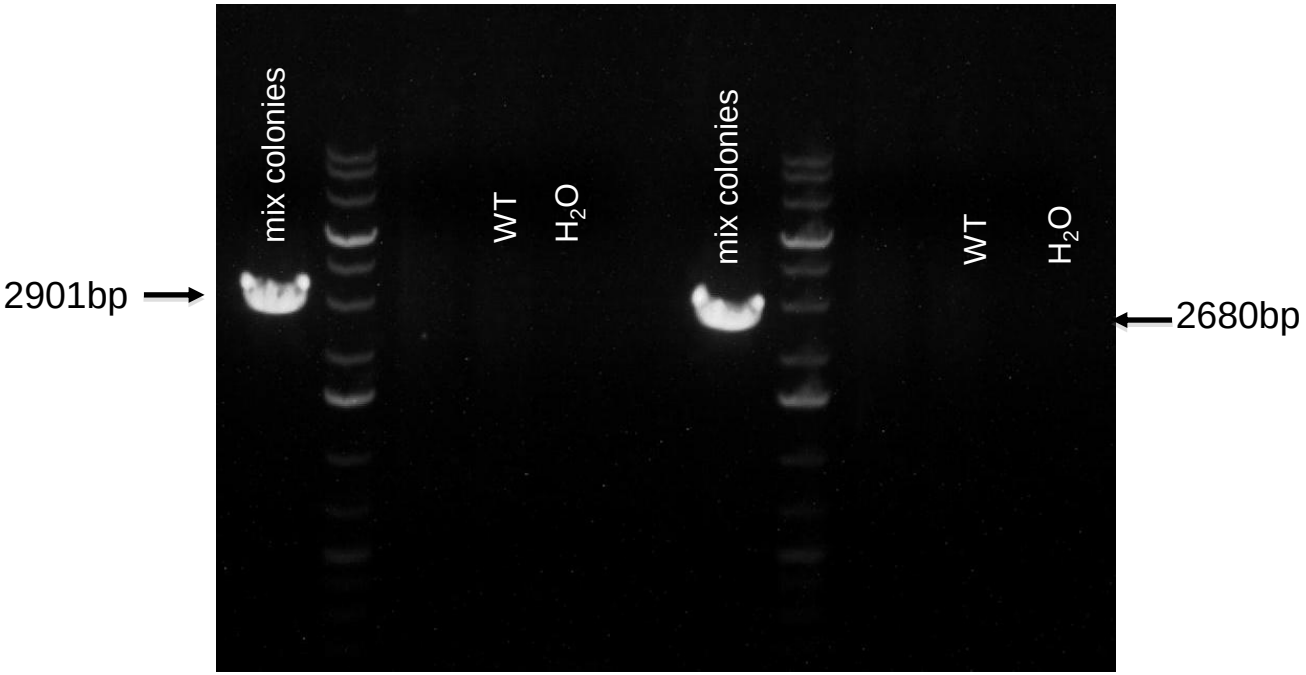


Positive colonies will be picked and expanded for further analysis.

Screening of mix colonies-Genotyping-piggyBac(the 3rd time)

Primers: CL-CYH-008-L-GT-F/CL-CYH-008-L-GT-R

Primers: CL-CYH-008-R-GT-F/ CL-CYH-008-R-GT-R

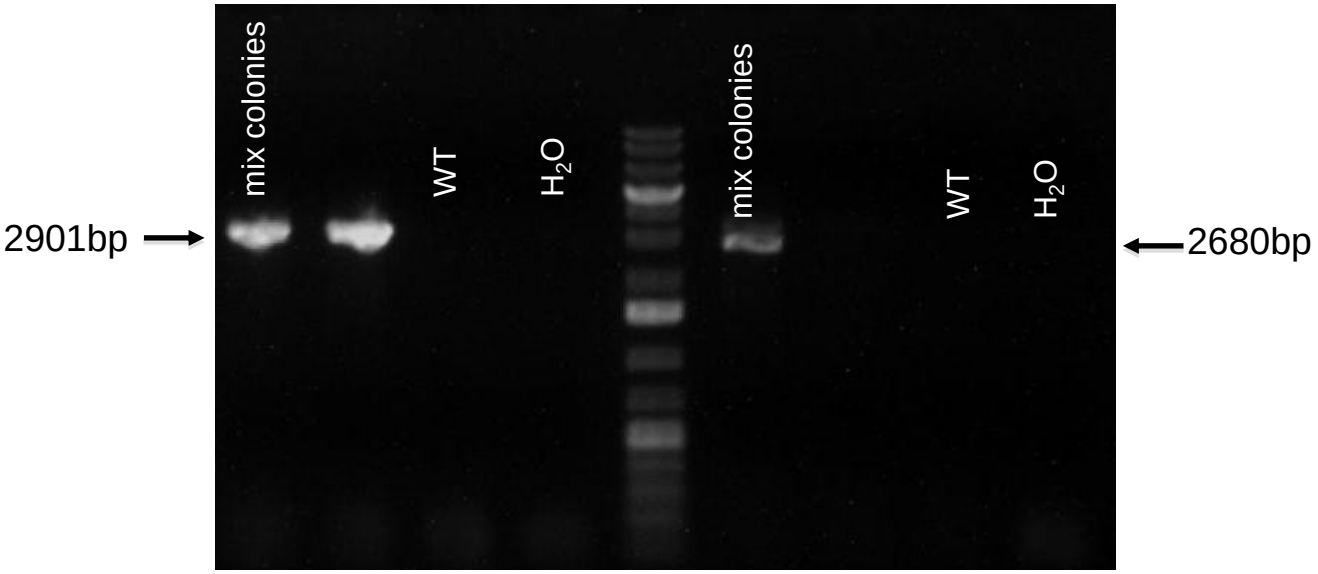


Positive colonies will be picked and expanded for further analysis.

Screening of mix colonies-Genotyping-piggyBac(the 4th time)

