



Curso de Genômica e Genética Funcional de Microrganismos

# Metodologias para edição de genomas (CRISPR-Cas)

Elvio H. Benatto Perino

[eperino@ipb-halle.de](mailto:eperino@ipb-halle.de)

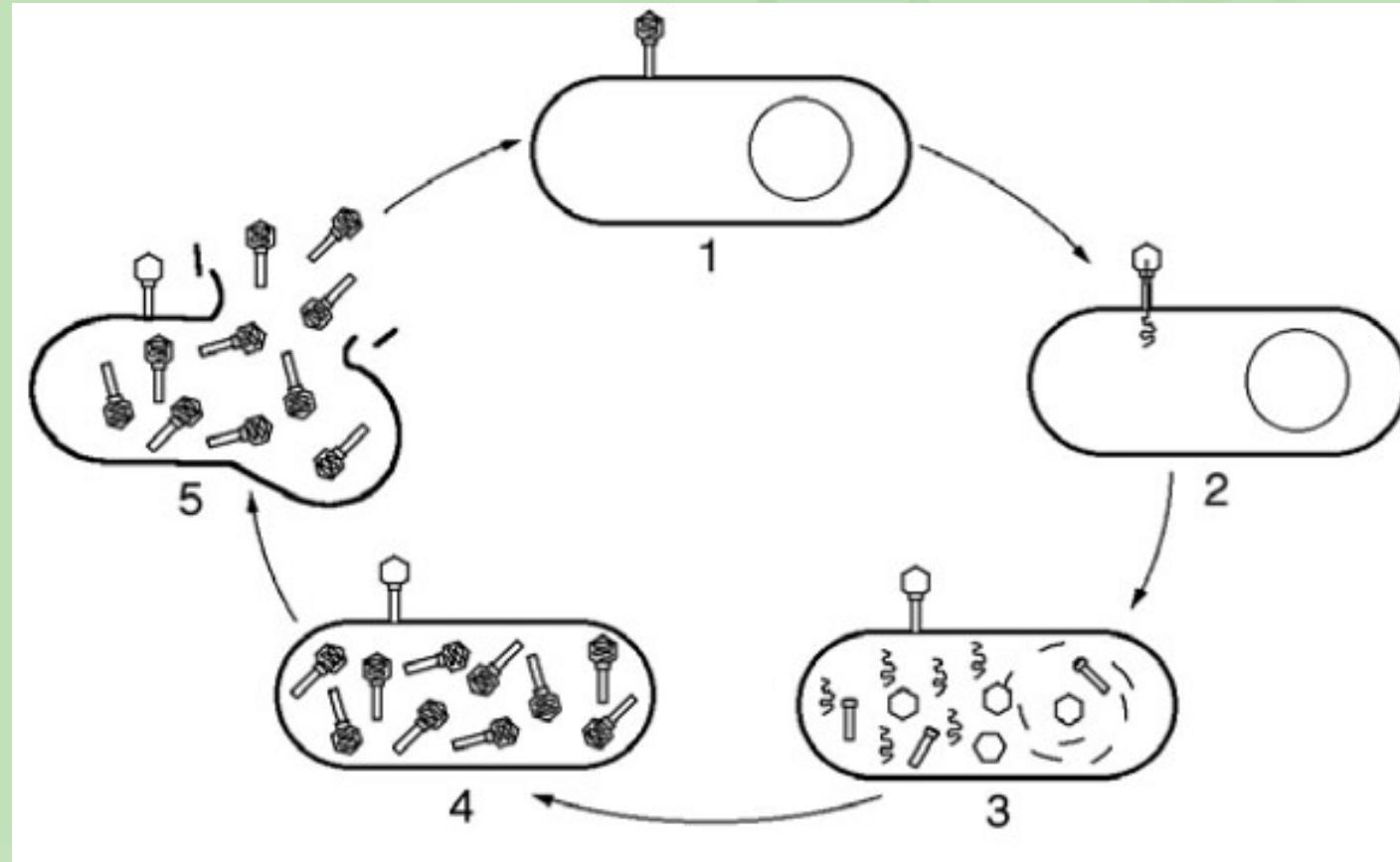


# Introdução

- CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)
- Proteína Cas (Crispr associated protein)

# Introdução

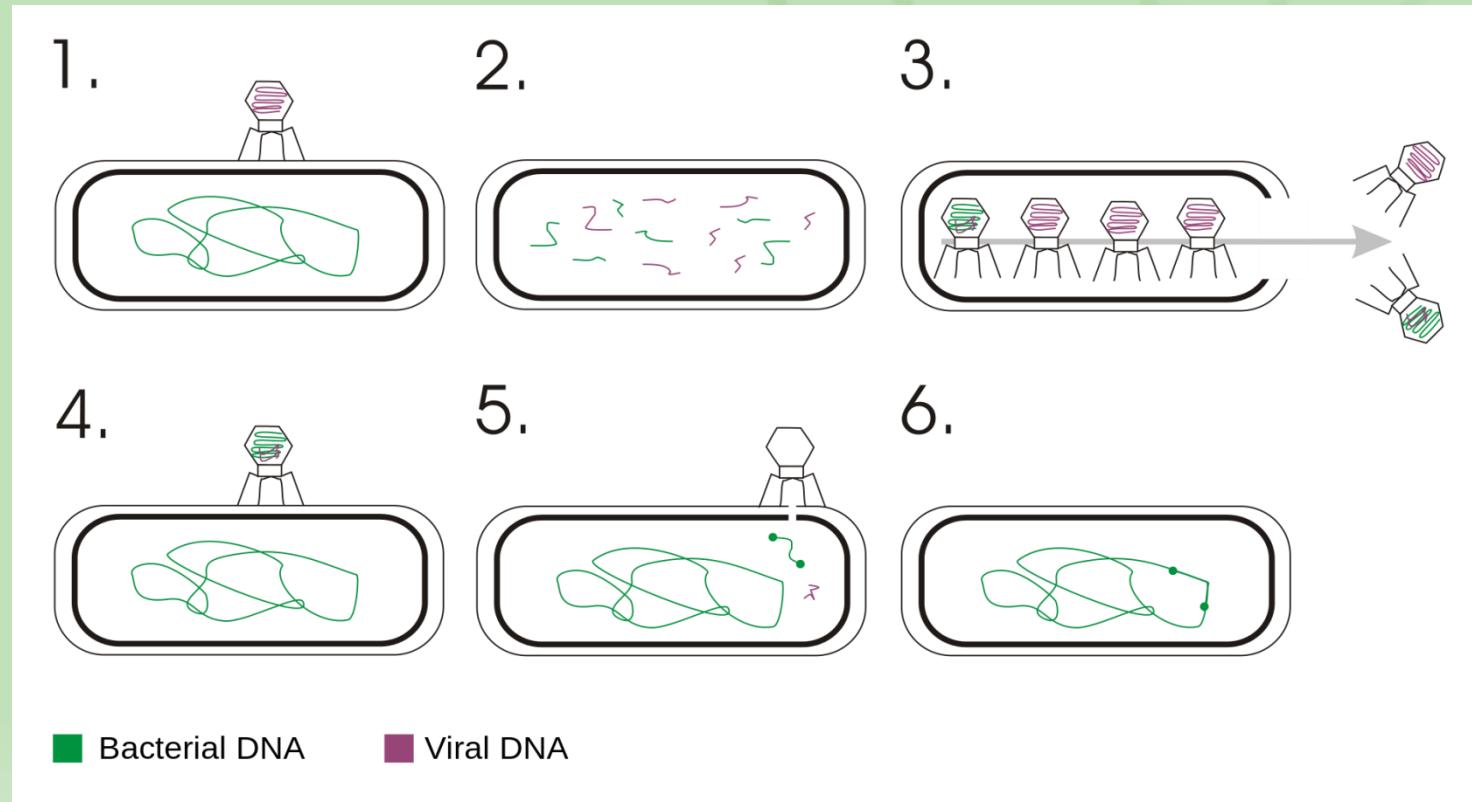
- Ciclo lítico



<https://www.flickr.com/photos/profesorjano/6968799251>

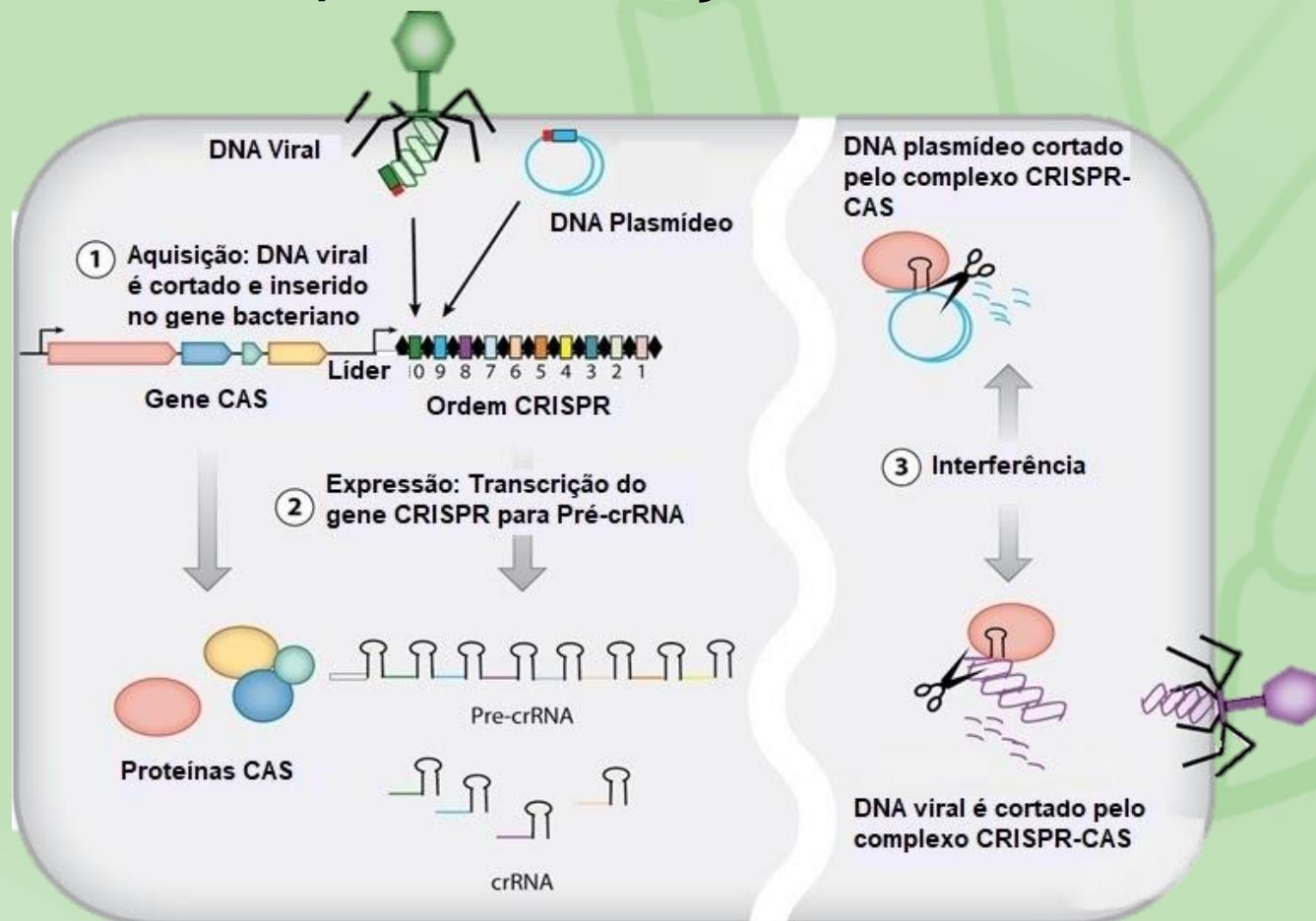
# Introdução

- Defesa em bactérias
  - Reprodução sexuada por transdução



# Introdução

- Defesa em bactérias
  - Reprodução sexuada por transdução
  - CRISPR



# Introdução

- Defesa em bactérias
  - Reprodução sexuada por transdução
  - CRISPR

JOURNAL OF BACTERIOLOGY, Dec. 1987, p. 5429-5433  
0021-9193/87/125429-05\$02.00/0  
Copyright © 1987, American Society for Microbiology

Vol. 169, No. 12

## Nucleotide Sequence of the *iap* Gene, Responsible for Alkaline Phosphatase Isozyme Conversion in *Escherichia coli*, and Identification of the Gene Product

YOSHIZUMI ISHINO, HIDEO SHINAGAWA, KOZO MAKINO, MITSUKO AMEMURA, AND ATSUO NAKATA\*

*Department of Experimental Chemotherapy, The Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan*

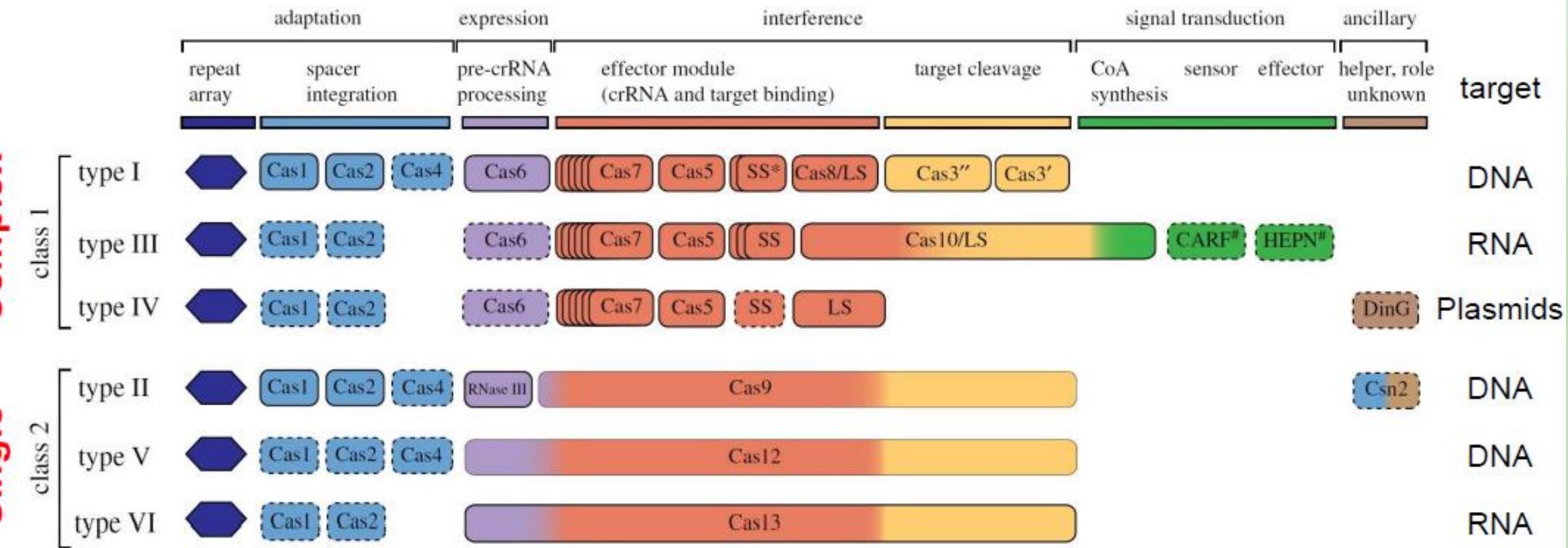
Received 1 May 1987/Accepted 22 August 1987

# Introdução

- Sistemas CRISPR
  - Mecanismos de CRISPR Classe 2 (Cas9, Cas12a, Cas13)
  - Como desenhar um gRNA
  - Clonagem por GoldenGate
  - Prática CRISPR/Golden Gate
  - Seleção de organismos editados

# Mecanismo

**Single**



# Mecanismo

	<b>Cas9</b>	<b>Cas12a</b>	<b>Cas13</b>
Organismos	<i>Streptococcus pyogenes</i> , <i>S. thermophilus</i> , <i>Staphylococcus aureus</i> , <i>Neisseria meningitidis</i> , <i>Campylobacter jejuni</i>	<i>Francisella novicida</i> , <i>Acidaminococcus sp.</i> , <i>Lachnospiraceae sp.</i> , <i>Prevotella sp.</i>	<i>Leptotrichia buccalis</i> , <i>Leptotrichia shahii</i> , <i>Ruminococcus flavefaciens</i> , <i>Bergeyella zoohelcum</i> , <i>Prevotella buccae</i> , <i>Listeria seeligeri</i> .
Size	1000-1600 aa	1100-1300 aa	900-1300 aa
gRNA (tamanho total)	18-24 nt (100 nt)	18-25 nt (42-44 nt)	22-30 nt (52-66 nt)
Cut	Blunt-ended dsDNA break	5 nt overhang dsDNA break	ssRNA



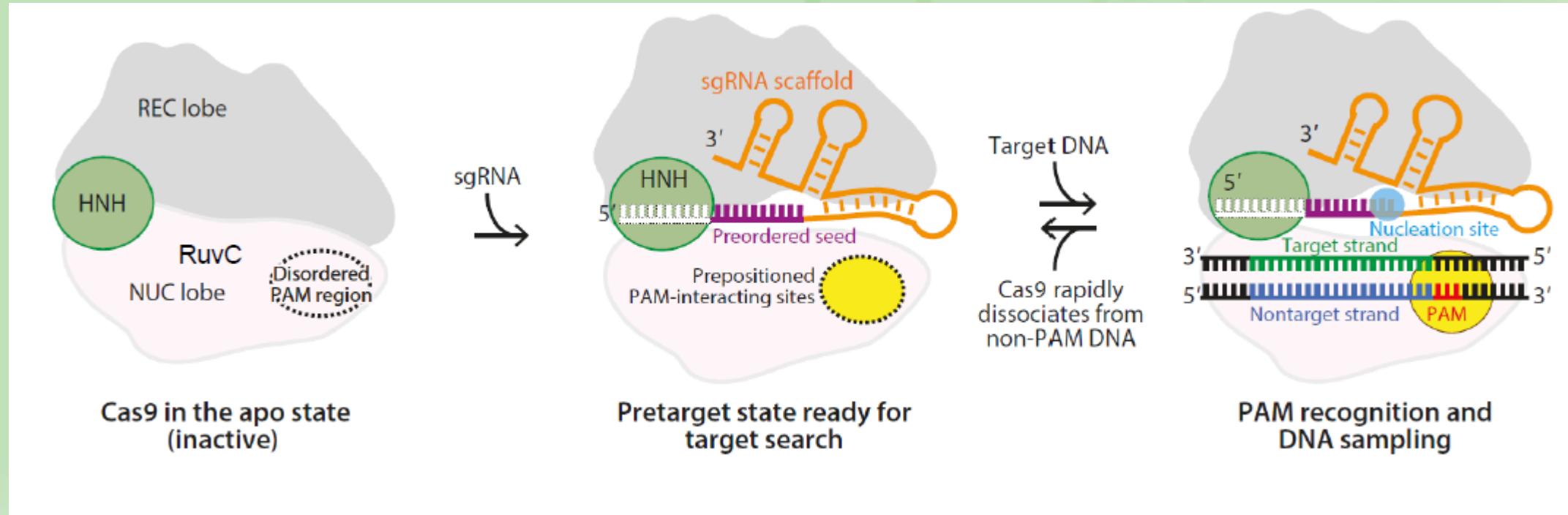
$\uparrow$  edições maiores  
 $\downarrow$  afinidade (17-20 matches)



NHEJ (larger deletions)