Primers: CL-CYH-008-L-GT-F/CL-CYH-008-L-GT-R

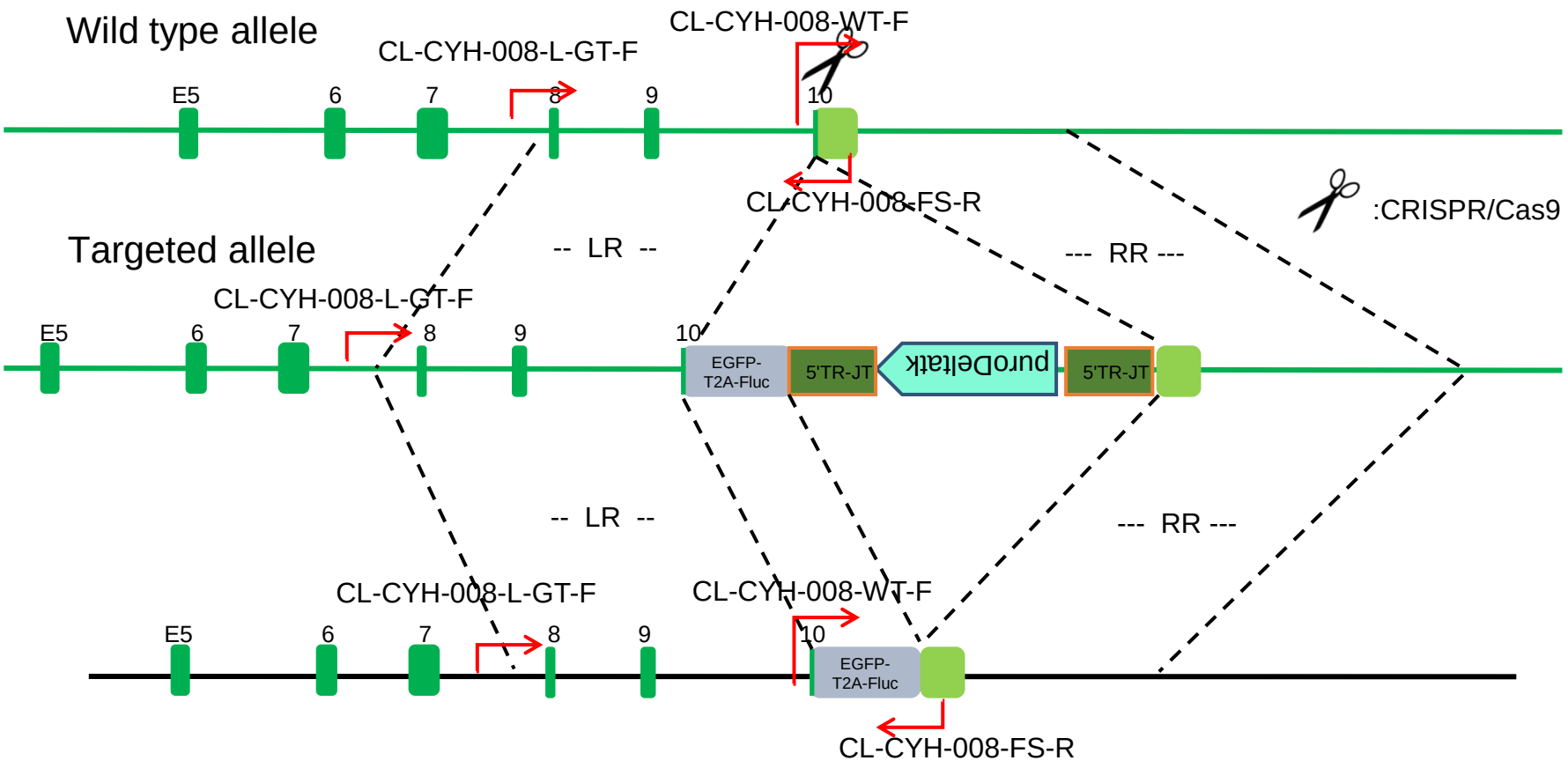
Primers: CL-CYH-008-R-GT-F/ CL-CYH-008-R-GT-R



Positive colonies will be picked and expanded for further analysis.

Part 6
Screening of positive colonies

I. Genotyping primer design



I. Genotyping primer design

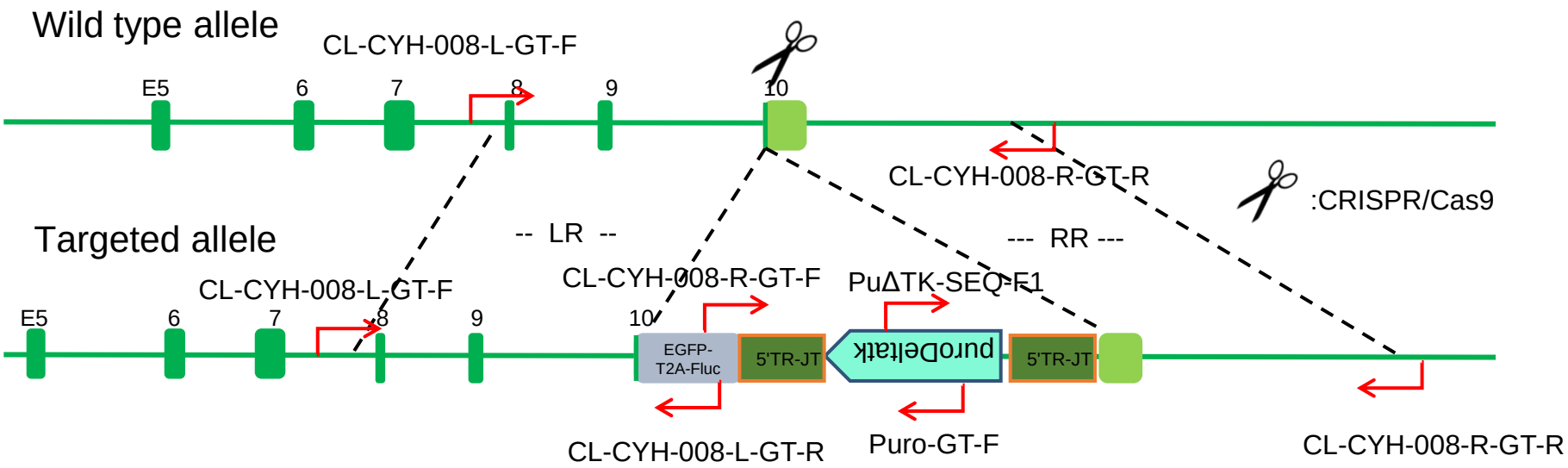
Primer	Sequence (5'-3')	Tm(℃)	Product size
CL-CYH-008-WT-F	GTCTTGTGTGTCTGCCCCTTCTAGT	62	Mut:6406bp WT:527bp Indel:~527bp
CL-CYH-008-FS-R	CCAAAGATTTATTGAAGCAGAACCAAGT	59	
CL-CYH-008-L-GT-F	TCCTGCTGCAAGTACTATCTCATCC	59	Mut:7378bp WT:1499bp Indel:~1499bp
CL-CYH-008-FS-R	CCAAAGATTTATTGAAGCAGAACCAAGT	59	

Enzyme: KOD-FX

Program: Touchdown PCR

94 ℃	5 min	
98 ℃	10 sec	15 cycles
67 ℃	30 sec（- 0.7℃/cycle）	
68 ℃	1 kb / min	
98 ℃	10 sec	25 cycles
57 ℃	30 sec	
68 ℃	1 kb / min	
68 ℃	10 min	
4 ℃	forever	

II. Genotyping primer design



II. Genotyping primer design

Primer	Sequence (5'-3')	Tm(℃)	Product size
CL-CYH-008-L-GT-F	TCCTGCTGCAAGTACTATCTCATCC	59	Mut: 2901bp
CL-CYH-008-L-GT-R	GTTGCTTAGGTCGTACTTGTCGATG	59	
CL-CYH-008-R-GT-F	ATGGATAGCAAGACCGACTACCAGG	61	Mut: 3301bp
Puro-GT-F	GCAACAGATGGAAGGCCTCCTGGCG	67	
PuΔTK-SEQ-F1	AGTAGCGTGGGCATGGATCC	61	Mut: 3113bp
CL-CYH-008-R-GT-R	CGAAGTTTTCACTCCAGAACACACA	59	

Enzyme: KOD-FX

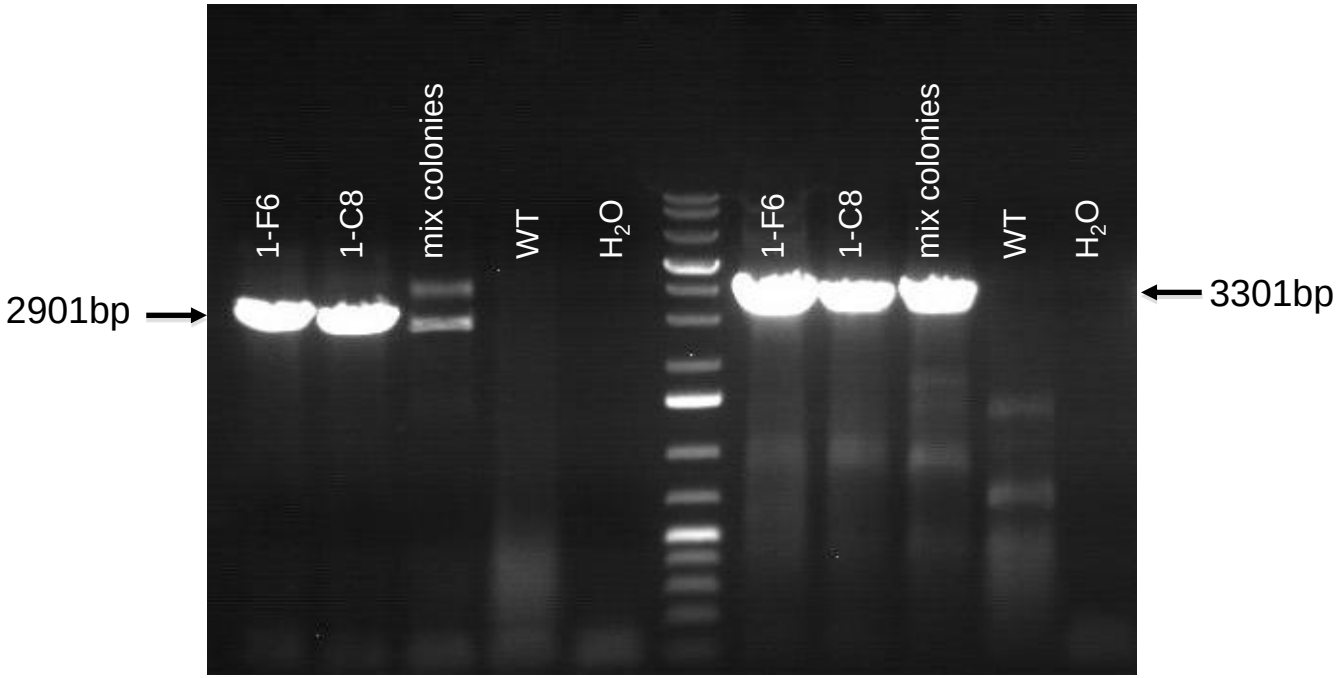
Program: Touchdown PCR

94 ℃	5 min	
98 ℃	10 sec	15 cycles
67 ℃	30 sec（- 0.7℃/cycle）	
68 ℃	1 kb / min	
98 ℃	10 sec	25 cycles
57 ℃	30 sec	
68 ℃	1 kb / min	
68 ℃	10 min	
4 ℃	forever	

Genotyping-Junction PCR

Primers: CL-CYH-008-L-GT-F/CL-CYH-008-L-GT-R

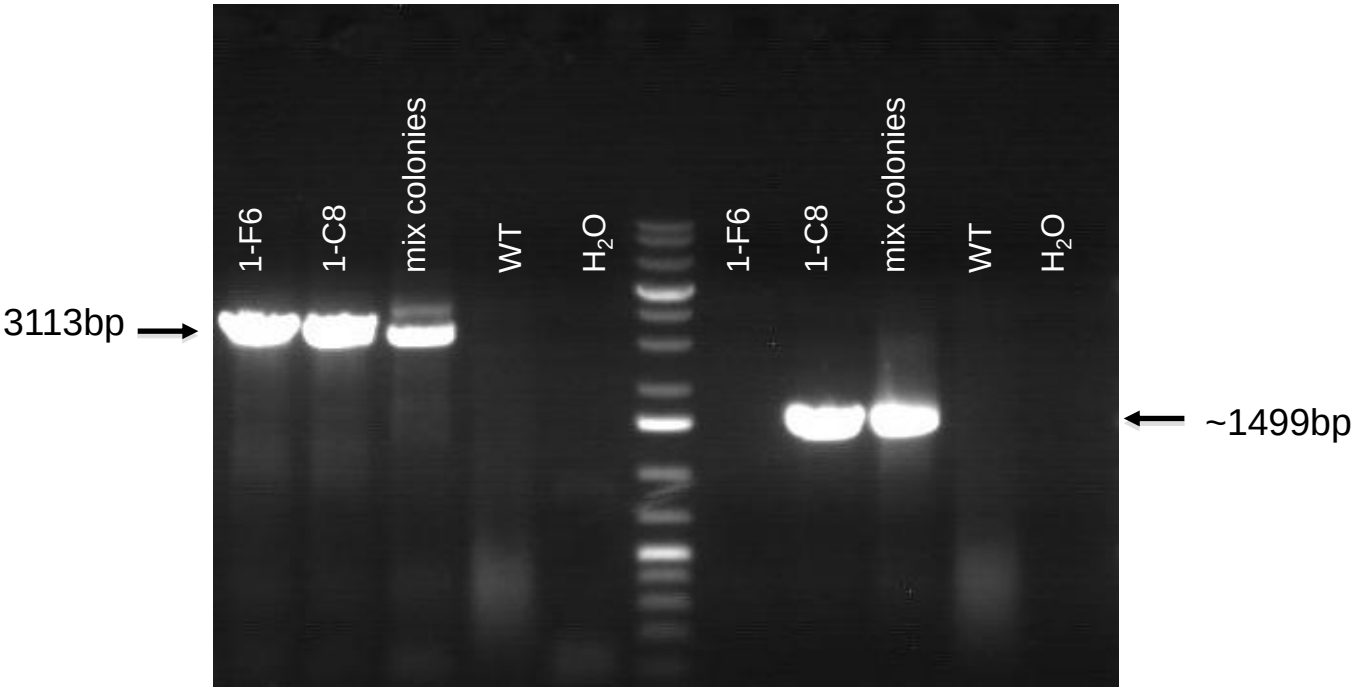
Primers: CL-CYH-008-R-GT-F/Puro-GT-F



Genotyping-Junction PCR

Primers: PuΔTK-SEQ-F1/CL-CYH-008-R-GT-R

Primers: CL-CYH-008-L-GT-F/ CL-CYH-008-FS-R



Colony screening by PCR and sequencing

Abbreviation:

“no band”: No expected band was detected in PCR screening, but integration band was detected in the same reaction.

HR: Homologous Recombination.

Δ： deletion.