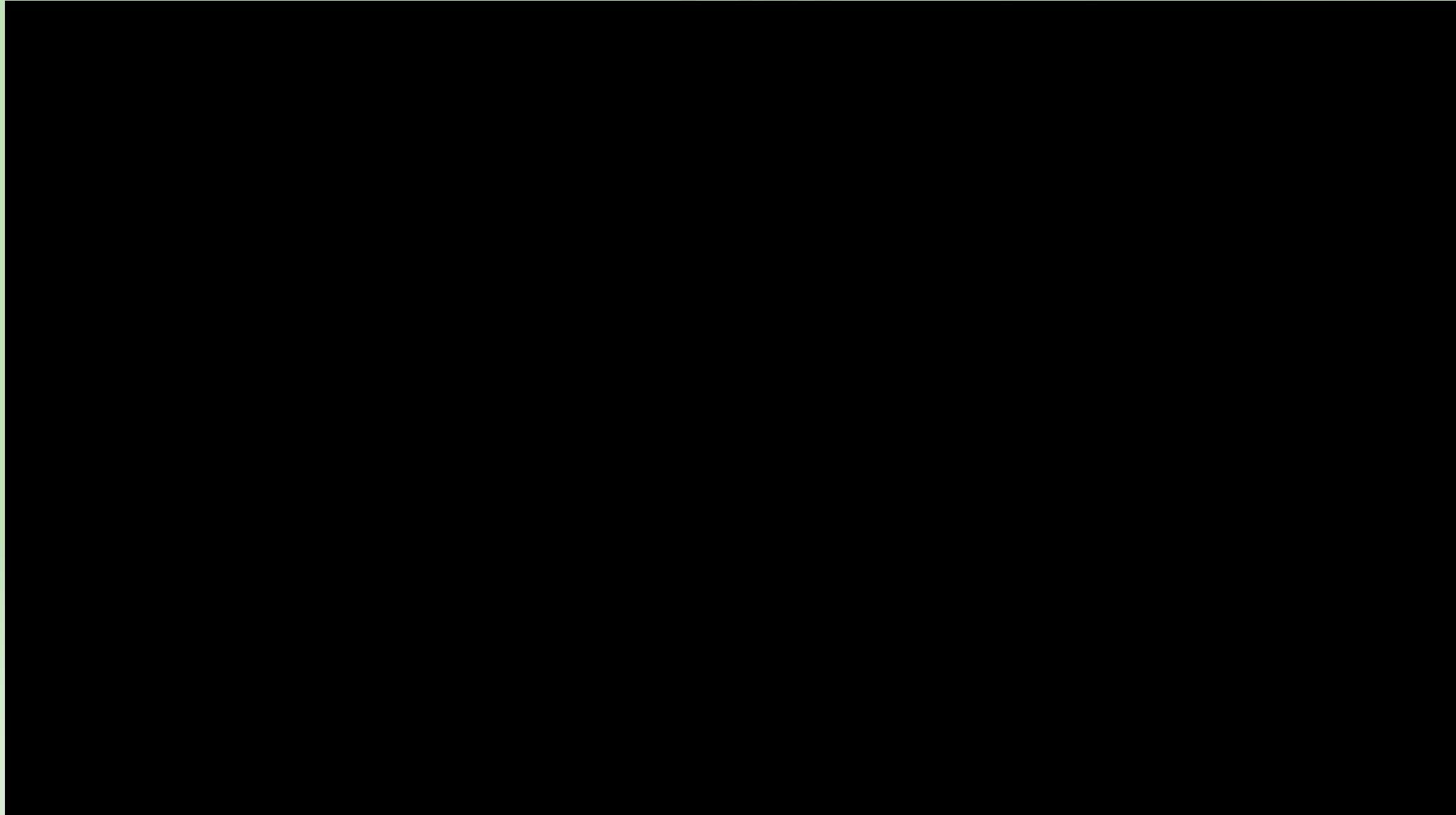
Antiviral (SHERLOCK)

# Mecanismo

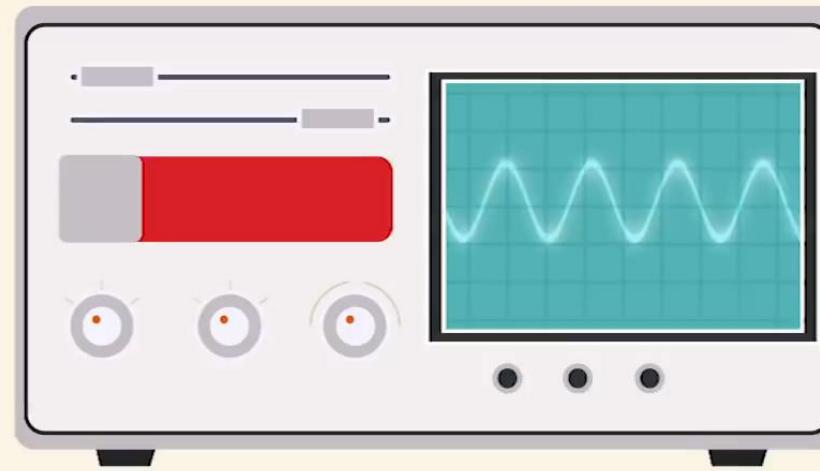


Sequência PAM (*Sp-Cas9*): 20pb - NGG

[www.youtube.com/watch?v=ZOoUllMxf4](https://www.youtube.com/watch?v=ZOoUllMxf4)



[www.youtube.com/watch?v=22F85FOAyik](https://www.youtube.com/watch?v=22F85FOAyik)



# Planejamento

## Organismo alvo

- Conhecer o organismo alvo
- Conhecer o genoma do organismo alvo
- Quantos alelos editar?

## Gene/Região alvo

- Sequenciar seu gene/região alvo
- Verificar splicing alternativos

## gRNA

- Verificar sequência PAM (depende da Cas)
- Verificar off-targets
- Knock-out/Knock-down/HDR

## Clonagem

- Escolher os plasmídeos e métodos de clonagem
- Fazer a clonagem *in silico*

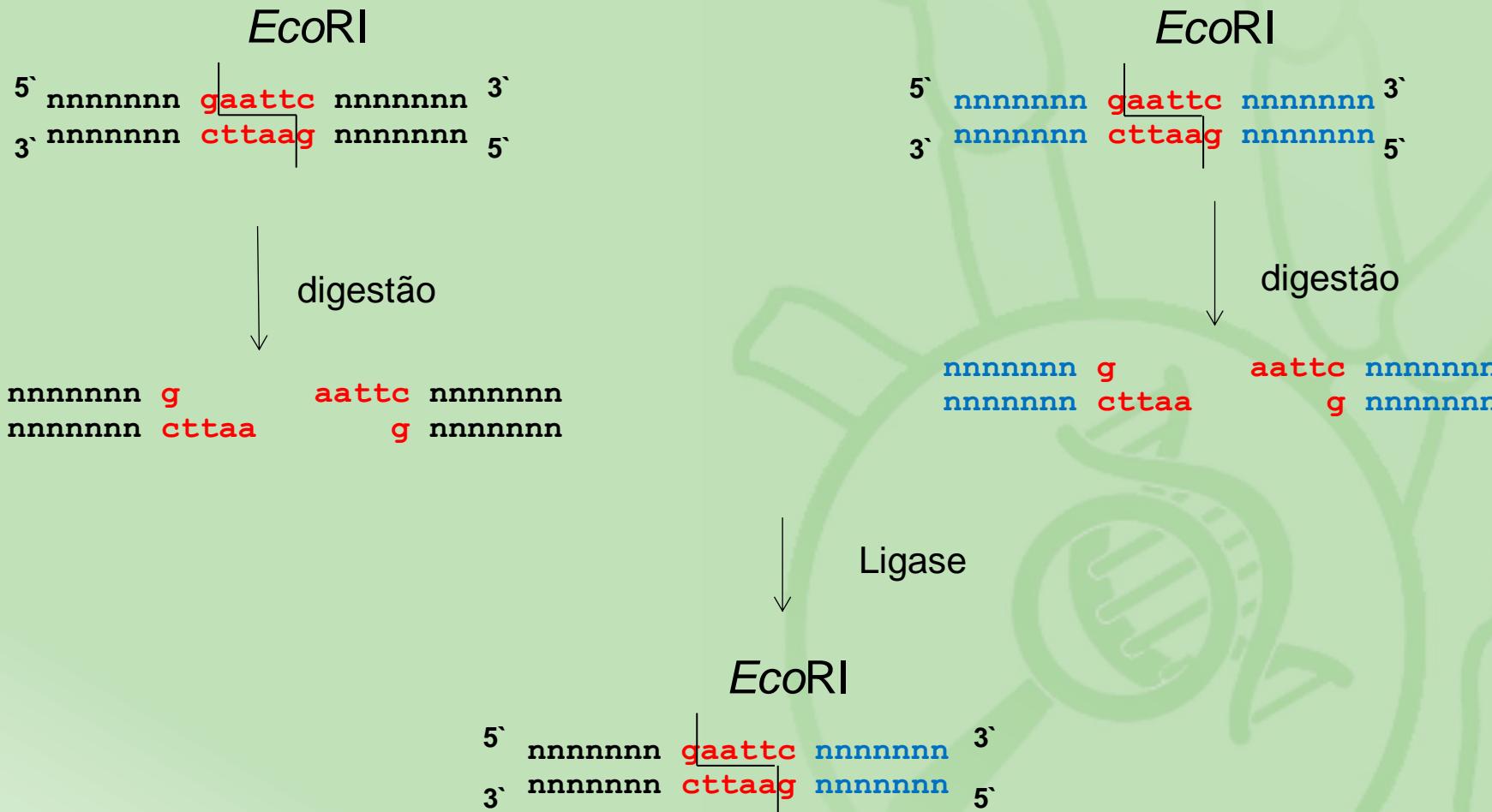
## Delivery da Cas e do gRNA

- Escolher o método de delivery
- Verificar se é compatível com seu sistema experimental