Colonies screening by PCR and sequencing

Colony	HR allele	non-HR allele	Genotype
1-F6	+	none	KI,KI
1-C8	+	△6bp	KI,△6

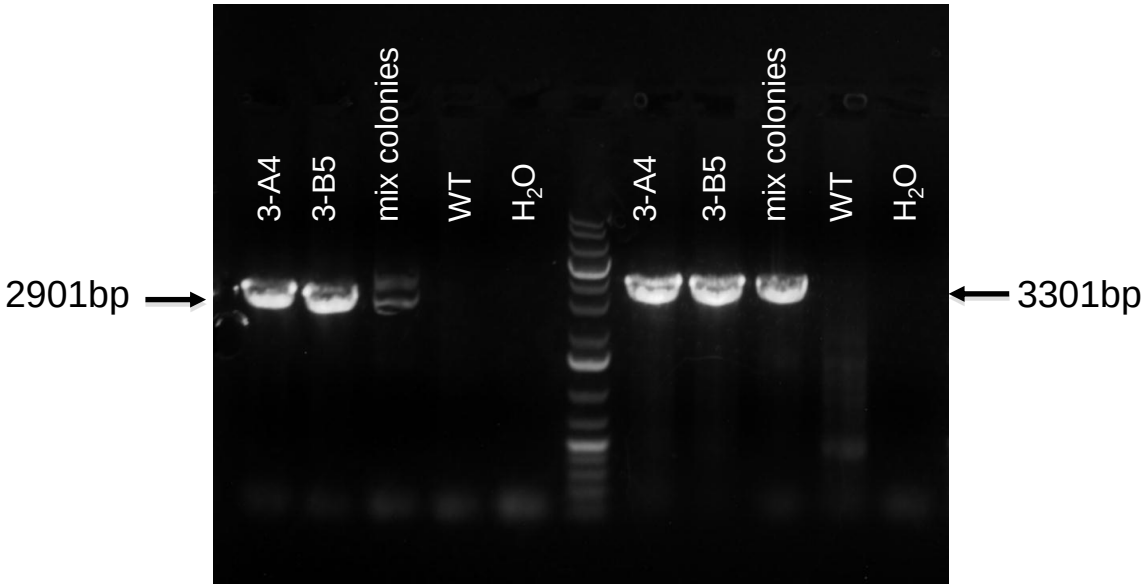
Conclusion

Genotyping results suggest that colonies 1-F6 and 1-C8 are positive *CL-CYH-008* KI cell lines. These colonies are ready for delivery and further analysis.

Genotyping-Junction PCR(the 2nd time)

Primers: CL-CYH-008-L-GT-F/CL-CYH-008-L-GT-R

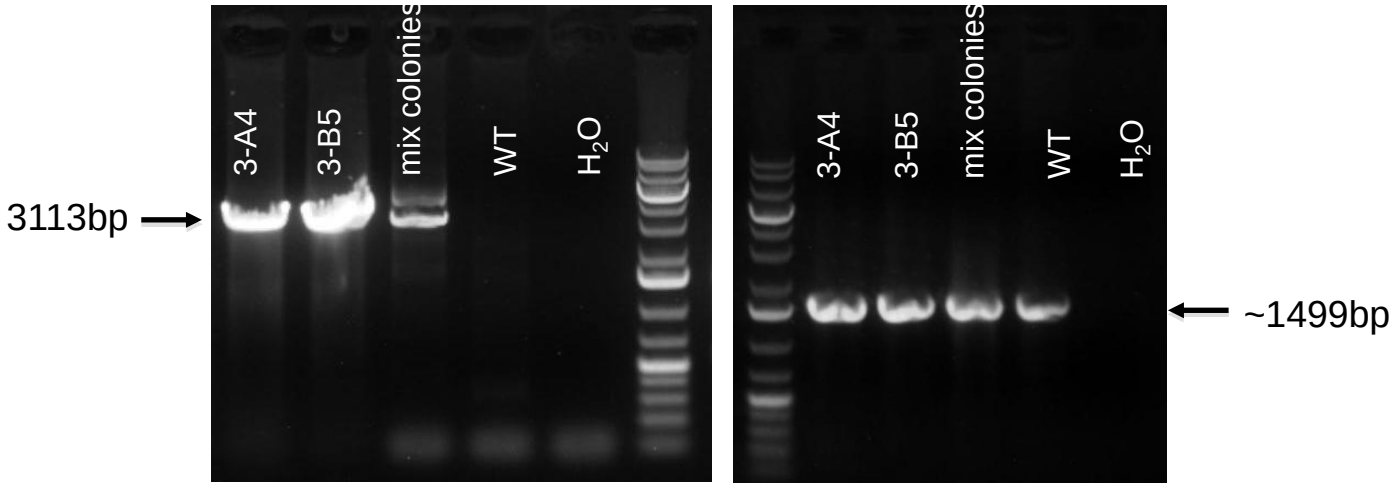
Primers: CL-CYH-008-R-GT-F/Puro-GT-F



Genotyping-Junction PCR(the 2nd time)

Primers: PuΔTK-SEQ-F1/CL-CYH-008-R-GT-R

Primers: CL-CYH-008-L-GT-F/ CL-CYH-008-FS-R



Colony screening by PCR and sequencing

Abbreviation:

HR: Homologous Recombination.

Δ： deletion.

WT: Wide type allele

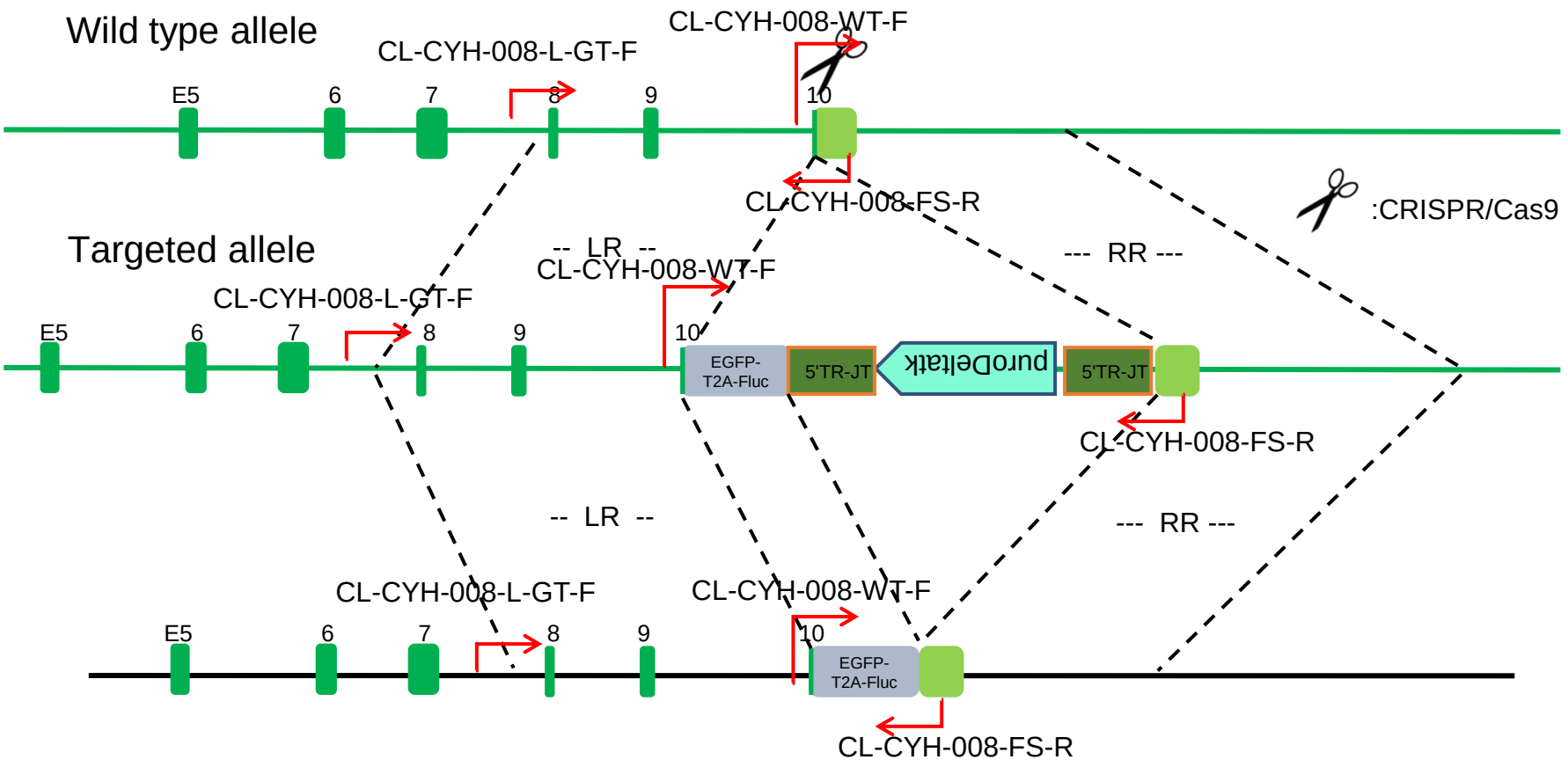
Colonies screening by PCR and sequencing(the 2nd time)

Colony	HR allele	non-HR allele	Genotype
3-A4	+	△11bp	KI, △11
3-B5	+	G→C	KI,WT(G→C)

Conclusion(the 2nd time)

Genotyping results suggest that colonies 3-A4 and 3-B5 are positive *CL-CYH-008* KI cell lines. These colonies are ready for delivery and further analysis.

III. Genotyping primer design-piggyBac transposase



III. Genotyping primer design-piggyBac transposase

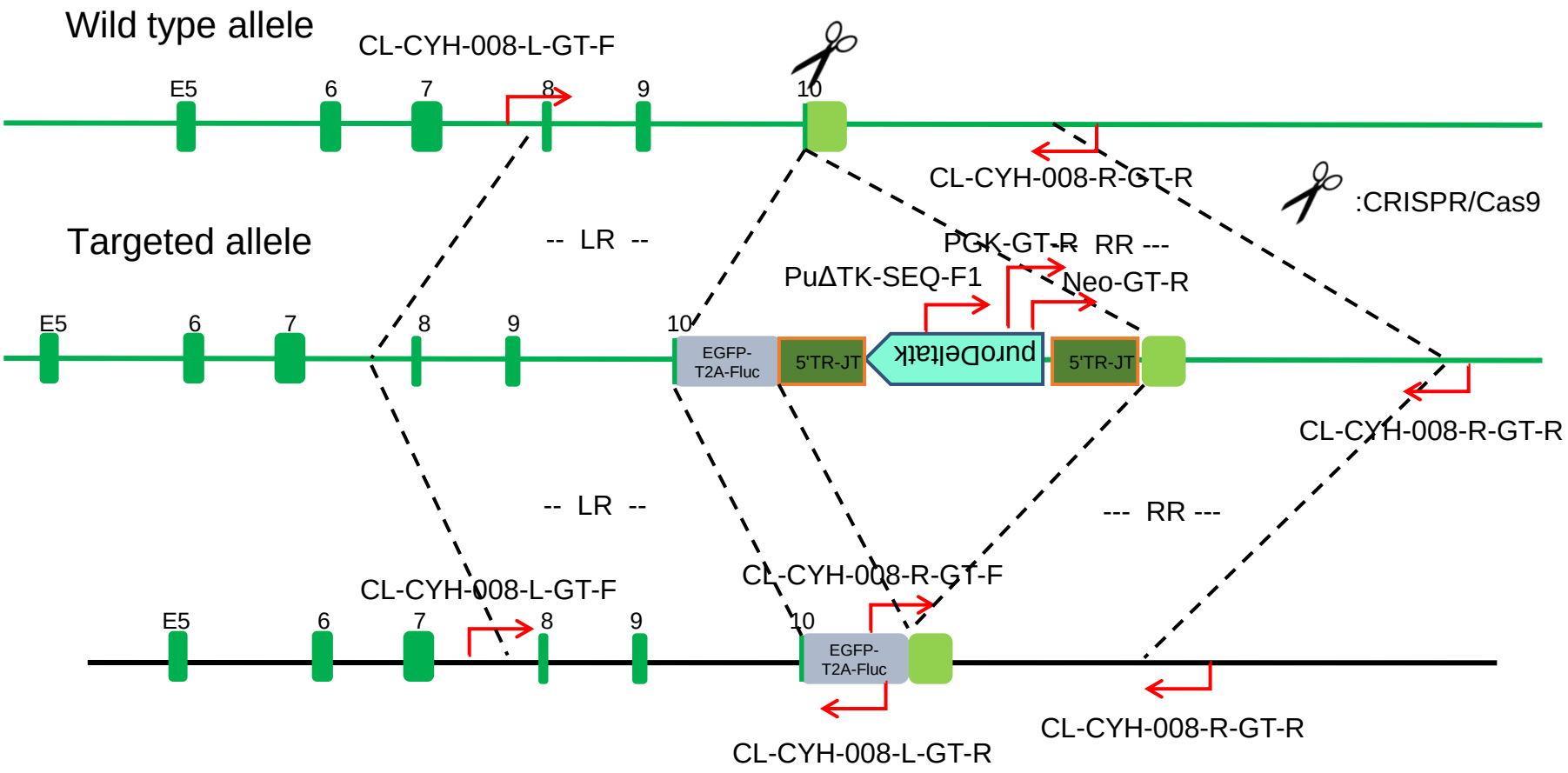
Primer	Sequence (5'-3')	Tm(℃)	Product size
CL-CYH-008-WT-F	GTCTTGTGTGTCTGCCCCTTCTAGT	62	Mut:2972bp WT:527bp Indel:~527bp
CL-CYH-008-FS-R	CCAAAGATTTATTGAAGCAGAACCAAGT	59	
CL-CYH-008-L-GT-F	TCCTGCTGCAAGTACTATCTCATCC	59	Mut:3944bp WT:1499bp Indel:~1499bp
CL-CYH-008-FS-R	CCAAAGATTTATTGAAGCAGAACCAAGT	59	

Enzyme: KOD-FX

Program: Touchdown PCR

94 ℃	2 min	
98 ℃	10 sec	15 cycles
67 ℃	30 sec（- 0.7℃/cycle）	
68 ℃	1 kb / min	
98 ℃	10 sec	25 cycles
57 ℃	30 sec	
68 ℃	1 kb / min	
68 ℃	10 min	
4 ℃	forever	

IV. Genotyping Junction PCR-piggyBac transposase



IV. Genotyping Junction PCR-piggyBac transposase

Primer	Sequence (5'-3')	Tm(℃)	Product size
CL-CYH-008-L-GT-F	TCCTGCTGCAAGTACTATCTCATCC	59	Mut: 2901bp
CL-CYH-008-L-GT-R	GTTGCTTAGGTCGTACTTGTCGATG	59	
CL-CYH-008-R-GT-F	ATGGATAGCAAGACCGACTACCAGG	61	Mut: 2680bp
CL-CYH-008-R-GT-R	CGAAGTTTTCACTCCAGAACACACA	60	

Enzyme: KOD-FX

Program: Touchdown PCR

94 ℃	2 min	
98 ℃	10 sec	15 cycles
67 ℃	30 sec（- 0.7℃/cycle）	
68 ℃	1 kb / min	
98 ℃	10 sec	25 cycles
57 ℃	30 sec	
68 ℃	1 kb / min	
68 ℃	10 min	
4 ℃	forever	