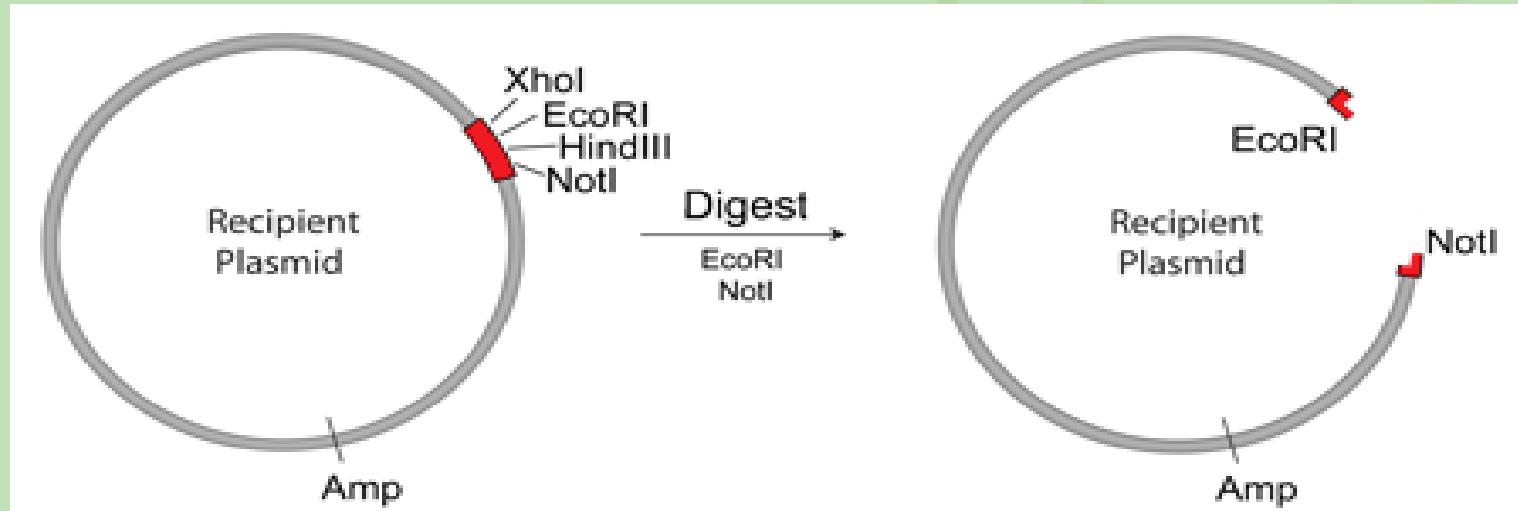
## Método de validação

- Escolher um ou mais métodos de validação

# Clonagem clássica



# Clonagem clássica

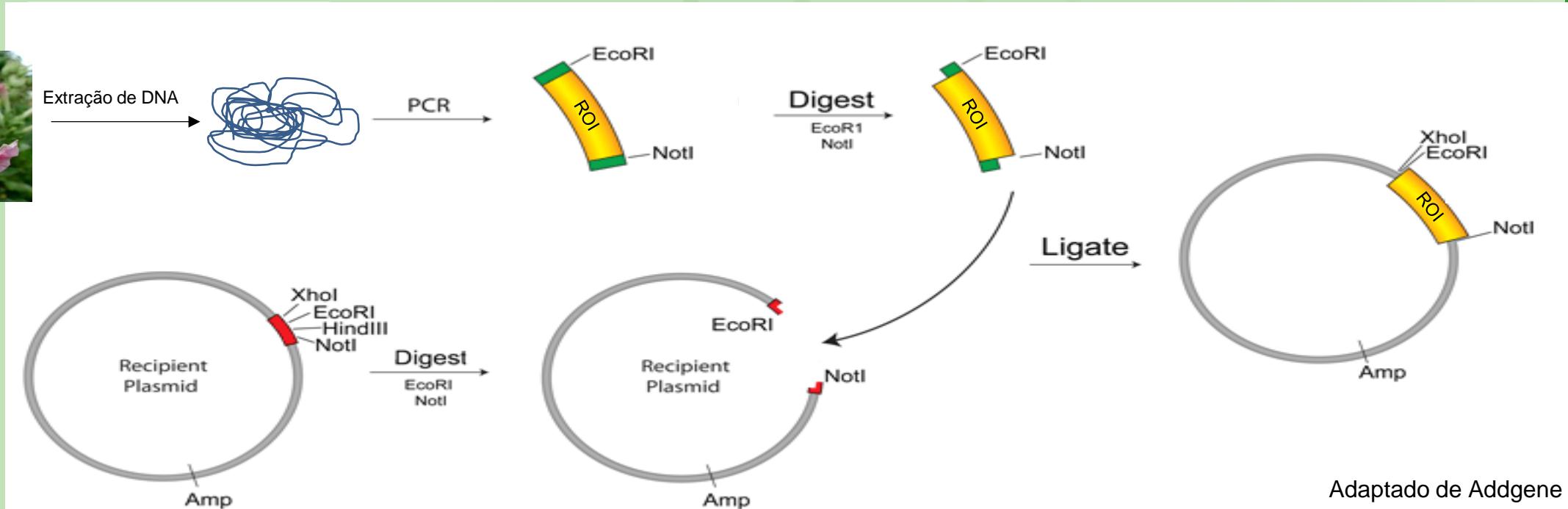


Adaptado de Addgene

- pPICZα
- pTYB21
- pUC19

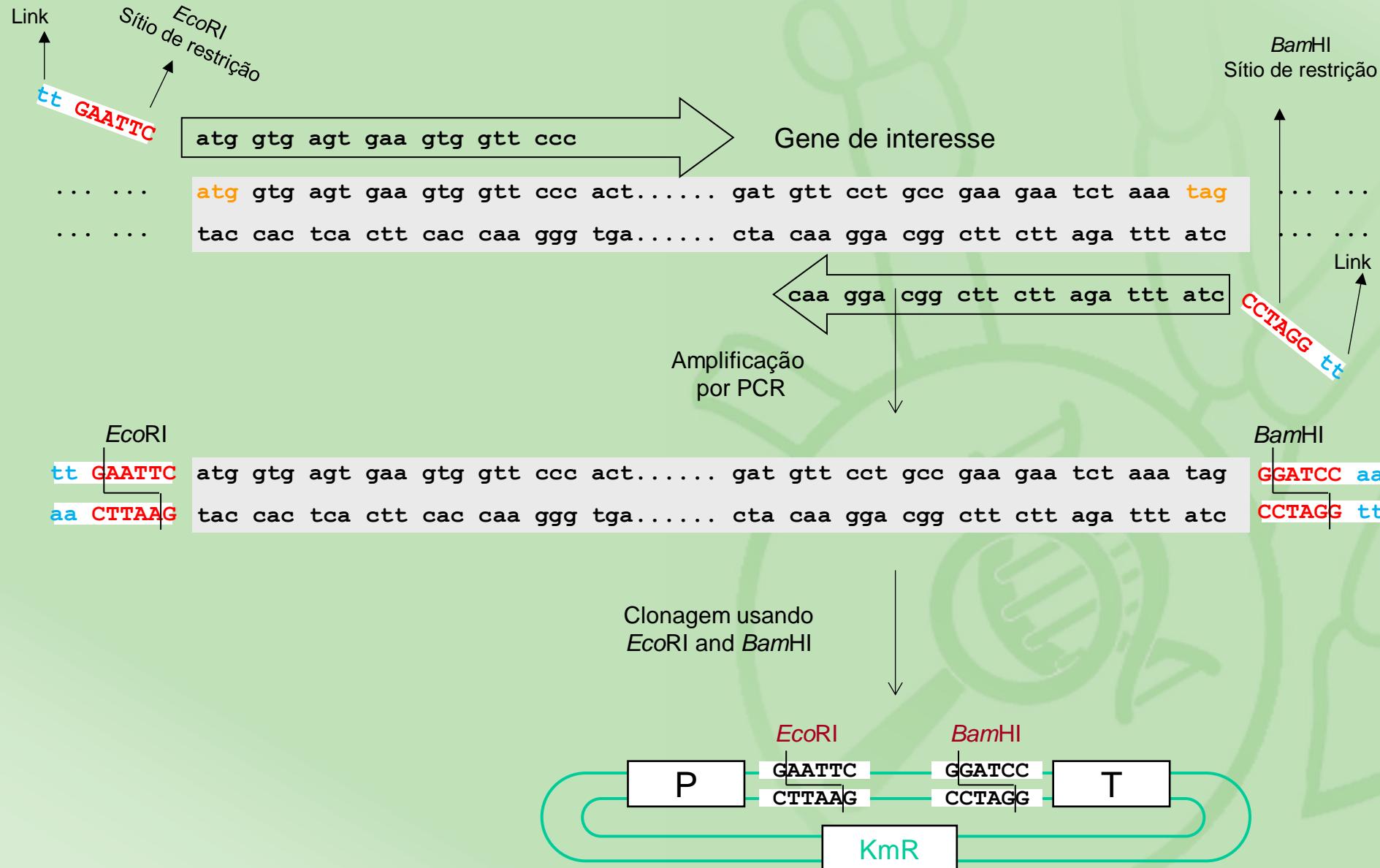
# Clonagem clássica

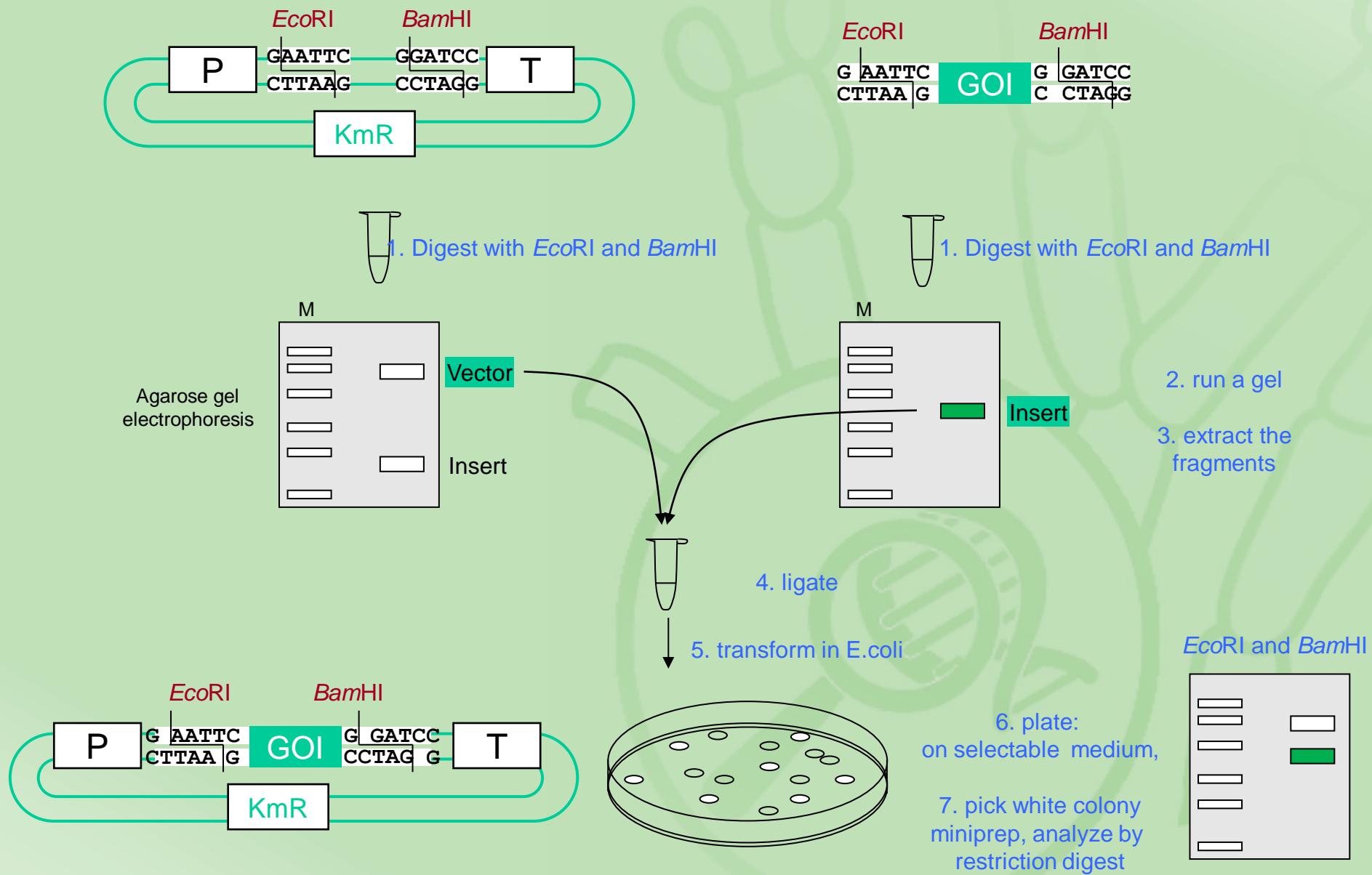
Organismo alvo



Adaptado de Addgene

# Amplificação por PCR do DNA genômico ou cDNA





# Clonagem por GoldenGate

Engler C. and Marillonnet S. 2014

Engler C. et al., 2008

Engler C. et al., 2009

Werner S. et al., 2012

Weber E. et al., 2011

# Enzimas de restrição

Blunt ends



Sticky ends



Sticky ends



*EcoRV*



*EcoRI*



*Bsal*



Type IIS

# Enzimas de restrição

*Bsal* (*Eco31I*)

5'... GGTCTC (N)<sub>1</sub>▼...3'  
3'... CCAGAG (N)<sub>5</sub>▲...5'

*BpiI* (*BbsI*)

5'... GAAGAC (N)<sub>2</sub>▼...3'  
3'... CTTCTG (N)<sub>6</sub>▲...5'



$$4^*4^*4^*4 = \textcolor{red}{256} \text{ possibilidades para sequências de overhang}$$

É possível fazer fusão de qualquer seguimento de DNA usando enzimas de restrição do tipo IIS?

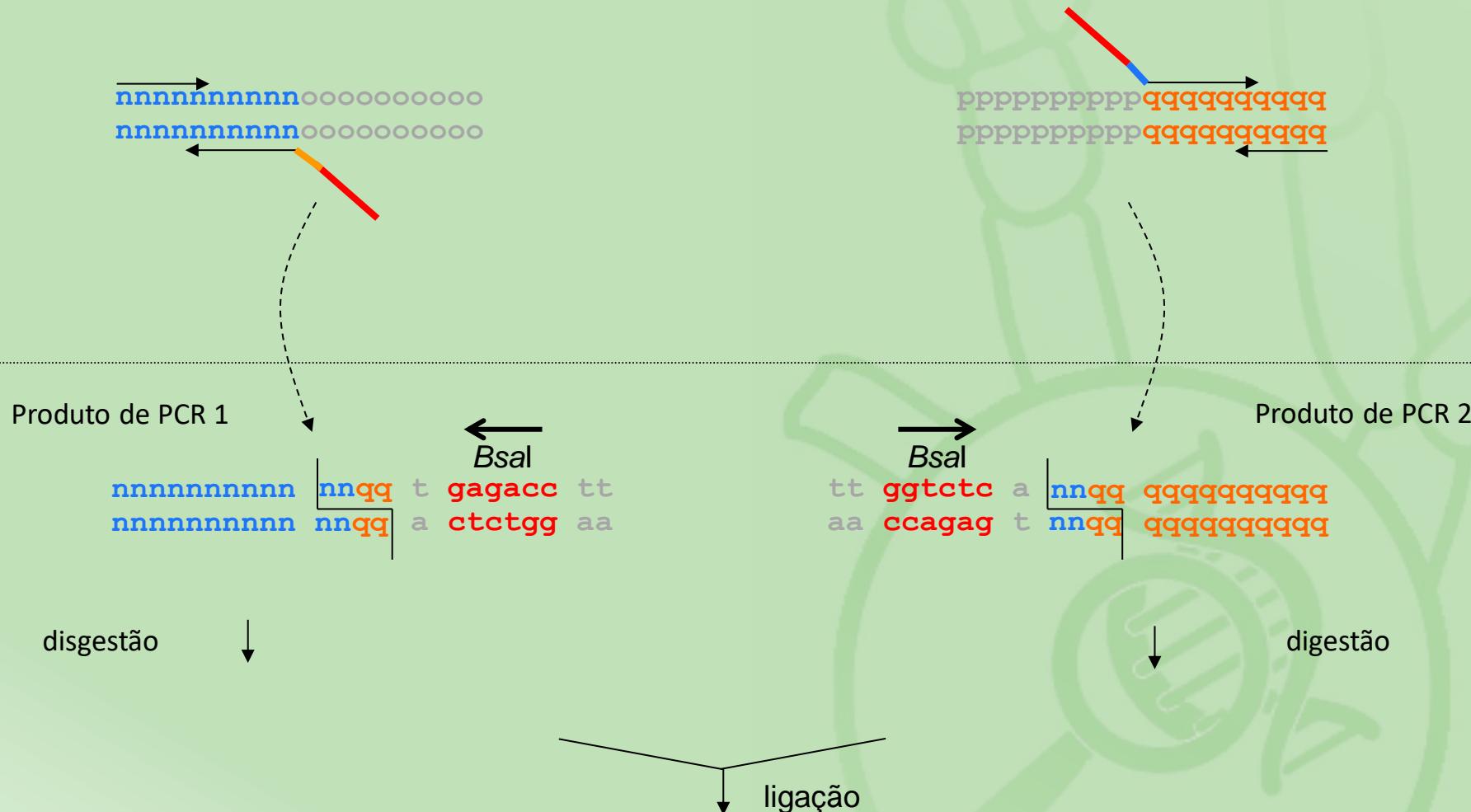
nnnnnnnnnnnnn oooooooooooooo  
nnnnnnnnnnnnn oooooooooooooo



pppppppppppqqqqqqqqqq  
pppppppppppqqqqqqqqqq

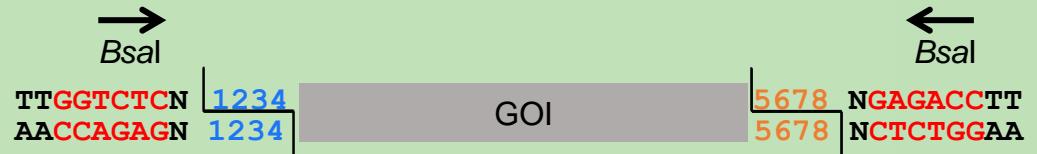
nnnnnnnnnnnnnqqqqqqqqqq  
nnnnnnnnnnnnnqqqqqqqqqq

É possível fazer fusão de qualquer seguimento de DNA usando enzimas de restrição do tipo IIS?

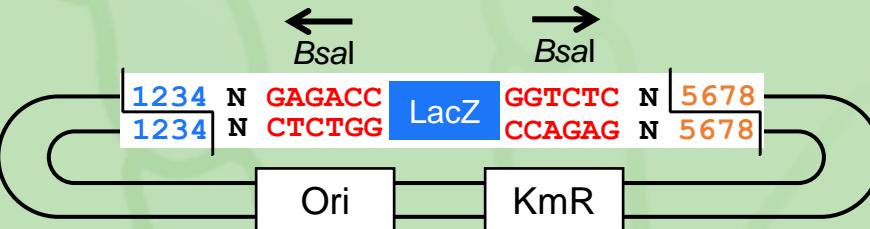


# Clonagem GoldenGate

Protudo de PCR      *Bsal + T4 Ligase*



Vetor de destino



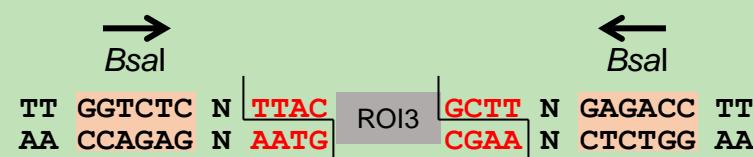
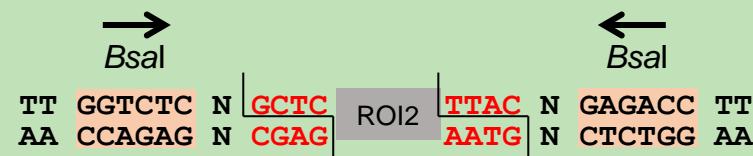
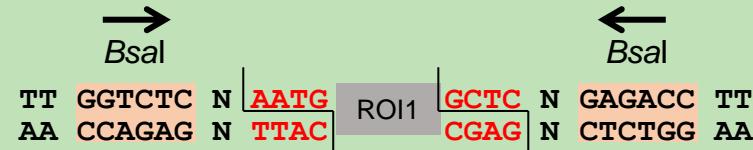
Ligaçāo estável



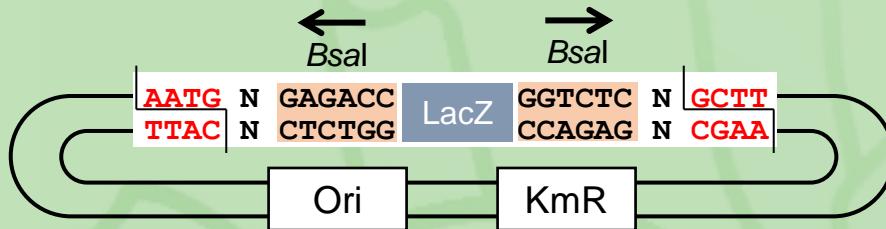
Antibótico + X-Gal para seleção azul-branco de alta eficiência

# Montando vários fragmentos em um vetor

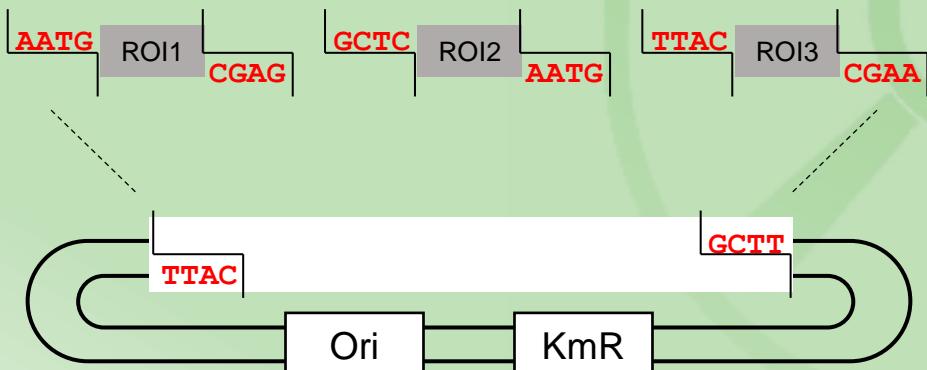
Produtos de PCR



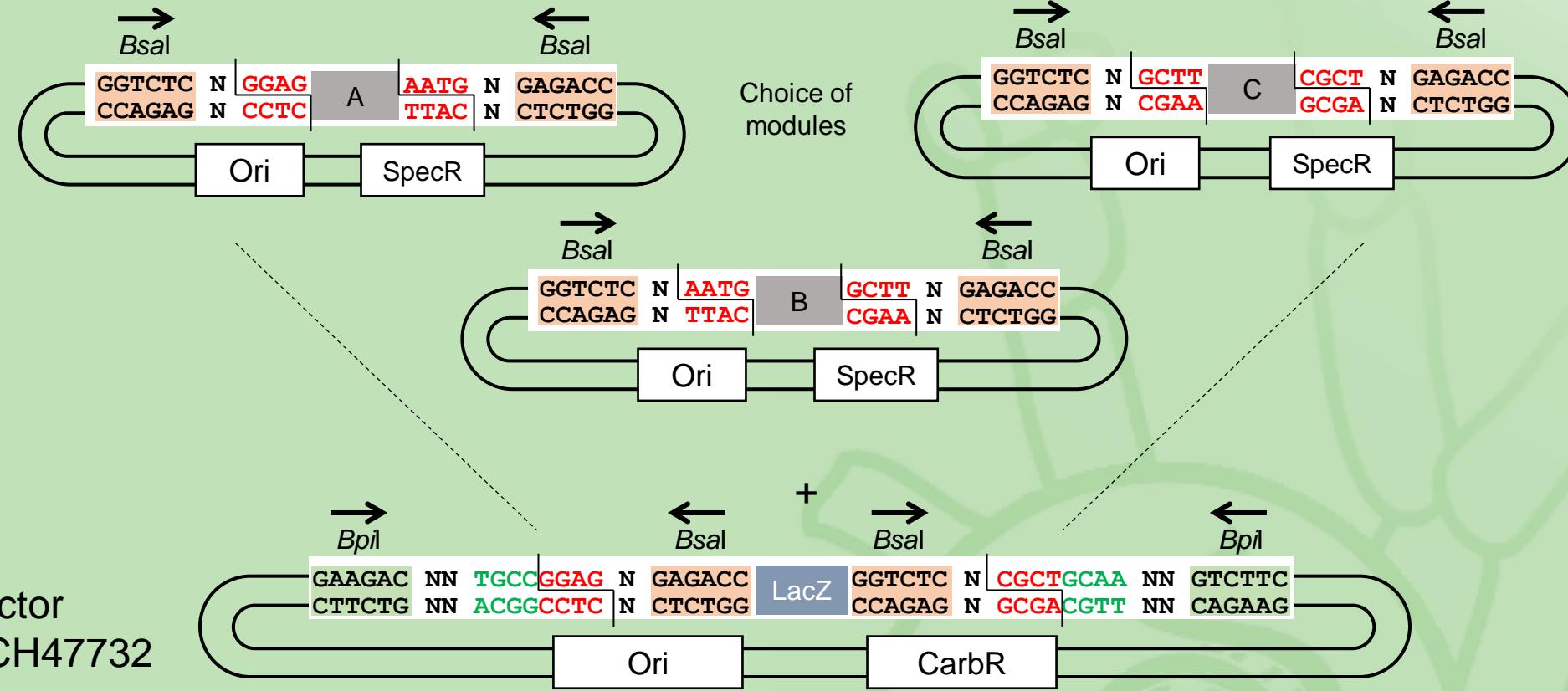
Vetor de Destino



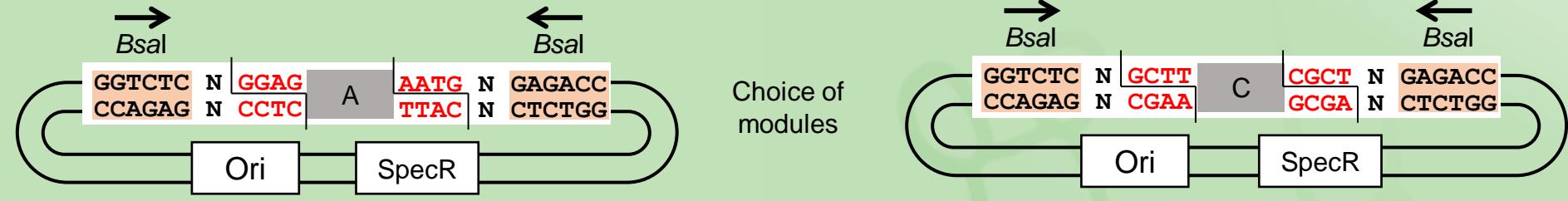
BsaI + T4 Ligase



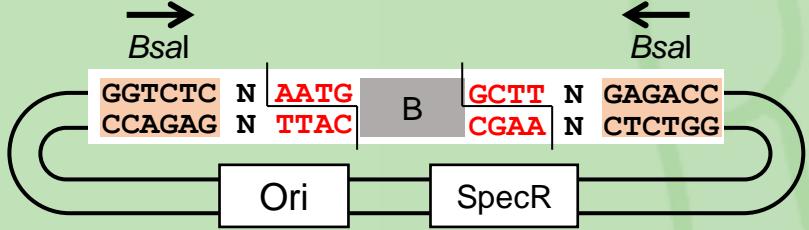
## GG-Modules



## GG-Modules

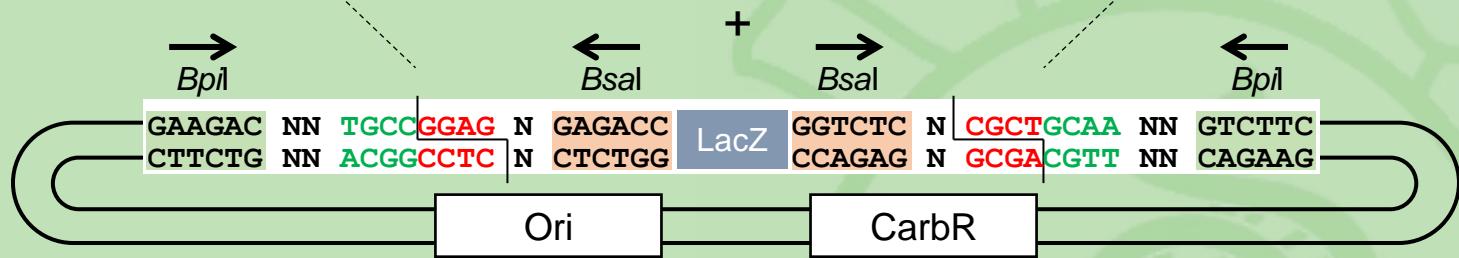


Choice of modules



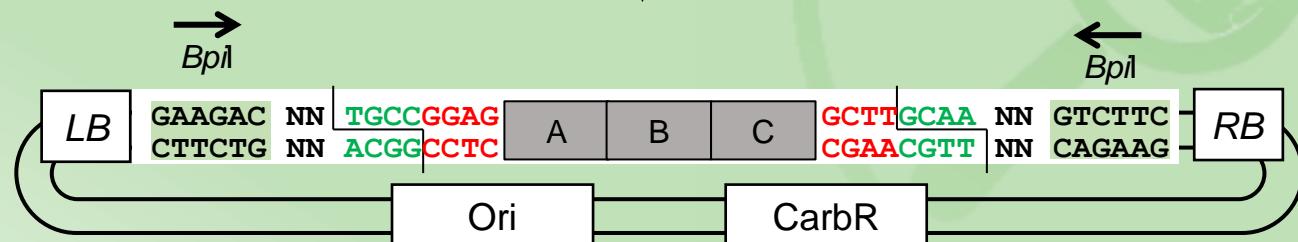
Level 0

Vector  
pICH47732



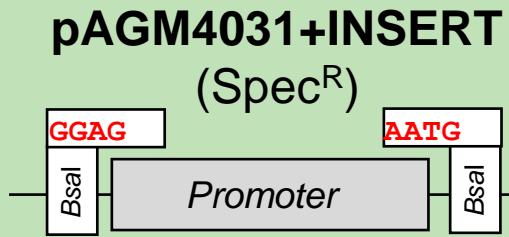
Level 1

Module



Level 1  
transcription units

Quais as mudanças dos vetores de Level 0 para o Level 1?