IV. Genotyping Junction PCR-piggyBac transposase

Primer	Sequence (5'-3')	Tm(℃)	Product size
Neo-GT-R	CAGAGGCCACTTGTGTAGCG	61	none(by piggyBac) Or 2062bp
CL-CYH-008-R-GT-R	CGAAGTTTTCACTCCAGAACACACA	59	
PuΔTK-SEQ-F1	AGTAGCGTGGGCATGGATCC	61	none(by piggyBac) Or 3113bp
CL-CYH-008-R-GT-R	CGAAGTTTTCACTCCAGAACACACA	59	
PGK-GT-R	AGAAAGCGAAGGAGCAAAGCTGCTA	63	none(by piggyBac) Or 2313bp
CL-CYH-008-R-GT-R	CGAAGTTTTCACTCCAGAACACACA	59	

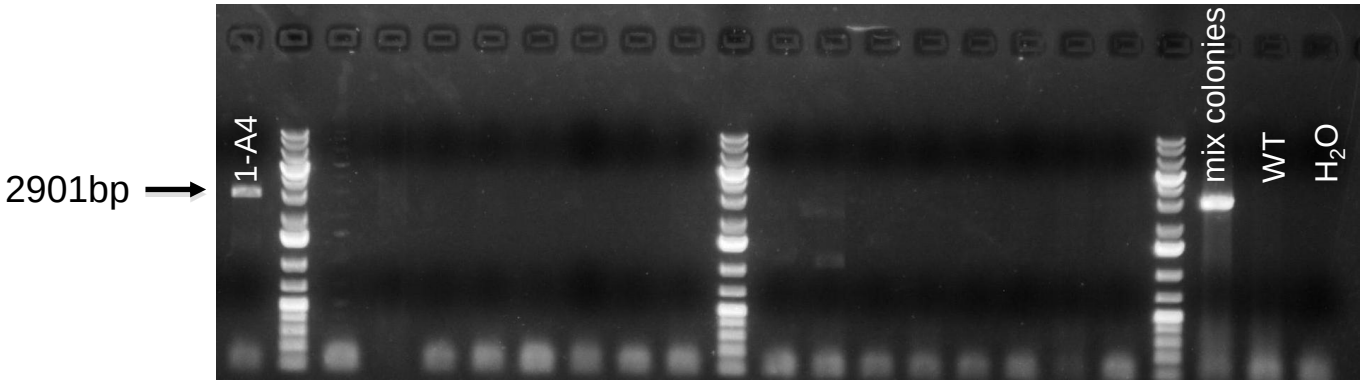
Enzyme: KOD-FX

Program: Touchdown PCR

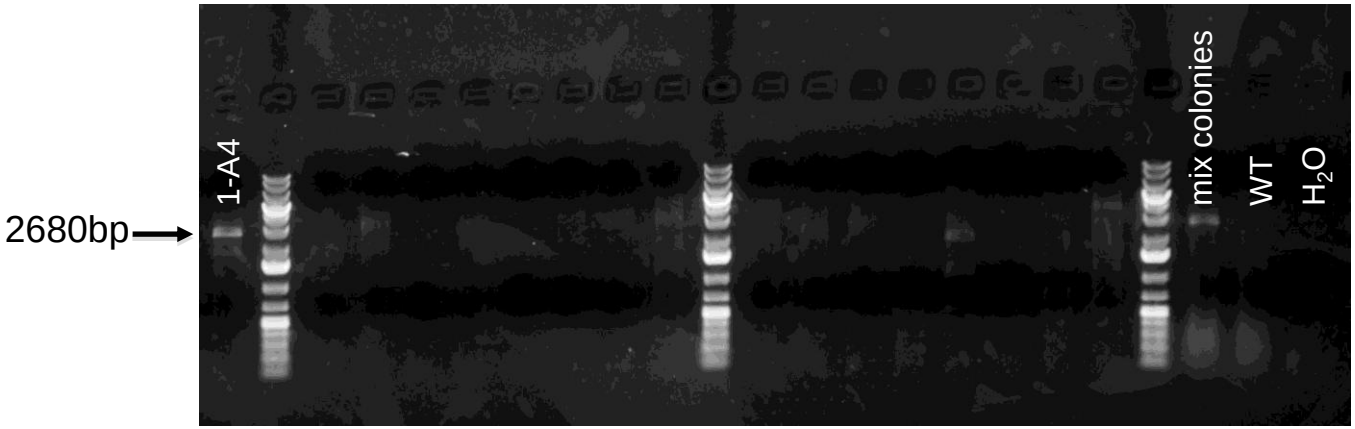
94 ℃	2 min	
98 ℃	10 sec	15 cycles
67 ℃	30 sec（- 0.7℃/cycle）	
68 ℃	1 kb / min	
98 ℃	10 sec	25 cycles
57 ℃	30 sec	
68 ℃	1 kb / min	
68 ℃	10 min	
4 ℃	forever	

Genotyping-Junction PCR-piggyBac

Primers: CL-CYH-008-L-GT-F/CL-CYH-008-L-GT-R

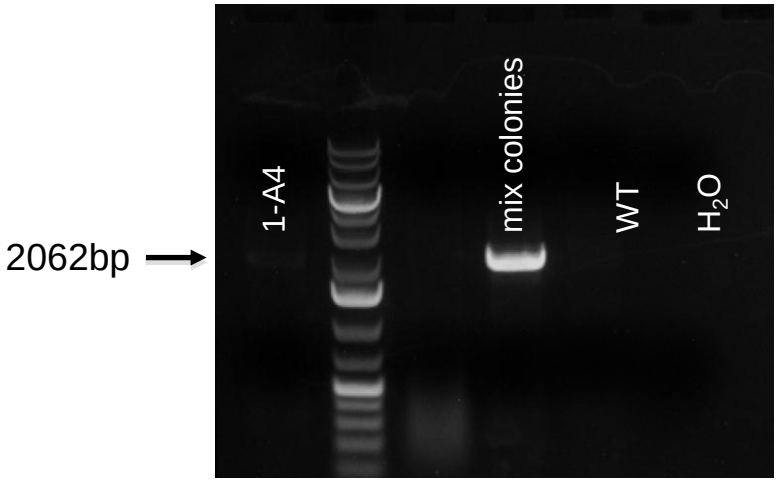


Primers: CL-CYH-008-R-GT-F/ CL-CYH-008-R-GT-R

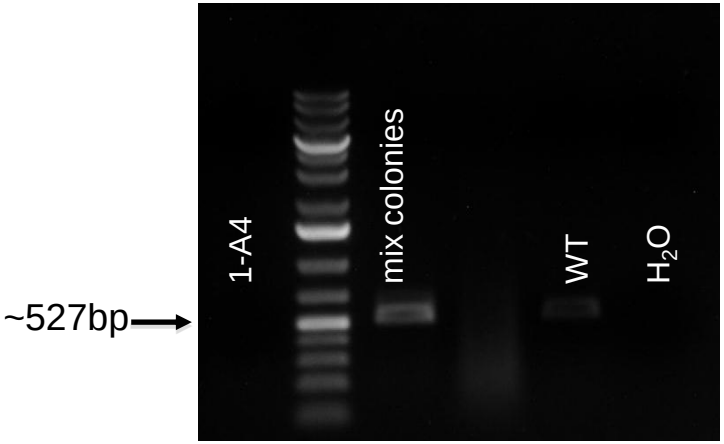


Genotyping-Junction PCR-piggyBac

Primers: Neo-GT-R/CL-CYH-008-R-GT-R



Primers: CL-CYH-008-WT-F/ CL-CYH-008-FS-R



Colony screening by PCR and sequencing

Abbreviation:

“no band”: No expected band was detected in PCR screening, but integration band was detected in the same reaction.

“none”: No WT/Indel sequence was detected in non-HR Allele PCR product.

HR: Homologous Recombination.

Colonies screening by PCR and sequencing

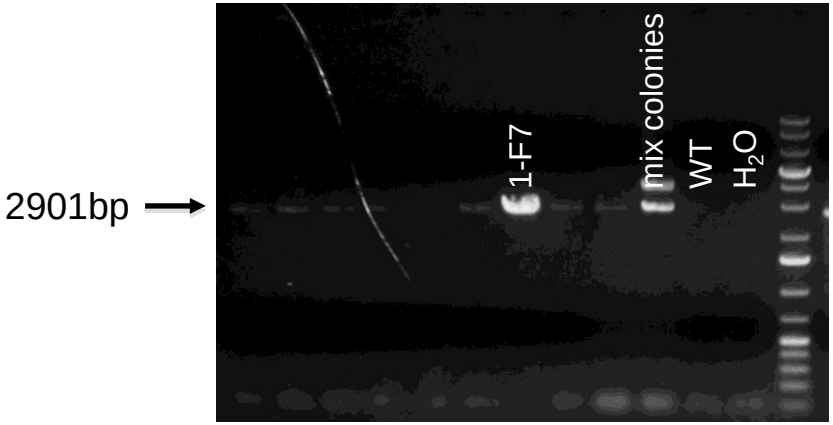
Colony	HR allele	non-HR allele	Genotype
1-A4	+	none	KI,KI

Conclusion

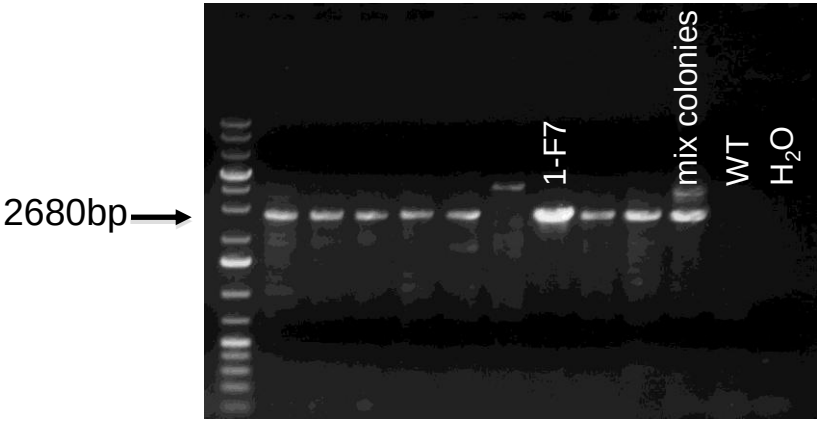
Genotyping results suggest that colony 1-A4 is a positive *CL-CYH-008* KI cell line. This colony is ready for delivery and further analysis.

Genotyping-Junction PCR-piggyBac(the 2nd time)

Primers: CL-CYH-008-L-GT-F/CL-CYH-008-L-GT-R

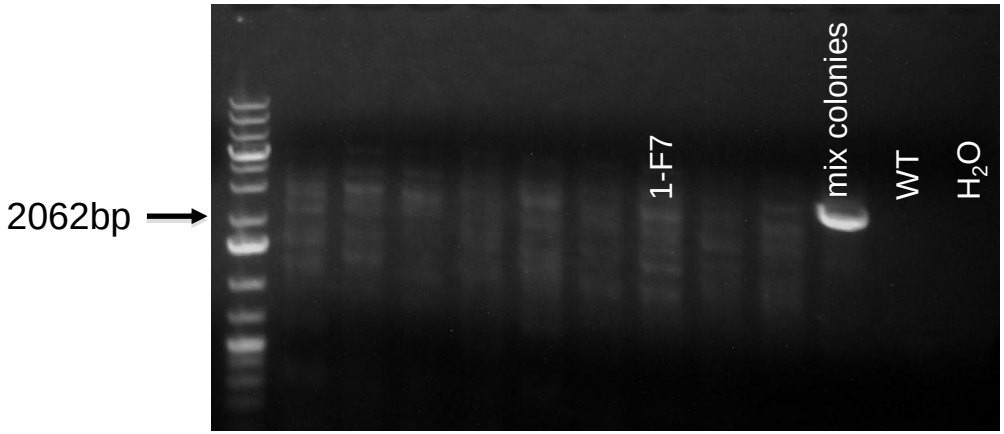


Primers: CL-CYH-008-R-GT-F/ CL-CYH-008-R-GT-R

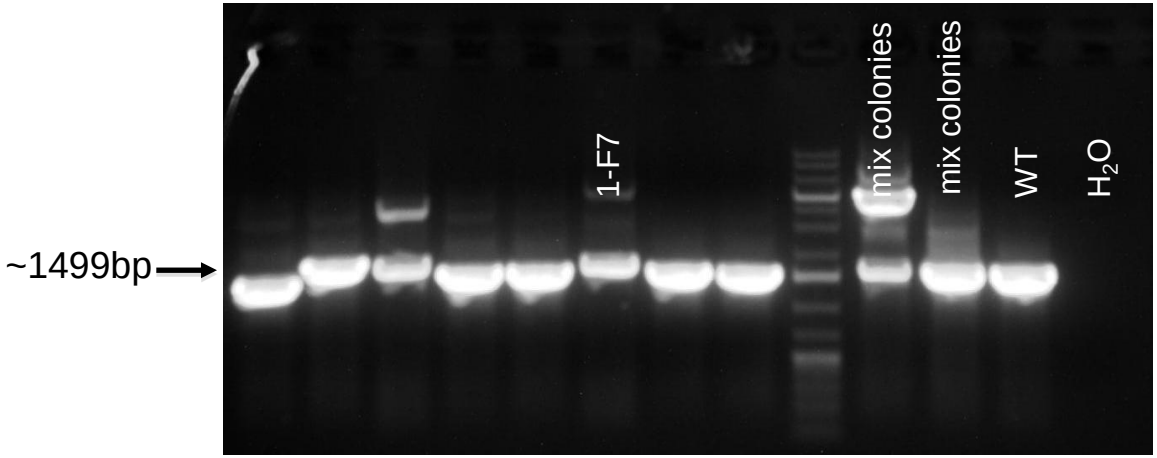


Genotyping-Junction PCR-piggyBac(the 2nd time)

Primers: Neo-GT-R/CL-CYH-008-R-GT-R



Primers: CL-CYH-008-L-GT-F/ CL-CYH-008-FS-R



Colony screening by PCR and sequencing

Abbreviation:

“no band”: No expected band was detected in PCR screening, but integration band was detected in the same reaction.

“none”: No WT/Indel sequence was detected in non-HR Allele PCR product.

FS: Frame shift.

HR: Homologous Recombination.