Module  
**Level 0**

**pAGM4031+INSERT**  
(Spec<sup>R</sup>)



+

**pICH47732**  
(Carb<sup>R</sup>)



**Level 1**

Bpil, T4 Ligase

**pICH47732+Inserts**  
(Carb<sup>R</sup>)



**Level 1**  
Transcriptional Unit

# Clonagem por GoldenGate

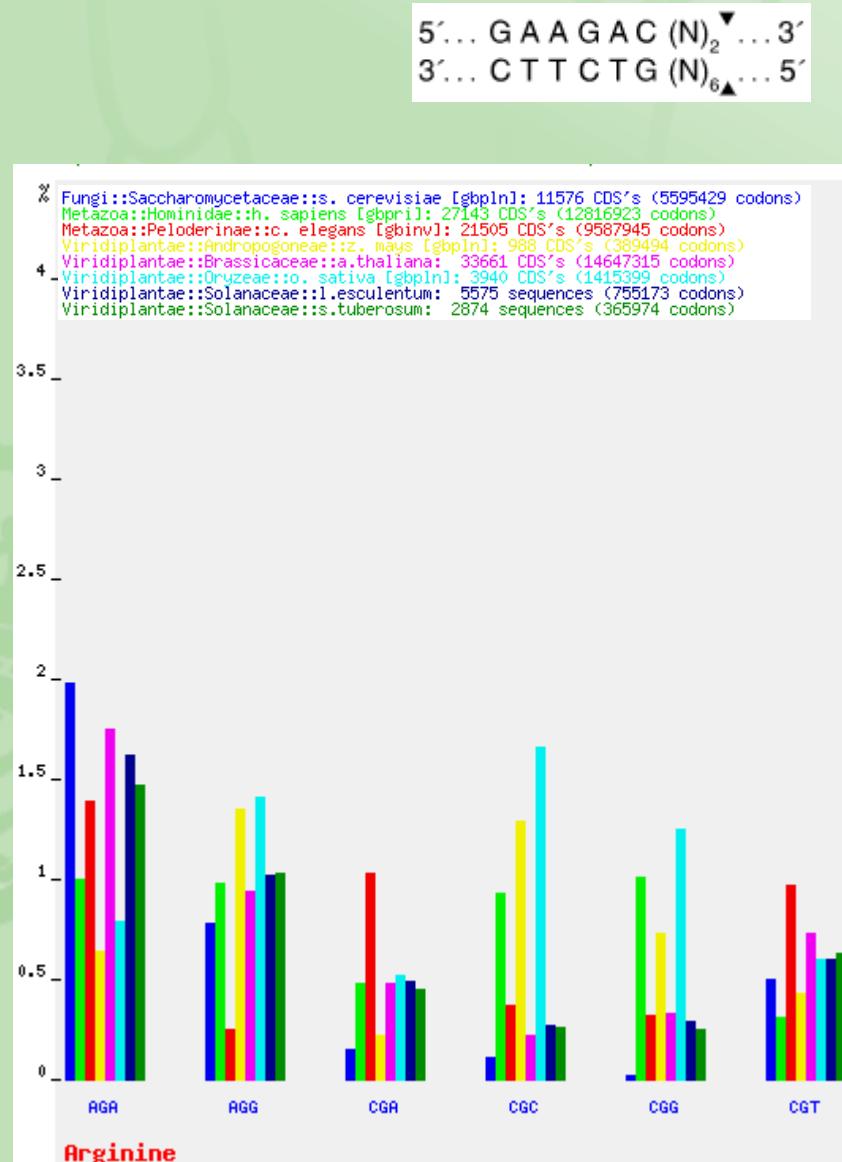
BpI (BbsI)

- Level 0: Preparar as regiões alvo

BpI

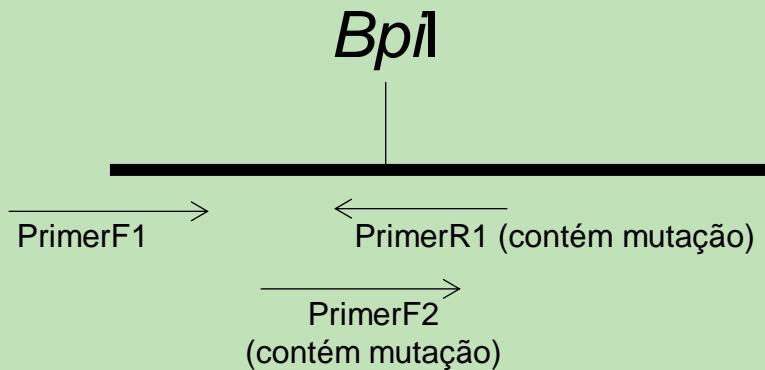
CGA\_AGA\_CGA

[triplet][frequency: per thousand]([number])			
TTT 24.3(8919)	TCT 24.9(9135)	TAT 17.1(6266)	TGT 10.6(3885)
TTC 17.8(6516)	TCC 12.4(4564)	TAC 11(4047)	TGC 7.3(2704)
TTA 13(4771)	TCA 21.3(7824)	TAA 1.6(593)	TGA 3.2(1175)
TTG 22.6(8279)	TCG 5.9(2192)	TAG 1.1(423)	TGG 12.8(4714)
CTT 24.8(9105)	CCT 20.1(7385)	CAT 15.6(5721)	CGT 7.8(2880)
CTC 12.7(4664)	CCC 7(2593)	CAC 8.6(3183)	CGC 4.1(1519)
CTA 9.5(3489)	CCA 18.1(6652)	CAA 21(7693)	CGA 6(2214)
CTG 11.1(4077)	CCG 5.7(2120)	CAG 15.2(5581)	CGG 4(1492)
ATT 27.1(9941)	ACT 19.9(7291)	AAT 28.1(10316)	AGT 14.9(5458)
ATC 13.8(5056)	ACC 9.7(3578)	AAC 15.9(5841)	AGC 10(3682)
ATA 12.6(4645)	ACA 16.5(6063)	AAA 30.7(11260)	AGA 16.2(5932)
ATG 27.3(10004)	ACG 4.5(1647)	AAG 29.6(10840)	AGG 11.8(4346)
GTT 26.9(9869)	GCT 29.6(10864)	GAT 36.8(13489)	GGT 20.9(7681)
GTC 10.2(3769)	GCC 11(4037)	GAC 13.8(5060)	GGC 9.6(3546)
GTA 10.5(3870)	GCA 20.7(7581)	GAA 33.8(12395)	GGG 22.1(8114)
GTG 15.1(5538)	GCG 5.4(1990)	GAG 25.7(9430)	GGG 10.6(3910)



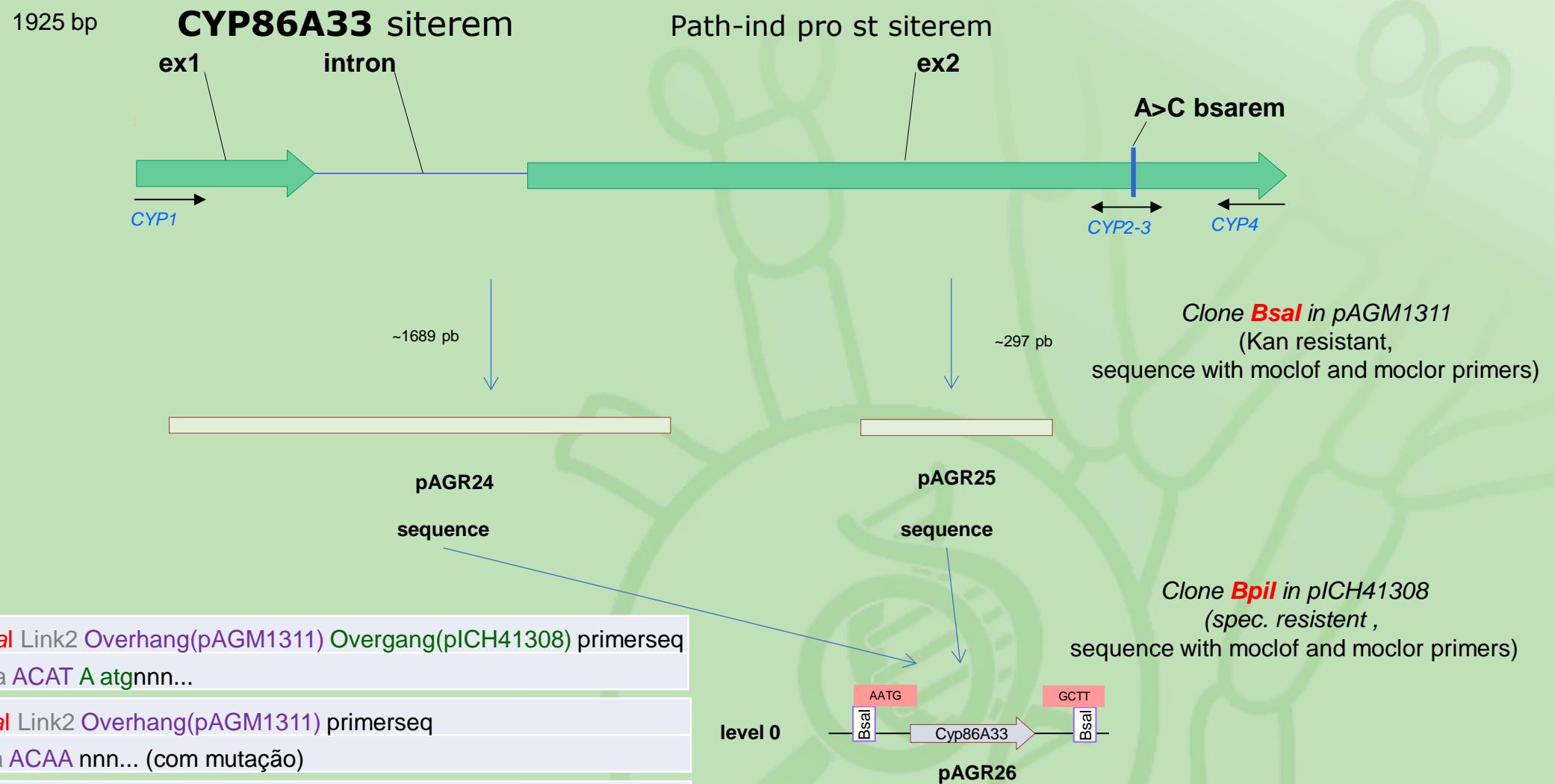
# Clonagem por GoldenGate

- Level 0: Preparar as regiões alvo



CGA\_AGA\_CGA  
↓  
CGA\_AGG\_CGA

# Exemplo com mutação



Nome	Link1 <b>Bsal</b> Link2 Overhang(pAGM1311) Overgang(pICH41308) primerseq
PrimerF1	tt ggtctc a ACAT A atgnnn...
Nome	Link1 <b>Bsal</b> Link2 Overhang(pAGM1311) primerseq
PrimerR2	tt ggtctc a ACAA nnn... (com mutação)
Nome	Link1 <b>Bsal</b> Link2 Overhang(pAGM1311) primerseq
PrimerF3	tt ggtctc a ACAT nnn... (com mutação)
Nome	Link1 <b>Bsal</b> Link2 Overhang(pAGM1311) Overgang(pICH41308) primerseq
PrimerR4	tt ggtctc a ACAA AAGC nnn...
Link1:	apoio da enzima
Link2:	nucleotídeo extra da enzima (1 para <i>Bsal</i> : 2 para <i>Bpil</i> )

CYP1 (31)	tt ggtctc a ACAT A atggatccatactagttattctgg
CYP2 (32)	tt ggtctc a ACAA cGccaccggcgagagccatc
CYP3 (33)	tt ggtctc a ACAT ggCgaccggttcgAACGCCAAAG
CYP4 (34)	tt ggtctc a ACAA AAGC tcatgcagacatagcaatcttggag

# Exemplo sem mutação

~ 2253 bp      **ABCG1**

ex3

*ABC1*

*ABC2*

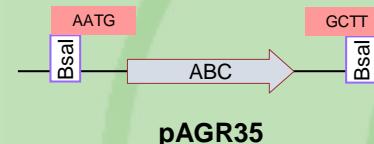
~2253 pb

Clone *BpI* in pICH41308

(spec resistant,

sequence with moclof and moclor primers)

level 0



Nome	Link1 <i>BpI</i> Link2 Overgang(pICH41308) primerseq
------	--

PrimerF1	tt gaagac aa A atgnnn...
----------	--------------------------

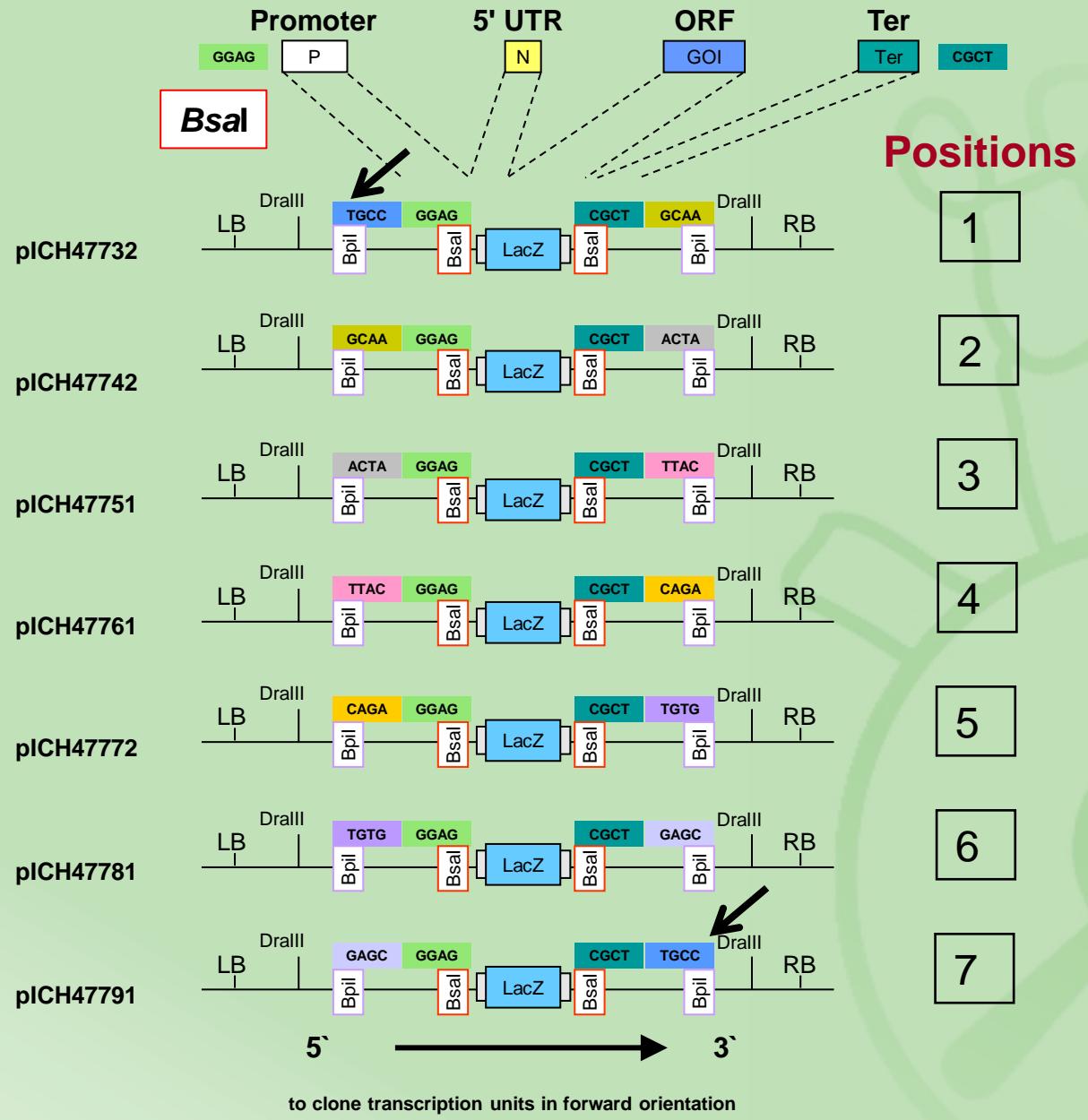
Nome	Link1 <i>BpI</i> Link2 Overgang(pICH41308) primerseq
------	--

PrimerR2	tt gaagac aa AAGC nnn...
----------	--------------------------

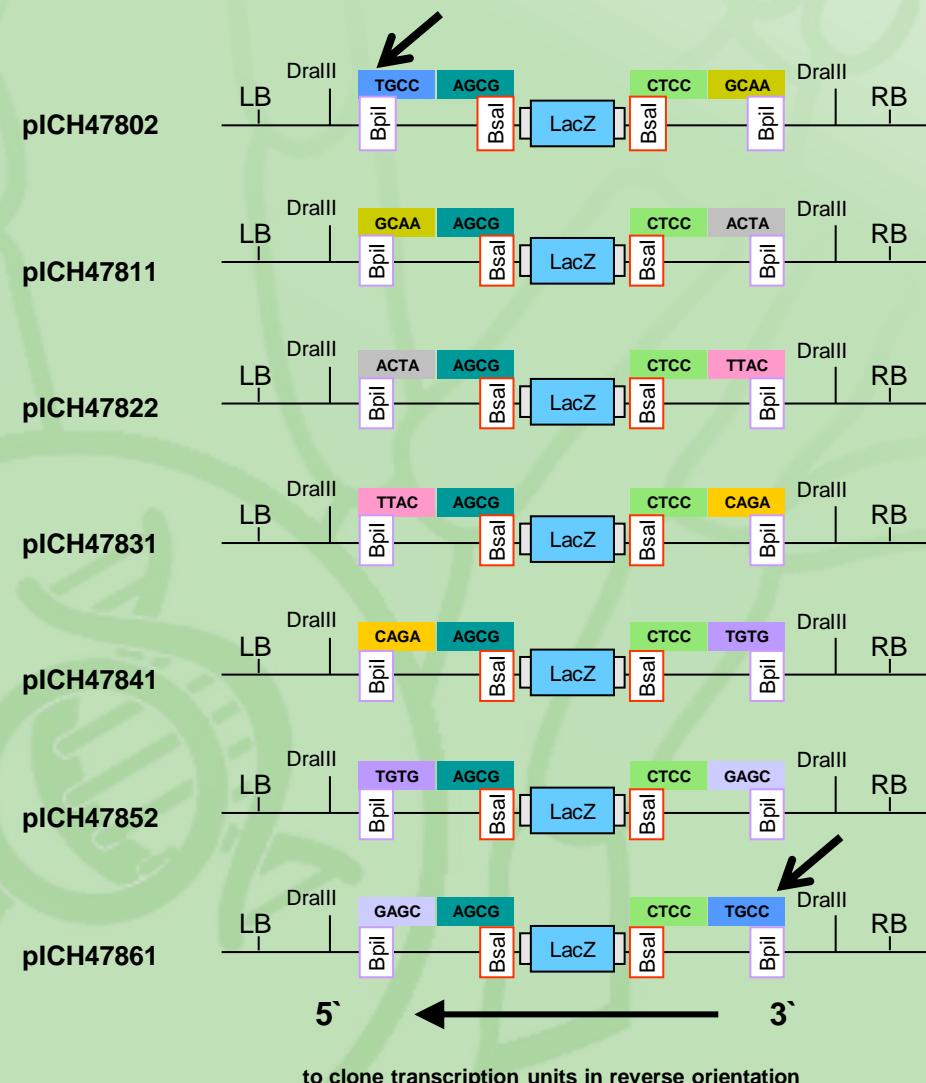
Link1: apoio da enzima

Link2: nucleotídeo extra da enzima (1 para *BsaI*: 2 para *BpI*)

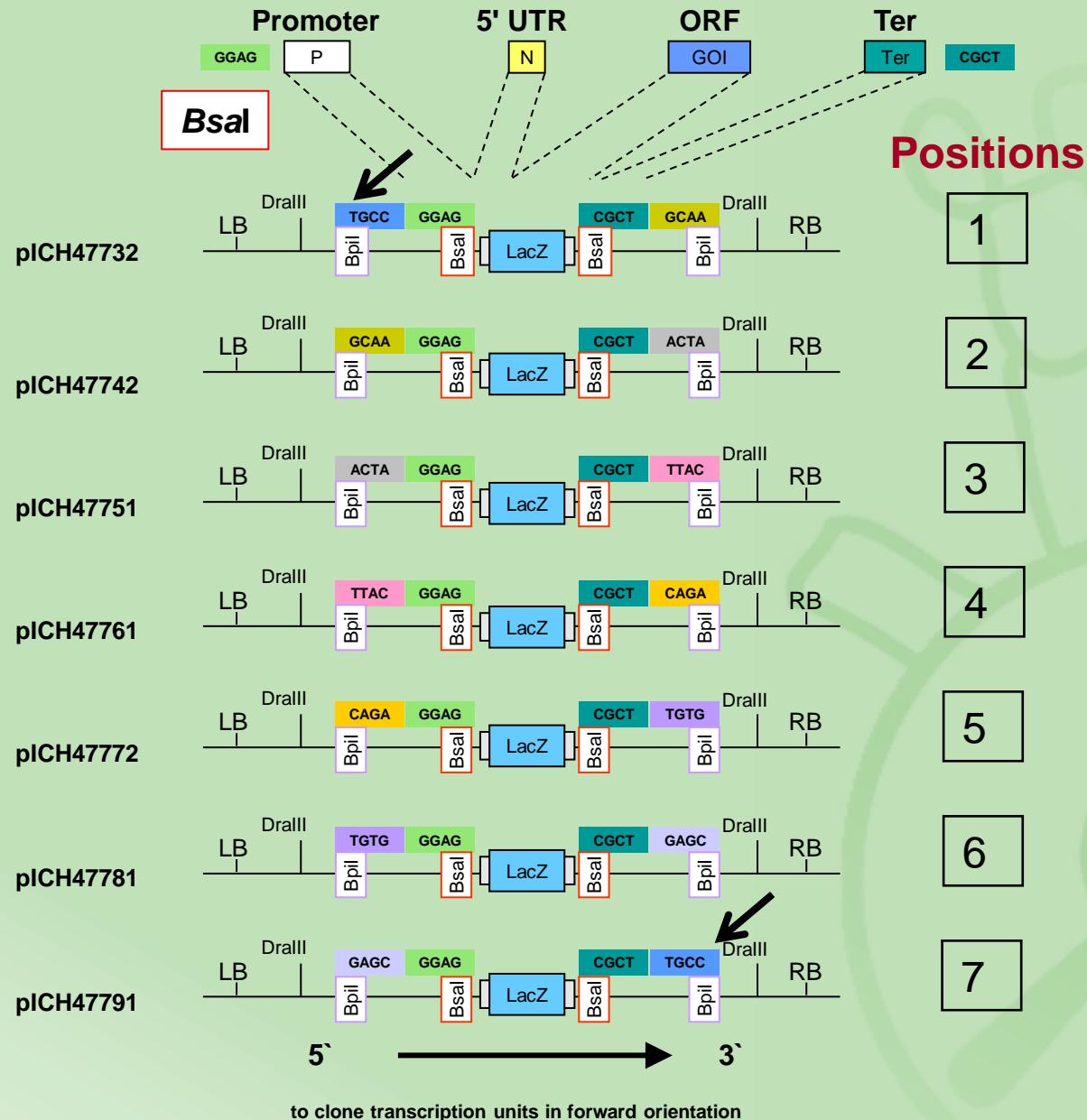
ABC1 (46)	tt gaagac aa A atgtcaaggatagtggcggaaaatatgttacaag
ABC2 (47)	tt gaagac aa AAGC tcaccttcttgttacttccaagcaac



## Level 1 cloning vectors



## Level 1 cloning vectors



pICH47732

pICH47742

pICH47751

pICH47761

pICH47772

pICH47781

pICH47791

TGCC

1

GCAA

2

ACTA

3

TTAC

4

CAGA

5

TGTG

6

GAGC

7

TGCC

pICH47732

pICH47742

pICH47751

pICH47761

pICH47772

pICH47781

pICH47791

TGCC

1

GCAA

2

ACTA

3

TTAC

4

CAGA

5

TGTG

6

GAGC

7

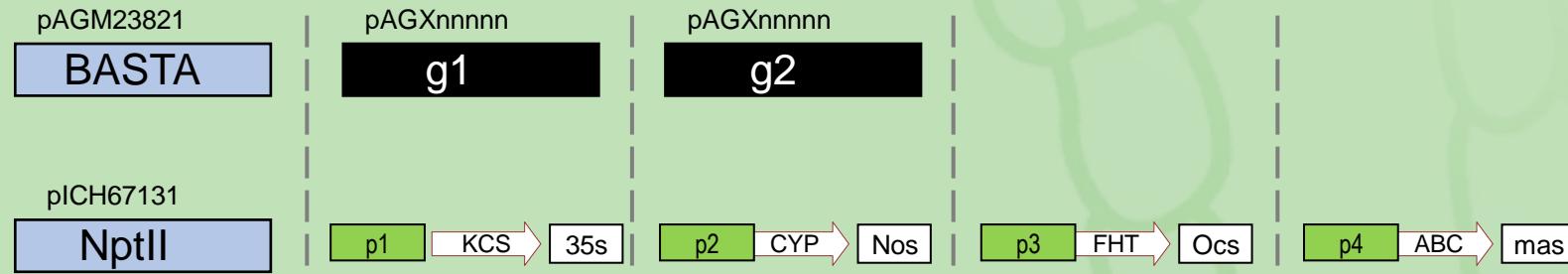
TGCC

pAGM23821

BASTA

pICH67131

NptII





pAGM23821

**BASTA**

pAGXnnnnn

**g1**

pAGXnnnnn

**g2**

pICH67131

**NptII**

p1 KCS 35s

p2 CYP Nos

p3 FHT Ocs

p4 ABC mas

pICH67131

**NptII**

pAGXnnnnn

**Cas9**

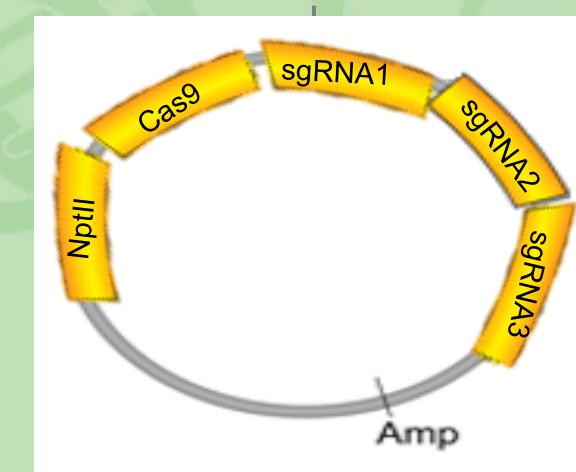
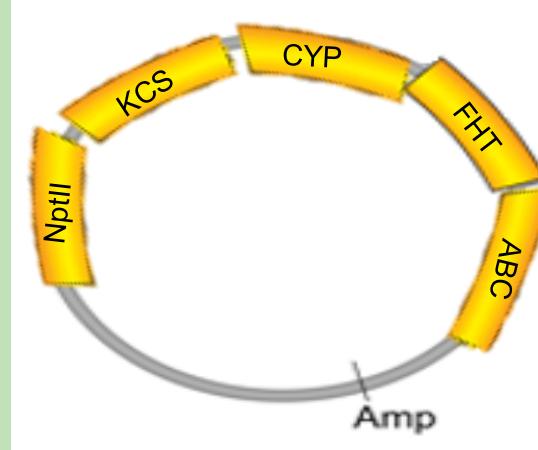
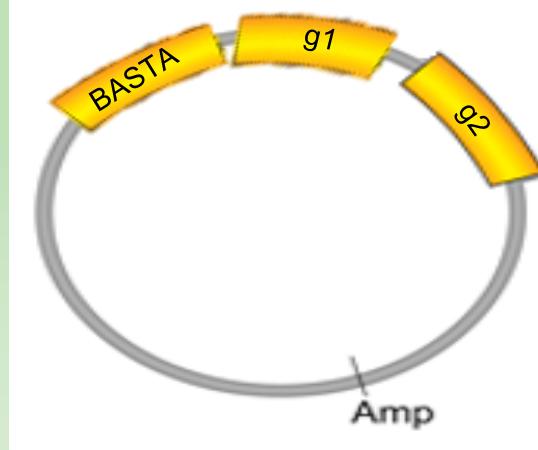
pAGXnnnnn

**sgRNA1**

pAGXnnnnn

**sgRNA2**

pAGXnnnnn

**sgRNA3**

# Ferramentas online úteis

## Sequence massager (attotron)

<http://biomodel.uah.es/en/lab/cybertory/analysis/massager.htm>

## Tm Calculators

<https://tmcalculator.neb.com/>

<https://www.thermofisher.com/de/de/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html>

## Oligo calc (Northwestern)

<http://biotools.nubic.northwestern.edu/OligoCalc.html>

## Oligoanalyzer (IDT) (Necessário Registro)

<https://www.idtdna.com/calc/analyzer/>

## NEBcutter (NEB)

<http://nc2.neb.com/NEBcutter2/>

# Golden Gate cloning: the assembly reaction

## Reaction mix

vector	20 fmol	x
insert 1, 20 fmol		x
insert 2, 20 fmol		x
.....		x
.....		x
insert n, 20 fmol		x
T4 Ligase *(Promega)	1.0 $\mu$ L	
Bsal (NEB) or BpiI (Fermentas)	0.5 $\mu$ L	
Ligase buffer (Promega)	1.5 $\mu$ L	
H <sub>2</sub> O		to 15 $\mu$ L

\*For 1 to 3 fragments:  
ligase 3 u/ $\mu$ L

For 4 or more fragments:  
ligase HC 10-20 u/ $\mu$ L  
(optional, normal ligase  
will work as well)

Enzyme + ligase:  
max volume up to 10% of final  
ligation volume

## Calculating the amount of DNA to pipet

For 20 fmol ( $E^{-15}$ ):

For example:

$$V (\mu\text{L}) = \frac{20 \text{ fmol} \times \text{Size (bp)}}{\text{conc (ng}/\mu\text{L}) \times 1520}$$

size 6939 bp  
Conc 385 ng/ $\mu$ L

--> Volume to pipet: 0.23  $\mu$ L

--> It is better to dilute the original DNA prep  
and pipet an appropriate higher volume

## Programs for restriction-ligation

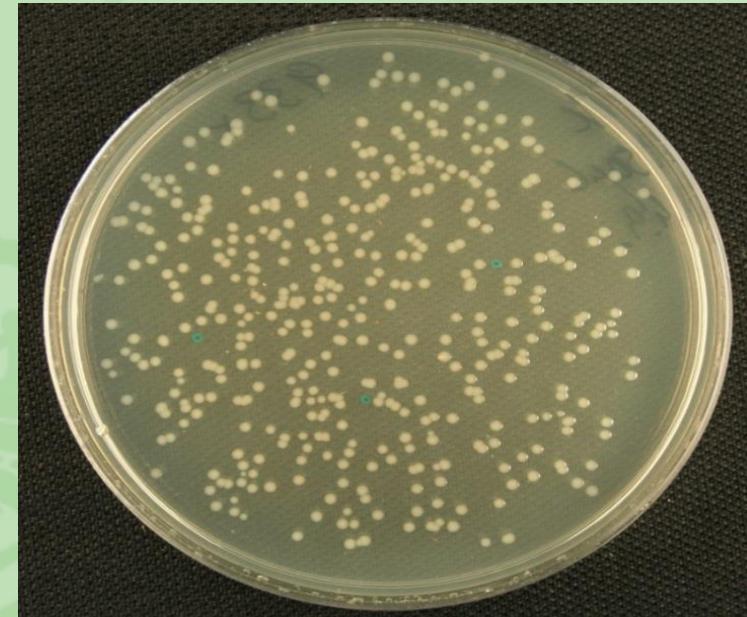
Many  
fragments

37 °C	5 min
16 °C	5 min
20 x	
50 °C	5 min
80 °C	5 min
4 °C	$\infty$

Assembly in a thermocycler

## **Transformation of *E. coli* :**

- Let the S.O.C./LB medium in RT
- Add 15 µl ligation to 50 µl chemically competent cells
- Let it 15 min in ice
- Heat shock 30-60 s at 42°C
- Transfer it immediately to ice
- Add 250 µl S.O.C./LB medium
- Shake it horizontally 1h – 100 rpm at 37°C
- Plate 30-50 µl in a plate with the right antibiotic + or – X-Gal



# Mais informações sobre GoldenGate

- **Thermo Fisher**

- [https://www.thermofisher.com/de/de/home/life-science/cloning/geneart-type-ii-assembly-kits.html?ef\\_id=Cj0KCQjwvYSEBhDjARIsAJMn0IhwLW-OVwAa\\_X-TYZ4mP0tpp5hYUluuB6nwD-JdQgQIJCdxS3ZnL0aAkg2EALw\\_wcB:G:s&s\\_kwcid=AL!3652!3!476186507167!e!!g!!golden%20gate%20assembly%20cloning&cid=bid\\_mol\\_gse\\_r01\\_co\\_cp1358\\_pjt0000\\_bid00000\\_0se\\_gaw\\_nt\\_pur\\_con&gclid=Cj0KCQjwvYSEBhDjARIsAJMn0IhwLW-OVwAa\\_X-TYZ4mP0tpp5hYUluuB6nwD-JdQgQIJCdxS3ZnL0aAkg2EALw\\_wcB](https://www.thermofisher.com/de/de/home/life-science/cloning/geneart-type-ii-assembly-kits.html?ef_id=Cj0KCQjwvYSEBhDjARIsAJMn0IhwLW-OVwAa_X-TYZ4mP0tpp5hYUluuB6nwD-JdQgQIJCdxS3ZnL0aAkg2EALw_wcB:G:s&s_kwcid=AL!3652!3!476186507167!e!!g!!golden%20gate%20assembly%20cloning&cid=bid_mol_gse_r01_co_cp1358_pjt0000_bid00000_0se_gaw_nt_pur_con&gclid=Cj0KCQjwvYSEBhDjARIsAJMn0IhwLW-OVwAa_X-TYZ4mP0tpp5hYUluuB6nwD-JdQgQIJCdxS3ZnL0aAkg2EALw_wcB)

- **BioLabs**

- <https://international.neb.com/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/golden-gate-assembly>

- **Addgene**

- <https://blog.addgene.org/plasmids-101-golden-gate-cloning>
- <https://www.addgene.org/>

- **Papers Sylvestre Marillonnet**

- Golden Gate cloning. Engler C, Marillonnet S. 2014
- A One Pot, One Step, Precision Cloning Method with High Throughput Capability. Engler C, Kandzia R, Marillonnet S. 2008
- Golden Gate Shuffling: A One-Pot DNA Shuffling Method Based on Type IIs Restriction Enzymes. Engler C, Gruetzner R, Kandzia R, Marillonnet S. 2009
- Fast track assembly of multigene constructs using Golden Gate cloning and the MoClo system. Werner S, Engler C, Weber E, Gruetzner R, Marillonnet S. 2012
- A Modular Cloning System for Standardized Assembly of Multigene Constructs. Weber E, Engler C, Gruetzner E, Werner S, Marillonnet S. 2011

# Construindo um Cassette CRISPR

- Dicas gRNA (*Sp-Cas9*)
  1. A sequência PAM é absolutamente necessária para a clivagem, mas NÃO faz parte da sequência do gRNA e, portanto, não deve ser incluída no sgRNA.
  2. Evitar PAM GGG ou NGGG
  3. A sequência alvo pode estar em qualquer uma das fitas de DNA, mas NÃO em um intron.
  4. Não é recomendado regiões no pepítideo-sinal
  5. 18-24 pb (ideal: 20 pb)
  6. Selecionar o gRNA com menos off-targets (com 3 ou mais mismatches)
  7. 40-80 % CG
  8. G no 5'-end (U6p)
  9. Evitar TTTT...
  10. Evitar hairpins ou outras estruturas secundárias (CC<sub>n</sub>nnnnnnnnnnnnnnnGGG)
  11. Recomendado um A/T 4 nt upstream da sequência PAM (nnnnnnnnnnnnnnn(A/T)nnn NGG)