Colonies screening by PCR and sequencing(the 2nd time)

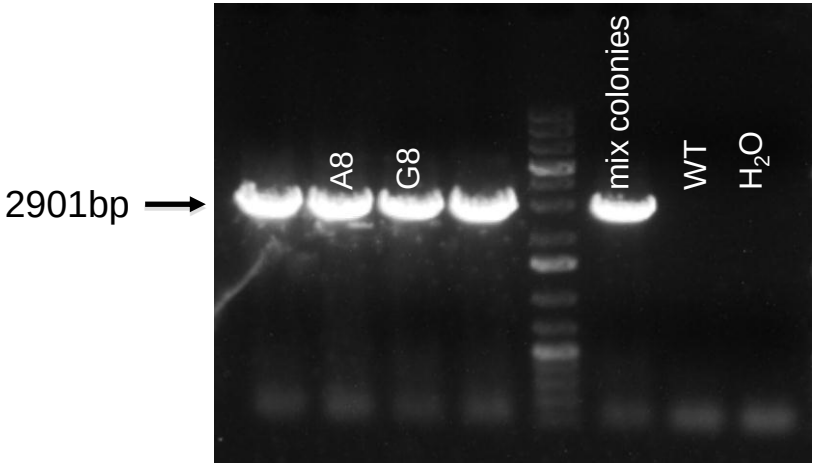
Colony	HR allele	non-HR allele	Genotype
1-F7	+	G→C	KI,WT(G→C)

Conclusion(the 2nd time)

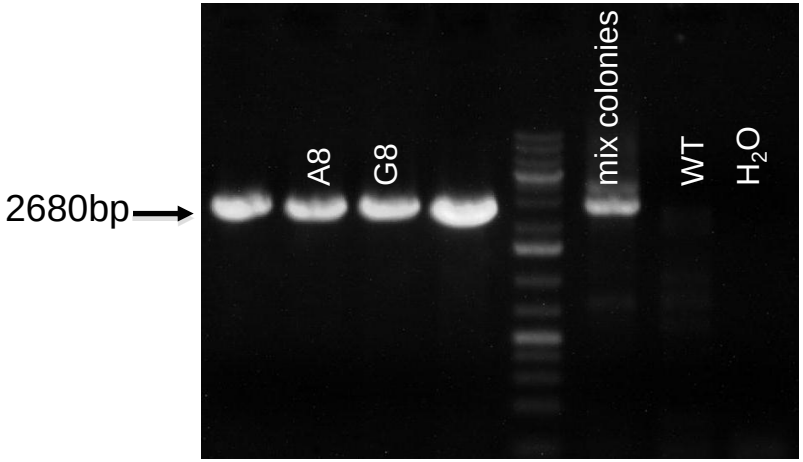
Genotyping results suggest that colony 1-F7 is a positive *CL-CYH-008* KI cell line. This colony is ready for delivery and further analysis.

Genotyping-Junction PCR-piggyBac(the 3rd time)

Primers: CL-CYH-008-L-GT-F/CL-CYH-008-L-GT-R

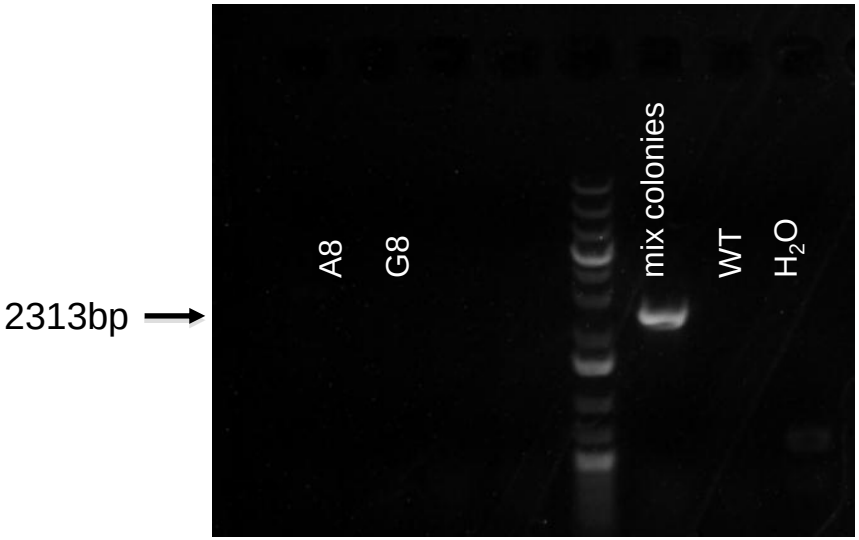


Primers: CL-CYH-008-R-GT-F/ CL-CYH-008-R-GT-R

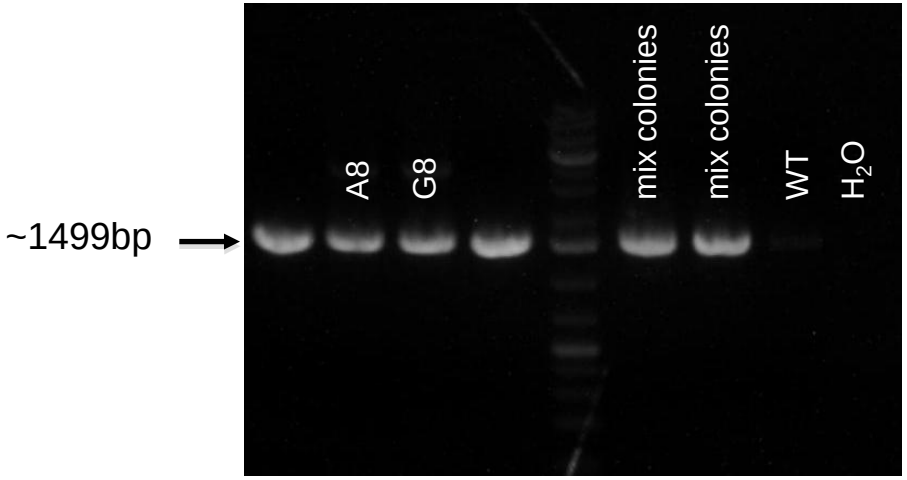


Genotyping-Junction PCR-piggyBac(the 3rd time)

Primers: PGK-GT-R/CL-CYH-008-R-GT-R



Primers: CL-CYH-008-L-GT-F/ CL-CYH-008-FS-R



Colony screening by PCR and sequencing

Abbreviation:

“no band”: No expected band was detected in PCR screening, but integration band was detected in the same reaction.

“none”: No WT/Indel sequence was detected in non-HR Allele PCR product.

HR: Homologous Recombination.

Colonies screening by PCR and sequencing(the 3rd time)

Colony	HR allele	non-HR allele	Genotype
A8	+	G→C	KI,WT(G→C)
G8	+	G→C	KI,WT(G→C)

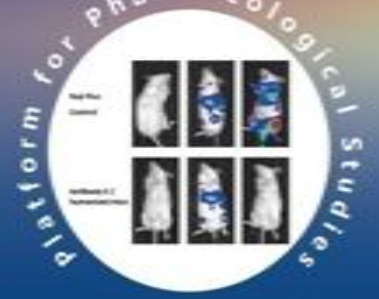
Conclusion(the 3rd time)

Genotyping results suggest that colonies A8 and G8 are positive *CL-CYH-008* KI cell lines. These colonies are ready for delivery and further analysis.

This project has been completed.

BIOCYTOGEN

Biocytogen was founded in 2008. Headquartered in Beijing, Biocytogen has two branches/facilities based in Haimen, Jiangsu China and Worcester MA, USA, also an administrative branch in Zhangjiang, Shanghai. Biocytogen is a global leader in providing high quality gene modified animal model generation and development, a top supplier of innovative human disease animal models in pharmaceutical research and a service provider of preclinical pharmacological/pharmadynamics studies.



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