# Lista de ferramentas online para desenhar o gRNA

Nome	Site	Genomas e observações
Green Listed – a CRISPR Screen Tool	<a href="http://greenlisted.cmm.ki.se/">http://greenlisted.cmm.ki.se/</a>	Rato e humano
EuPaGDT	<a href="http://grna.ctegd.uga.edu/">http://grna.ctegd.uga.edu/</a>	Patógenos em geral + Genoma customizado
<b>CRISPOR</b>	<b><a href="http://crispor.tefor.net/">http://crispor.tefor.net/</a></b>	<b>Alta variedade</b>
sgRNA Scorer	<a href="https://sgrnascorer.cancer.gov/">https://sgrnascorer.cancer.gov/</a>	14 diferentes (humano, camundongo, rato, elefante, gato, cachorro, vaca...)
Cas-Designer	<a href="http://www.rgenome.net/cas-designer/">http://www.rgenome.net/cas-designer/</a>	59 diferentes
CRISPR-ERA	<a href="http://crispr-era.stanford.edu/">http://crispr-era.stanford.edu/</a>	9 diferentes (Humano, camundongo, rato, zebrafish, levedura, <i>E. coli</i> ...)
CCTOP'	<a href="https://cctop.cos.uni-heidelberg.de/">https://cctop.cos.uni-heidelberg.de/</a>	Alta variedade
Off-Spotter	<a href="https://cm.jefferson.edu/Off-Spotter/">https://cm.jefferson.edu/Off-Spotter/</a>	Humano, camundongos e levedura
CRISPR Multitargeter	<a href="http://www.multicrispr.net/index.html">http://www.multicrispr.net/index.html</a>	12 diferentes (Humano, camundongo, rato, arabidopsis, arroz, milho, drosophila...)
ZiFiT Targeter	<a href="http://zifit.partners.org/ZiFiT/">http://zifit.partners.org/ZiFiT/</a>	Sem organismos alvo, porém também é usado para TALEN
CRISPRdirect	<a href="http://crispr.dbcls.jp/">http://crispr.dbcls.jp/</a>	Alta variedade
<b>CHOPCHOP</b>	<b><a href="http://chopchop.cbu.uib.no">chopchop.cbu.uib.no</a></b>	<b>Alta variedade</b>
E-Crisp	<a href="http://www.e-crisp.org/E-CRISP/designcrispr.html">http://www.e-crisp.org/E-CRISP/designcrispr.html</a>	55 diferentes
CasFinder	<a href="https://arep.med.harvard.edu/CasFinder/">https://arep.med.harvard.edu/CasFinder/</a>	Humano e camundongos (tem que fazer o download)
FlyCrispr	<a href="https://flycrispr.org/">https://flycrispr.org/</a> - <a href="http://targetfinder.flycrispr.neuro.brown.edu/">http://targetfinder.flycrispr.neuro.brown.edu/</a>	37 diferentes, dos quais 19 são de Drosophilas
<b>Synthego</b>	<b><a href="http://Design.synthego.com/#/">Design.synthego.com/#/</a></b>	<b>Alta variedade</b>
<b>CRISPick</b>	<b><a href="https://portals.broadinstitute.org/gppx/crispick/public">https://portals.broadinstitute.org/gppx/crispick/public</a></b>	<b>Humano, camundongo e rato</b>
<b>WashU gRNA Designer</b>	<b><a href="http://crispr.wustl.edu/">http://crispr.wustl.edu/</a></b>	<b>Humano e camundongo</b>

# Construindo um Cassete CRISPR

- Escolher o gene ou região de interesse
    - *ABCG1* de *Solanum tuberosum* (cDNA)

**ATG**TCAAGGATAGTGGCGGAAAATATGTTACAAGGGGGAGAAAATGTACAATTATAATCAAAGAGTACAACAAAGCCATGGAGATGTCACAAGCCAGCGCGTA  
CTCTCACCCACCCTAGGCCAAATGCTAAAGCGCGTGGGAGACGTGAGAAAGGAAGGCCACCGGCACGAAACTCCGGTGCACCGGATTCTCGATATGAGTGATA  
CTCAAAGCATATCATCTCACTCTCCTTTGTACTCTCCTCAACAACCTCACCTACAGCGTAAAAGTCCGCCGAAAATGCCTTCCAGCGATACTCCGG  
CGACCGGCCGGAGTTCCACCGGTATCCCCTGCCGGAGAAAATCTGTTCACGAACACAAAATTCCCTGAACAAATATCTCCGGGAGGCCGGGACGG  
CGAGATAGTCGCCGTCTGGGTGCATCAGGGTCGGGAAATCGACCCCTGATCGATGCCCTCGCGAATAGGATCGCGAAGGAGAGTTAAAAGGAACGATAACGT  
TAAACGGAGAGCCACTTGAGATTGAAAGTAATCTCAGCATATGTAATGCAAGATGATCTTTATATCCAATGTTGACAGTTGAAGAACACATTAATG  
TTTGCAGCTGAATTCAAGATTGCCACGTACTTCATCAAAATCAAAAAAGAAAATGAGAGTTCAAGCTTGATTGATCAATTAGGACTACGAAATGCTGAAAAAC  
AATCATTGGTATGAGGGTATCGTGGAGTGTGGTGGTAAAGACGACGAGTTCGATTGAAATTGATATTATTGATATTGACCTATCATATTGTTTAGACG  
AGCCAACCTCAGGTCTGACTCGACTAGTCATATGGTGGTAAAGGTTCTACACGAATTGCTCAAAGTGGAAAGTATTGTTATCATGTCAATTCACTCAGCCA  
AGTTATCGAATTCTCGGGTTATTGGATGGATGCTCTTGTCCCCTGGTCAAACGGTTATAGTGGTCACCTATGAACCTCCCACATTGTTGCTGATTT  
TGGTCACCCAATACCGGATAGTAAAATAGAACAGAGTTGCTCTGGATCTAATTGCGAACTAGAAGGGTCCCCCTGGAGGGACAAAAAGTTGGTTGAGTTCA  
ACAAAACATGGAAAATACTAAAAGGAGTAATGAAAATCCAAACACCTACTCATGGATTGTCATTGAAAGAACATTAGCGCGAGTATTCAAGA  
GGGAAGTTGGTTCAAGGACAACGAGTGATATTGATACTAGTCCAGCATCAATGGTCCAACCTACGCGAATCCATTGGATTGAAATGCTGTGTTGCTCAA  
GAGATCATTACGAATTCTGGAGGGTGCCAGAGTTATTGGTATTGCTAGGGCAATCGTGGTCACGGGTTCATCCTAGCTACCATGTTGGCAACTTG  
ATGATTCCCCTAAAGGGTTCAAGAAAGGTTGGTTCTTGCTATGTCAACAACTTCTATACTTGCGCGGACGCGTGCCTGTGTTCTCCAAGAG  
AGGTACATTTCATGAGGGAGACTGCTTATAATGCTTATAGGAGATCTCCTATTGTCTATCTCATGCTATAGTTCTTGCCAGCATTGATCTTCTAGCTT  
TGCATTGCTGCTATAACTTTGGGCTGTAGGCCTGTAGGTGGATTTCGGGCTTTGTTCTATTGCGATAACTAGCCTCCTGGGCCGGAAATT  
CATTGTCACGTTCTCCGGTAGTTCTAGTGTATGTTAGGTTACACCATAGTGGTCGCGATCCTAGCCTATTGCGCTTCTCAGGATTCTCATC  
AATCGCGATAGGATTCCACCTATTGGATATGGTTCACTACCTGTCTGGTAAATATCCTATGAAGCTGTGTTACAAAATGAATTGATGCAACAAA  
GTGTTTGTCAAAGGGATTCAATTGTTGATAATTCAACCACTGGAAATGTGCCTAATGCATTGAAGGAAAATTGTTGAGTACAATGAGTAACACATTAAATG  
TCAAAATTACAAGTTAACATGTGTGACTACTGGGGCTGATATTGGTTCAACAAAGGGATTACTGATTAAAGTAAGTGGATTGTTGAGTACAATGAGTAACACATTAAATG  
TGGGGTTTCTTTAGGGTTTACTTAGCTTGTGCTTGGAAAGTAAGAACAAAGAGAAGG**TGA**

# Construindo um Cassette CRISPR

- Escolher o gene ou região de interesse

- ABCG1 de *Solanum tuberosum*
  - Procurar sequencias PAM (5'-NGG)
  - gRNA para Cas9:

- 20 pb + PAM

- xxxxxxxxxxxxxxxxxxxxNGG ou CCNxxxxxxxxxxxxxx

# Construindo um Cassette CRISPR

ATG **1** TCAAGGATAGGGCGGAAAATATGTTACAAGGGGAGAAAATGTACAATTATAATCAAAGAGTACAACAAAGCCATGGAGATGTCACAAGCCAGCGCGT  
ACTCTCACCCACCCTAGGCCAATGCTAAAGCGCGTGGGAGACGTGAGAAAGGAAGGCCACCGGCACGAAACTCCGGTGCACCGGATTCTCGATATGAGTGA  
TACTCAAAGCATATCATCTACTCTCTTGTACTCTCCTTCAACAAACCTCACCTACAGCGTAAAGTCCGCCGGAAAATGCCTTCCAGCGATACTC  
CGCGACCGGCCGGAGTTCCACCGGTGATCCCGTCGCCGGAGAAAATCTACAGGAACACAAAATTCCCTCTGAACAATATCTCCGGCAGGCCGGG  
ACGGCGAGATAGTCGCCGTCTGGTGCATCAGGTCGGGGAAATCGACCCCT **4** CGATGCCCTCGCGAATAGGATCGCGAAGGAGAGTTAAAAGGAACGAT  
AACGTTAACGGAGAGCCACTTGATTGAGATTGTTGAAAGTAATCTCAGCATATGTAATGCAAGATGATCTTATATCCAATGTTGACAGTTGAAGAAACA  
TTAATGTTGCAGCTGAATT CAGATTGCCACGTACTTCATCAAAATCAAAAAAGAAAATGAGAGTTCAAGCTTGATTGATCAATTAGGACTACGAAATGCTG  
CAAAAACAATCATTGGT GATGAGGGT CATCGTGGAGTGTCTGGTGGAAAGACGACGAGTTCGATTGGAATTGATATTATTATGACCCCT **5** CATATTGTT  
TTTAGACGAGCCAATT CAGGTCTTGACTCGACTAGTGCATATATGGTGGTAAGGTTCTACAACGAATTGCTCAAAGTGGAGTTGTTAATTGCAATT  
CATCAGCCAAGTTATCGAATTCTCGGTTATTGGATCGGATGCTCTTGTCCC GTGGTCAAACGGTTATAGTGGTCACCTATGAACCTCCACATTTT  
TTGCTGATTTGGTCACCCAATACCGGATAGT GAAAATAGAACAGAGTTGCTCTGGATCTAATT CGCGAACTAGAAGGGTCCCCTGGAGGGACAAAAGTT  
GGTTGAGTTCAACAAAACATGGGAAAATACTAAAAGGAGTAATGAAAATCCTGAAATCCAAACACCTACTCATGGATTGTCATTGAAAGAACGAAATTAGCGCG  
AGTATTCAAGAGGGAGTTGGTTCAAGGACAACCGAGTGATATT CATACTAGTCCAGCAGTCAATGGTTCAAACCCATTACGCGAATCCATTGGATTGAAATGC  
TTGTGTTGTCCAAGAGAGATCATTA CGAATTCTTGAGGGT GCCAGAGTTATTGGTATT CGTCTAGGGCAATCGTGGTCACGGGTTCATCCTAGCTACC  
GTTTGCAACTTGATGATTCCCCTAAAGGGTTCAAGAAAGGCTTGGTTCTTGCTATTGCTATGTCAACAACTTCTATACTTGCACGGGACGCCTGCCT  
GTGTTCTCCAAGAGAGGTACATTTCATGAGGGAGACTGCTTATAATGCTTATAGGAGATCTCCTATTGCTATCTCATGCTATAGTTCTTGCAGCAT  
TGATCTTCTTAGCTTGCATTGCTGCTATAACTTTGGCTGTAGGCCTTGTAGGTGGATTTCGGCTTTGTTCTATT CGCGATAATACTAGCCTC  
CTTCTGGCCGGAATT CATTGTCACGTTCTCCGGTGTAGTTCTAGTGTCTAGTTACACCATAGTGGTCGCGATCCTAGCCTATTCCCTCCTC  
TTCTCAGGATTCTTCAATCGCGATAGGATTCCACCTATTGGATATGGTTCACTACCTGTCTGGT GAAATATCCTTATGAAGCTGTGTTACAAAATG  
AATTTGATGATGCAACAAAGTGTGTTCAAGGGATTCAATTGTTGATAATT CACCACTGGAAATGTGCCTAATGCATTGAAGGAAAATTGTTGAGTAC  
AATGAGTAACACATTAAATGTCAAAATTACAAGTTCAACATGTGTGACTACTGGGCTGATATATTGGTTCAACAAGGGATTACTGATTAAAGTAAGTGGAAAT  
TGTGTTGGATTACTATTGCATGGGGTTTCTTAGGGTTTACTTAGCTTGTGCTTGGAAAGTAAGAACAGAGAAGG**TGA**

# Construindo um Cassette CRISPR

- Escolher o gene ou região de interesse
  - ABCG1 de *Solanum tuberosum*
  - Procurar sequencias PAM (5'-NGG)
    - CRISPOR (<http://crispor.tefor.net/>)

tefor infrastructure

CRISPOR ([citation](#)) is a program that helps design, evaluate and clone guide sequences for the CRISPR/Cas9 system. [CRISPOR Manual](#)  
June 2020: *saCas9 primer fixes, Snapgene/Geneious improvements, better export for Cpf1* [Full list of changes](#)

**Step 1**  
Planning a lentiviral gene knockout screen? Use [CRISPOR Batch](#)  
Sequence name (optional):   
Enter a single genomic sequence, < 2000 bp, typically a [protospacer](#).  
Clear Box - Reset to default  

```
cttcctttgtccccaaatctggggcgccgcggccggcccccctggcgccctaaggactcgccgcggaaagtggcc  
agggcgggggcgacctcgctcacagcgcccccggctattctcgccagctaccatgATGAAGATATCGCCGCG  
CTCGTCGTGACAACGGCTCGGCATGTGCAAGGCCGGCTCGCGGGCGACGATGCCCGGGCCGTCCTCCCC  
CTCCATCGTGGGGCGCC
```

**Inserir a sequência ABCG1 (cDNA) menor que 2000 bp**

**Step 2**  
Select a genome

**Step 3** ⓘ  
Select a Protospacer Adjacent Motif (PAM)  
  
**SUBMIT**

Text case is preserved, e.g. you can mark ATGs with lowercase.  
Instead of a sequence, you can paste a chromosome range, e.g. chr1:11,130,540-11,130,751

Version 4.98 - [Documentation](#) - [Contact us](#) - [Downloads/local installation](#) - [Citation](#) - [License](#)

# Construindo um Cassette CRISPR

- Escolher o gene ou região de interesse
  - ABCG1 de *Solanum tuberosum*
  - Procurar sequencias PAM (5'-NGG)
    - CRISPOR (<http://crispor.tefor.net/>)

 CRISPOR ([citation](#)) is a program that helps design, evaluate and clone guide sequences for the CRISPR/Cas9 system. [Crispor Manual](#)  
June 2020: saCas9 primer fixes, Snapgene/Geneious improvements, better export for Cpf1 [Full list of changes](#)

**Step 1**  
Planning a lentiviral gene knockout screen? Use [CRISPOR Batch](#)  
Sequence name (optional):   
Enter a single genomic sequence, < 2000 bp, typically an exon   
[Clear Box](#) - [Reset to default](#)  

```
cttcctttgtcccaatctggcgcgccccctggcgccctaaggactggcgccggaaagtggcc  
agggcgggggcgacctcggtcacagcgccggctatttcgcagtcaccatgATGATGATATGCCCG  
CTCGTCGTGACAAACGGCTCGGCATGTGCAAGGCCGGCTCGCGGGCGACGATGCCCCCGGGCGTCTTCCC  
CTCCATCGTGGGCGCC
```

Text case is preserved, e.g. you can mark ATGs with lowercase.  
Instead of a sequence, you can paste a chromosome range, e.g. chr1:11,130,540-11,130,751

**Step 2**  
Select a genome

**Selecionar o organismo  
(*Solanum tuberosum* (SolTub\_3.0))**

Halyomorpha halys - yellow-brown stink bug - NCBI GCF\_000696795.2 (Hhal\_2.0)

**Solanum** 

Solanum lycopersicum - Solanum lycopersicum - Phytozome V9, Dec 2012  
Solanum lycopersicum - Tomato - Solgenomics.net SL3.0 ITAG3.0  
Solanum lycopersicum - tomato - solgenomics.net M82 1.3  
Solanum melongena - eggplant/aubergine - NCBI GCA\_000787875.1 (SME\_r2.5.1)  
Solanum tuberosum - Solanum tuberosum - Phytozome V9, Dec 2012  
Solanum tuberosum - potatoes - NCBI GCF\_000226075.1 (SolTub\_3.0)

**SUBMIT**

Version 4.98 - [Documentation](#) - [Contact us](#) - [Downloads/local installation](#) - [Citation](#) - [License](#)

# Construindo um Cassette CRISPR

- Escolher o gene ou região de interesse
  - ABCG1 de *Solanum tuberosum*
  - Procurar sequencias PAM (5'-NGG)
    - CRISPOR (<http://crispor.tefor.net/>)

 CRISPOR (citation) is a program that helps design, evaluate and clone guide sequences for the CRISPR/Cas9 system. [CRISPOR Manual](#)  
June 2020: saCas9 primer fixes, Snapgene/Geneious improvements, better export for Cpf1 [Full list of changes](#)

**Step 1**  
Planning a lentiviral gene knockout screen? Use [CRISPOR Batch](#)  
Sequence name (optional):   
Enter a single genomic sequence, < 2000 bp, typically an exon   
[Clear Box - Reset to default](#)  

```
cttcctttgtccccaaatctggggcgccgcgccccctggcggcctaaggactggcgccggaaagtggcc  
aaggcgggggcgacctcgctcacagcgcccccggatatctcgacgtaccatgATGATGATATCGCCGCG  
CTCGTCGTGACAACGGCTCGGCATGTGCAAGGCCGGCTTCGGGGCGACGATGCCCGGGCGTCTTCCC  
CTCCATCGTGGGGCGCC
```

Text case is preserved, e.g. you can mark ATGs with lowercase.  
Instead of a sequence, you can paste a chromosome range, e.g. chr1:11,130,540-11,130,751

**Step 2**  
Select a genome  
  
Note: pre-calculated exonic guides for this species are on the [UCSC Genome Browser](#).  
We have 660 genomes, but not yours? Search [NCBI assembly](#)

**Escolher o tipo de Cas/PAM**

**Step 3**   
Select a Protospacer Adjacent Motif (PAM)  
  
  
**SUBMIT**

Version 4.98 - [Documentation](#) - [Contact us](#) - [Downloads/local installation](#) - [Citation](#) - [License](#)

# Construindo um Cassete CRISPR

- Escolher o gene ou região de interesse
    - ABCG1 de *Solanum tuberosum*
    - Procurar sequencias PAM (5'-NGG)
      - CRISPOR (<http://crispor.tefor.net/>)

*S. tuberosum* tem uma alta taxa de heterozigozidade

**S. tuberosum tem uma alta taxa de h**

**Query sequence, not found in the selected genome, Solanum tuberosum (GCF\_000226075.1)**

**Warning:** The query sequence was not found in the selected genome. This can be a valid query, e.g. a GFP sequence. If not, you might want to check if you selected the right genome for your query sequence. Use a tool like [BLAT](#) to check if the sequence really has a 100% identical match in the target genome.

When reading the list of guide sequences and off-targets below, bear in mind that in case that the input sequence is really in the genome and just has a few differences, the software will use the first found match as the on-target as it cannot distinguish 0-mismatch off-targets from 0-mismatch on-targets. In this case, the specificity scores of guide sequences are too low. In other words, some guides may be fine, the problem may just be that the on-target is shown as an off-target. Because there is no flanking sequence available, the guides in your sequence that are within 50bp of the ends will have no efficiency scores. The efficiency scores will instead be shown as '--'. Include more flanking sequence > 50bp to obtain these scores.

Your input sequence is 1920 bp long. It contains 204 possible guide sequences. Shown below are their PAM sites and the expected cleavage position located -3bp 5' of the PAM site. Click on a match for the PAM NGG below to show its 20 bp-long guide sequence. (Need help? Look at the [CRISPOR manual](#)) Colors green, yellow and red indicate high, medium and low specificity of the PAM's guide sequence in the genome.

Input sequence not in genome, cannot show genome variants.

Position	0	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
Sequence	ATGTCAAGGATAGTGGCGGAAATATGTTACAAGGGGGAGAAAATGTACAATTATAATCAAAGAGTACAACAAGCCATGGAGATGTCACAAGCCAGCGCGTACTCTTCACCCACCTTAGGCCAAATGCTAAAGCGCTGGAGACGTCAGA															
	---AGG								CCA---		CCA---	CCC---	CCA---	---	TGG	
	---GGG								---TGG			CCA---		---	GGG	
	---GGG										CCC---	CCT---				
	---GGG										---	AGG				

# Construindo um Cassette CRISPR

- Escolher o gene ou região de interesse
  - ABCG1 de *Solanum tuberosum*
  - Procurar sequencias PAM (5'-NGG)
    - CRISPOR (<http://crispor.tefor.net/>)

Show results for [ABCg1](#). Click here to include more matching sequences > 50bp to obtain these scores.

Your input sequence is 1920 bp long. It contains 204 possible guide sequences.  
Shown below are their PAM sites and the expected cleavage position located -3bp 5' of the PAM site.  
Click on a match for the PAM NGG below to show its 20 bp-long guide sequence. (Need help? Look at the [CRISPOR manual](#))  
Colors green, yellow and red indicate high, medium and low specificity of the PAM's guide sequence in the genome.

Input sequence not in genome, cannot show genome variants.

Position	0	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
Sequence	ATGTCAAGGATAGTGGCGGAAATATGTTACAAGGGGAGAAAATGTACAATTATAATCAAAGAGTACAACAAGCCATGGAGATGTCACAAGCCAGCGCGTACTCTTCACCCACCCCTAGGCCAAATGCTAAAGCGCGTGGGAGACGTGAGAAAGGA															
	---AGG								CCA---		CCA---		CCC---	CCA---	---TGG	---AGG
	---GGG								---TGG			CCA---		---GGG		
	---GGG										CCC---		CCT---			
	---GGG										CCT---		---AGG			

Download for: SerialCloner (free) - ApE (free) - GenomeCompiler - Benchling - SnapGene - Geneious - Vector NTI - LaserGene - Genbank - FASTA

Predicted guide sequences for PAMs

# Construindo um Cassette CRISPR

- Escolher o gene ou região de interesse
  - ABCG1 de *Solanum tuberosum*
  - Procurar sequencias PAM (5'-NGG)
    - CRISPOR (<http://crispor.tefor.net/>)

**Predicted guide sequences for PAMs**

Ranked by default from highest to lowest specificity score (Hsu et al., Nat Biot 2013). Click on a column title to rank by a score.  
If you use this website, please cite our paper in NAR 2018. Too much information? Look at the CRISPOR manual.

Download as Excel tables: Guides / Guides, all scores / Off-targets / Saturating mutagenesis assistant

Position/ Strand	Guide Sequence + PAM + Restriction Enzymes	MIT Specificity Score	CFD Spec. score	Predicted Efficiency	Outcome	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score
95 / rev	GGTGGGTGAAGAGTACGCCT TGG Enzymes: <i>HinP1I</i> , <i>BspFNI</i> , <i>BstCBI</i> <b>Cloning / PCR primers</b>	100	100	55	24	58 74 0-0-0-0-3 0-0-0-0-0	4:exon:XM_015307842.1 4:exon:XM_006347900.2 4:exon:XM_006358104.2/XM_006358103.2/XM_006358102.2 3 off-targets
117 / rev	CGCGCTTAGCATTTGGCT AGG ⚠ Inefficient Enzymes: <i>BshFI</i> , <i>MaeI</i> , <i>BstI</i> , <i>BseDI</i> , <i>EriI</i> , <i>XmaII</i> <b>Cloning / PCR primers</b>	100	99	32	28	73 75 0-0-0-0-5 0-0-0-0-0	4:intergenicGap:XM_006363972.2 4:intergenic:XM_015308516.1-XR_001473402.1/XR_001473401.1-XR_001473400.1 4:exon:XM_006355583.2 show all...
140 / fw	AGGCCAAATGCTAACGCGCT TGG Enzymes: <i>HinP1I</i> , <i>BspFNI</i> <b>Cloning / PCR primers</b>	100	100	53	63	60 80 0-0-0-0-4 0-0-0-0-0	4:intergenic:XM_006341275.2-XM_006341276.2 4:intergenic:XM_015314926.1-XM_015314842.1 4:intergenic:XM_015313558.1-XM_015313559.1 show all...
155 / fw	GCGCGTGGGAGACGTGAGAA AGG <b>Cloning / PCR primers</b>	100	100	58	77	63 81 0-0-0-0-3 0-0-0-0-0	4:exon:LOC102596699/XM_006359628.2 4:intron:XM_006364240.2/XM_006364239.2 4:intergenic:XM_015307059.1-XM_006343675.2 3 off-targets
263 / rev	CCGGCGGACTTTACGCTGT AGG ⚠ Not with Hinf I?	100	100	42	56	50 72 0-0-0-0-0	

# Construindo um Cassette CRISPR

- Escolher o gene ou região de interesse
  - ABCG1 de *Solanum tuberosum*
  - Procurar sequencias PAM (5'-NGG)
    - CRISPOR (<http://crispor.tefor.net/>)

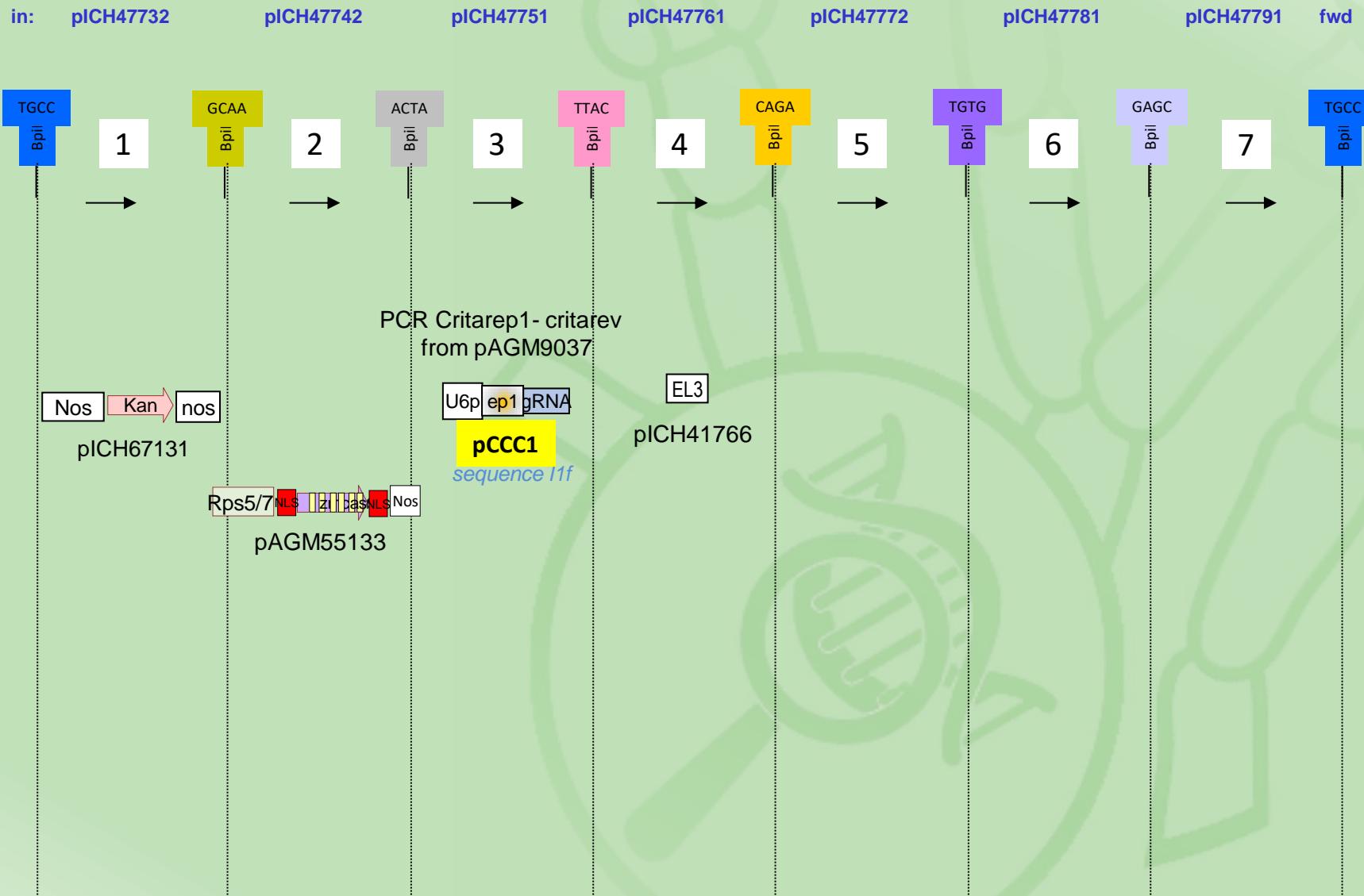
Position/ Strand	Guide Sequence + PAM + Restriction Enzymes	MIT Specificity Score	CFD Spec. score	Predicted Efficiency	Outcome	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score
		Show all scores	Doeinch '16	Mor-Mateos	Out-of-Frame	Lindel	<input type="checkbox"/> exons only No match, no chrom filter
343 / rev	AACAGATTTCTCGGGGAC GGG ⚠ Not with U6/U3 Enzymes: <i>Bsp</i> P1, <i>Hpy</i> 99I, <i>Nde</i> I, <i>Bsi</i> I <b>Cloning / PCR primers</b>	100	100	52	54	47 64	0 - 0 - 0 - 0 - 3 0 - 0 - 0 - 0 - 1  3 off-targets
351 / fw	TCCACCGGTGATCCCGTCGC CGG Enzymes: <i>Msp</i> I, <i>Hpy</i> 99I, <i>Lpn</i> P1, <i>Bsi</i> I <b>Cloning / PCR primers</b>	100	100	56	66	47 68	0 - 0 - 0 - 1 - 2 0 - 0 - 0 - 0 - 0  3 off-targets
408 / rev	CGGCCGACTATCTCGCGTCC CGG Enzymes: <i>Bsh</i> F1, <i>Bme</i> T110I, <i>Mnl</i> I, <i>Msp</i> I, <i>Bse</i> D1, <i>Psp</i> P1, <i>Nci</i> I, <i>Lpn</i> P1, <i>Sty</i> D4I, <i>Cfr</i> 9I <b>Cloning / PCR primers</b>	100	100	47	69	48 76	0 - 0 - 0 - 0 - 0 0 - 0 - 0 - 0 - 0  0 off-targets
434 / fw	CGGGAGAGATACTCGCGTCC TGG Enzymes: <i>Sty</i> D4I, <i>Lpn</i> P1, <i>Bse</i> D1, <i>Bst</i> NN, <i>Hpy</i> CH4V <b>Cloning / PCR primers</b>	100	100	42	64	72 69	0 - 0 - 0 - 0 - 3 0 - 0 - 0 - 0 - 0  3 off-targets
435 / fw	GGCGAGAGATACTCGCGTCT CGG ⚠ Inefficient Enzymes: <i>Sty</i> D4I, <i>Lpn</i> P1, <i>Bse</i> D1, <i>Bst</i> NN, <i>Hpy</i> CH4V <b>Cloning / PCR primers</b>	100	99	50	57	71 80	0 - 0 - 0 - 0 - 4 0 - 0 - 0 - 0 - 0  4 off-targets
444 / fw	GTCGCCGTCTGGGTGCATC AGG Enzymes: <i>Lwa</i> I, <i>Hpy</i> CH4V <b>Cloning / PCR primers</b>	100	100	36	31	62 83	0 - 0 - 0 - 1 - 7 0 - 0 - 0 - 0 - 0  8 off-targets
445 / fw	TEGGGGTCTTGGGTGCACTA GGG	100	100	50	54	59 84	0 - 0 - 0 - 1 - 4 0 - 0 - 0 - 0 - 0  4 off-targets
446 / fw	TEGGGGTCTTGGGTGCACTA GGG	100	100	50	54	59 84	0 - 0 - 0 - 1 - 4 0 - 0 - 0 - 0 - 0  4 off-targets

# Construindo um Cassette CRISPR

**ATG**TCAAGGATAGTGGCGGAAAATATGTTACAAGGGGGAGAAAATGTACAATTATAATCAAAGAGTACAACAAGCCATGGAGATGTCACAAGCCAGCGCGT  
ACTCTTCACCCACCCTAGGCCAAATGCTAAAGCGCGTGGGAGACGTGAGAAAGGAAGCCACC GGCGACGAAACTCCGGTGCACCGGATTCTCGATATGAGTGA  
TACTCAAAGCATATCATCTACTCTCTTGTACTCTCCTTCAACAAACCTCACCTACAGCGTAAAAGTCCGCCGGAAAATGCCTTCCAGCGATACTC  
CGGCGACCGGCCGGAGTTCCACCGGTGATCCCGTCGCCGGAGAAAATCTGTTACGAACACAAAATTCCCTGAACAATATCTCCGGCGAGGCCCGGG  
ACGGCGAGATAGTCGCCGTCC**TGG**TGCATCAGGGTCGGGGAAATCGACCGTGATCGATGCCCTCGGAATAGGATCGCGAAGGAGAGTTAAAAGGAACGAT  
AACGTTAACGGAGAGCCACTGATTGAGATTGTTGAAAGTAATCTCAGCATATGTAATGCAAGATGATCTTATATCCAATGTTGACAGTTGAAGAAACA  
TTAATGTTGCAGCTGAATT CAGATTGCCACGTACTTCATCAAAATCAAAAAAGAAAATGAGAGTTCAAGCTTGATTGATCAATTAGGACTACGAAATGCTG  
CAAAAACAATCATTGGT GATGAGGGT CATCGTGGAGTGTCTGGTGGAAAGACGACGAGTTCGATTGGAATTGATATTATTCA T GACCCTATCATATTGTT  
TTTAGACGAGCCA ACTTCAGGTCTTGACTCGACTAGTGCATATATGGTGGTGAAGGTTCTACAACGAATTGCTCAAAGT GGAAGTATTGTTATCATGTCAATT  
CATCAGCCAAGTTATCGAATTCTCGGTTATTGGATCGGATGCTCTTGTCCC GTGGTCAAACGGTTATAGTGGTCACCTATGAACCTCCCACATTTT  
TTGCTGATTTGGTCA CCCAATACCGGATAGT GAAAATAGAACAGAGTTGCTCTGGATCTAATT CGCAACTAGAAGGGTCCCCTGGAGGGACAAAAGTTT  
GGTTGAGTTCAACAAAACATGGGAAAATACTAAAAGGAGTAATGAAAATCCTGAAATCCAAACACCTACTCATGGATTGTCATTGAAAGAAGCAATTAGCGCG  
AGTATTCAAGAGGGAGTTGGTT CAGGGACAACGAGT GATATTCAACTAGTCCAGC ATCAATGGTCCA ACTTACGCGAATCCATTGGATTGAAATGC  
TTGTGTTGTCCAAGAGAGATCAT TACGAATTCTGGAGGGT GCCAGAGTTATTGGTATT CGTCTAGGGCAATCGTGGTCACGGGTTCATCCTAGCTACCAT  
GTTTGGCAACTTGATGATTCCCCTAAAGGGTTCAAGAAAGGCTTGGTTCTTGCTATTGCTATGTCAACAACATTCTATACTTGC GCGGACGCGTTGCCT  
GTGTTCTCCAAGAGAGGTACATTTCATGAGGGAGACTGCTTATAATGCTTATAGGAGATCTCCTATTGCTATCTCATGCTATAGTTCTTGCAGCAT  
TGATCTTCTTAGCTTGCATTGCTGCTATAACTTTGGCTGTAGGCCTTGTAGGTGGATTTCGGCTTTGTTCTATT CGCATAATACTAGCCTC  
CTTCTGGGCCGGAATT CATTGTCACGTTCTCCGGTGTAGTCTAGTGTCTAGGTTACACC ATAGTGGTCGCGAT CCTAGCCTATTCCCTCCTC  
TTCTCAGGATTCTTCAATCGCGATAGGATTCCACCTTATTGGATATGGTTCACTACCTGTCTGGT GAAATATCCTTATGAAGCTGT TACAAAATG  
AATTTGATGATGCAACAAAGT TGTCAAAGGGATTCAATTGTTGATAATT CACCACTGGAAATGTGCCTAATGCATTGAAGGAAAATTGTTGAGTAC  
AATGAGTAACACATTAAATGTCAAACATGTGTGACTACTGGGCTGATATATTGGTCAACAAGGGATTACTGATTAAAGTAAGTGAAG  
TGTGGATTACTATTGCATGGGGTTTCTTAGGGTTTACTTAGCTTGTGCTTGGAAAGTAAGAACAGAGAAGG**TGA**

# Overview of Level 1 (carb), *Clone Bsal*

Crispr Potato

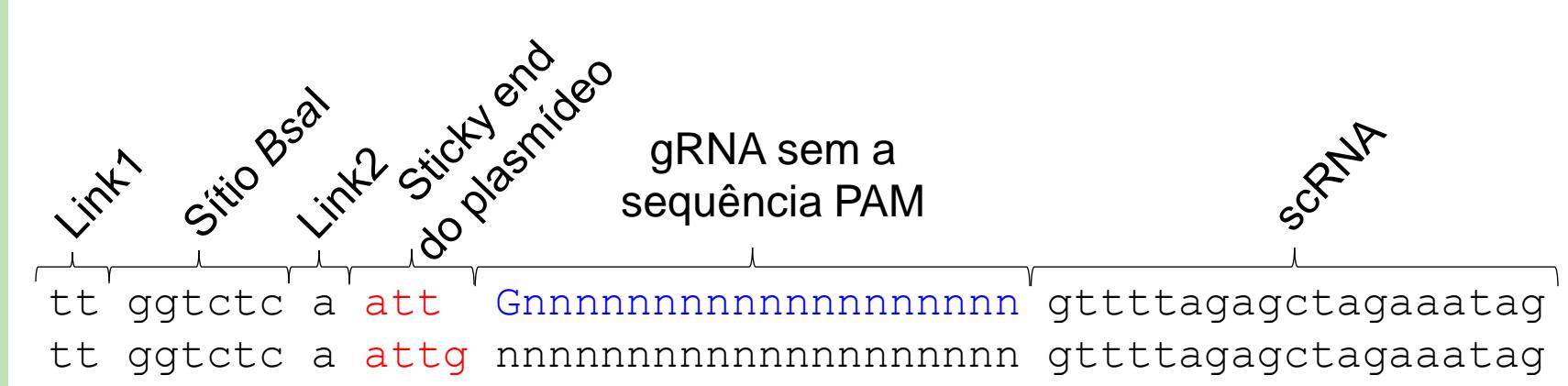


Primer l1f – GTGGTGTAAACAAATTGACGC

# Construindo um Cassette CRISPR

**gRNA**  
CGGCGAGATAGTCGCCGTCC

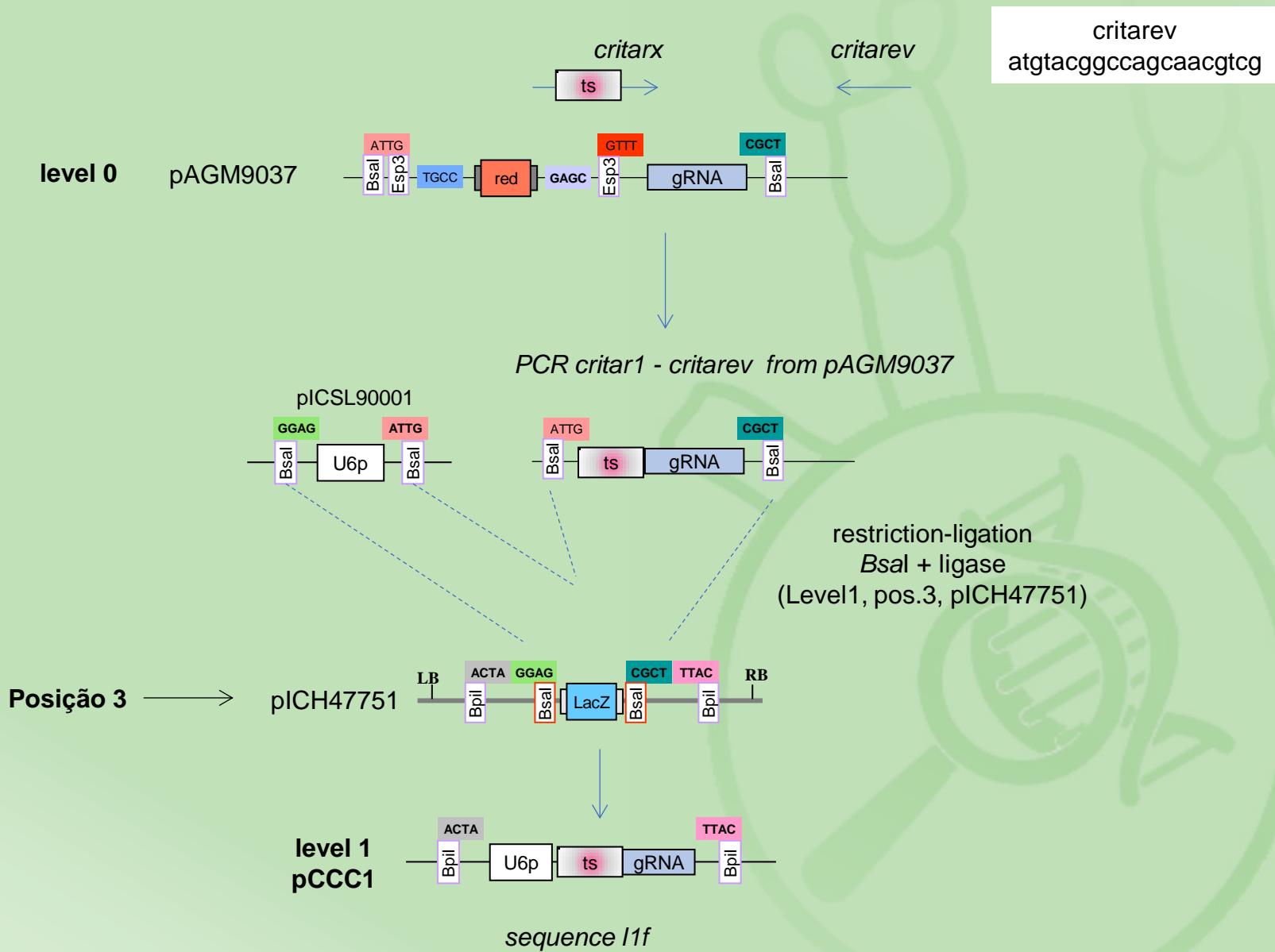
**PAM**  
TGG



**Bsal**  
5' ... GGTCTCN... 3'  
3' ... CCAGAGNNNN... 5'

tt ggtctc a **attg** CGGCGAGATAGTCGCCGTCC gtttagagctagaaatag      PrimerF: *citar1*

## Cloning of guide RNA, level 1

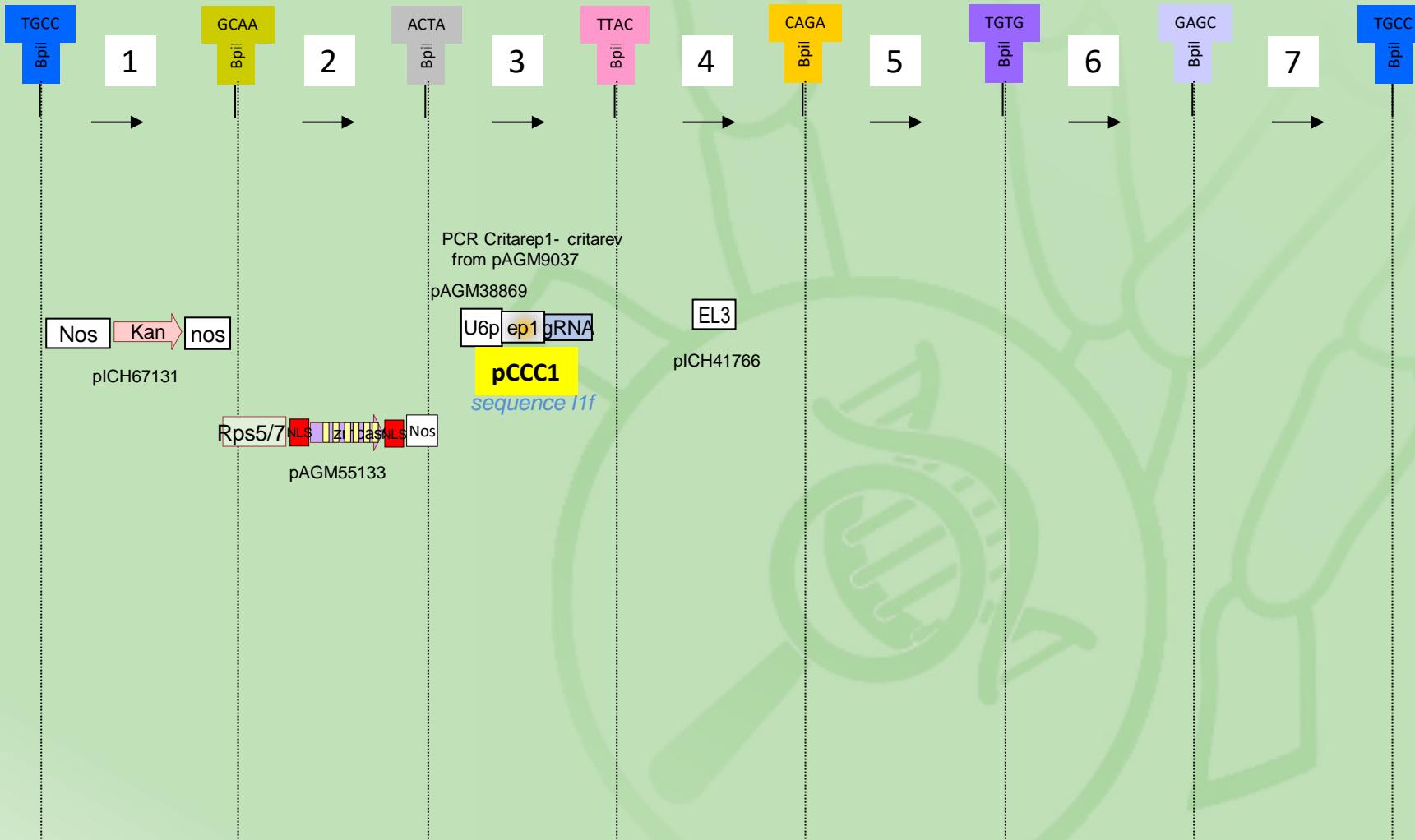


Vídeo  
pCCC1

# Overview of Level 1 (carb), Clone Bsal

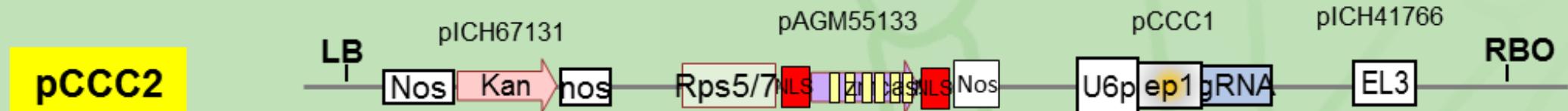
Crispr Potato

in:	pICH47732	pICH47742	pICH47751	pICH47761	pICH47772	pICH47781	pICH47791	fwd
in:	pICH47802	pICH47811	pICH47822	pICH47831	pICH47841	pICH47852	pICH47861	rev



Primer l1f – GTGGTGTAAACAAATTGACGC

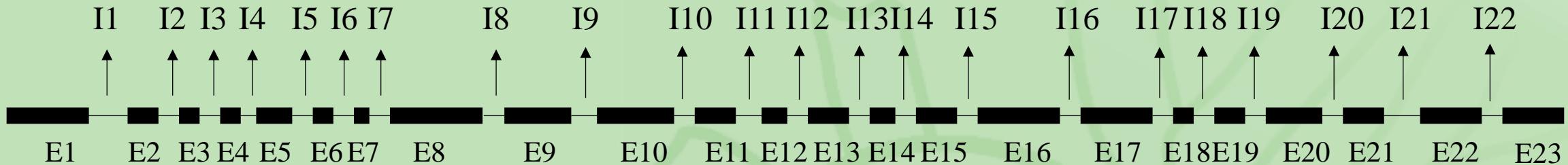
# Overview of Level 2 (kan), [Clone Bpil](#) in pAGM35831



Vídeo  
pCCC2

# Exercício de Clonagem

PDR1 (6412 pb) (*Solanum stoloniferum*)

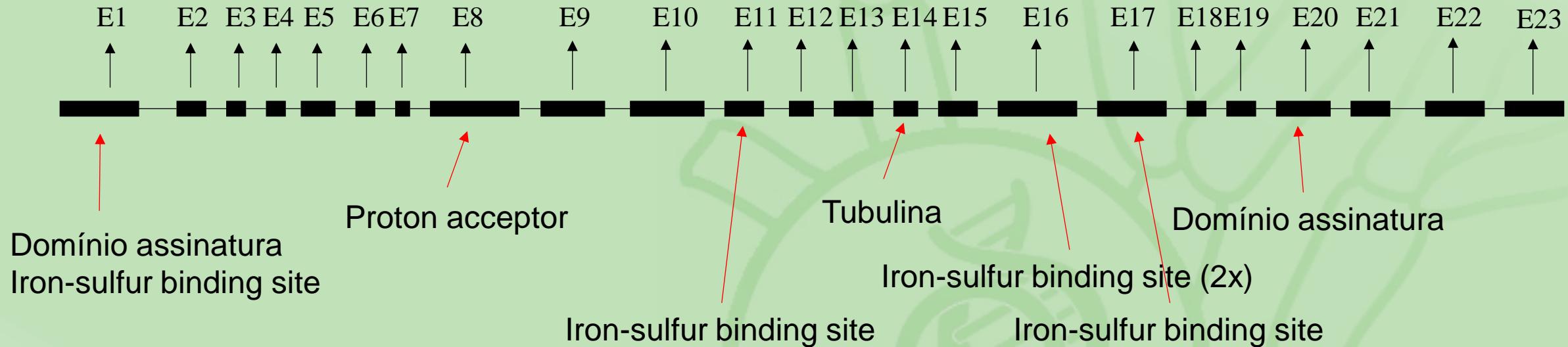


Traduzir o cDNA (<https://web.expasy.org/translate/>)

Verificar domínios (com o cDNA e com a sequência de aminoácidos) (<https://prosite.expasy.org/>)

# Exercício de Clonagem

## PDR1 (6412 pb) (*Solanum stoloniferum*)



- Traduzir o cDNA (<https://web.expasy.org/translate/>)
- Verificar por pepitídeo sinal (com a sequência de aminoácidos) (<http://www.cbs.dtu.dk/services/SignalP/>)

- CRISPOR (<http://crispor.tefor.net>)
  - Adicionar o Exon importante

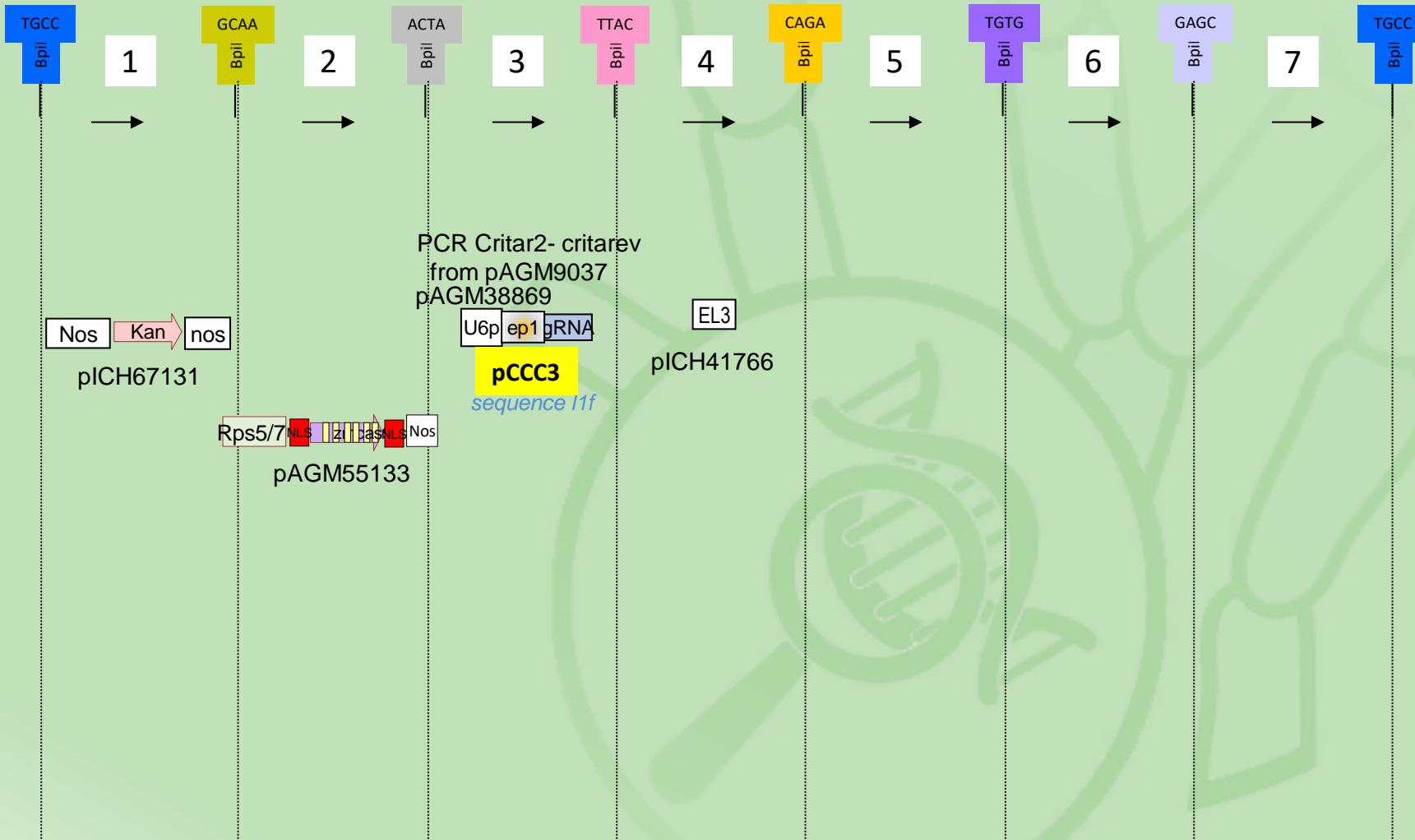
Download as Excel tables: [Guides](#) / [Guides, all scores](#) / [Off-targets](#) / [Saturating mutagenesis assistant](#)

Position/ Strand	Guide Sequence + PAM + Restriction Enzymes <input type="checkbox"/> Only G- <input type="checkbox"/> Only GG- <input type="checkbox"/> Only A-	MIT Specificity Score <input type="checkbox"/>	CFD Spec. score 	Predicted Efficiency		Outcome Doench '16 Mor.-Mateos	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score <input type="checkbox"/> exons only No match, no chrom filter
				Doench '16	Mor.-Mateos			
157 / rev	CTCCTTATACGACGATAAGT TGG <b>Cloning / PCR primers</b>	99	99	39	28	56	73	0 - 0 - 1 - 0 - 7 0 - 0 - 0 - 0 - 0 8 off-targets  4:intergenic:XM_015303173.1-XM_015303174.1 4:intergenic:XM_006352229.1-XM_015310817.1 4:intergenic:XM_015307004.1-XR_001473023.1 2:exon:XM_006366016.2 4:intergenic:XM_015313152.1-XR_001474658.1/XR_001474657.1/XR_001474656.1 /XR_001474655.1/XR_001474654.1/XR_001474653.1/XR_001474652.1/XR_001474651.1 /XR_001474650.1/XR_001474649.1 4:intergenic:XM_006352417.1-XM_015310883.1 4:exon:XM_006355965.2 4:exon:XM_015314024.1 show less...
136 / fw	ATGATGAAGAGGCCTTAAGA TGG Enzymes: <i>Tru1I</i> , <i>ApeKI</i> , <i>Fsp4HI</i> <b>Cloning / PCR primers</b>	96	96	43	30	47	85	0 - 0 - 1 - 3 - 39 0 - 0 - 0 - 0 - 0 43 off-targets  3:exon:XM_006360285.2 3:exon:XM_006360286.2/NM_001287891.1 4:intergenic:XM_015313145.1-XM_015313146.1 show all...

# Overview of Level 1 (carb), Clone Bsal

Crispr Potato

in:	pICH47732	pICH47742	pICH47751	pICH47761	pICH47772	pICH47781	pICH47791	fwd
in:	pICH47802	pICH47811	pICH47822	pICH47831	pICH47841	pICH47852	pICH47861	rev

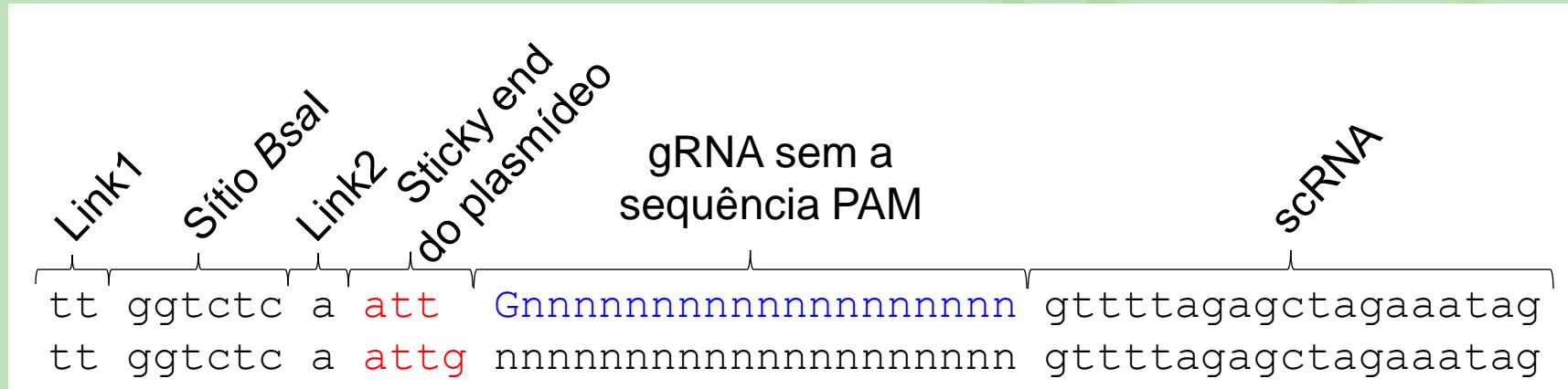


Primer 11f – GTGGTGTAAACAAATTGACGC

# Construindo um Cassette CRISPR

**gRNA**  
CGGCGAGATAGTCGCCGTCC

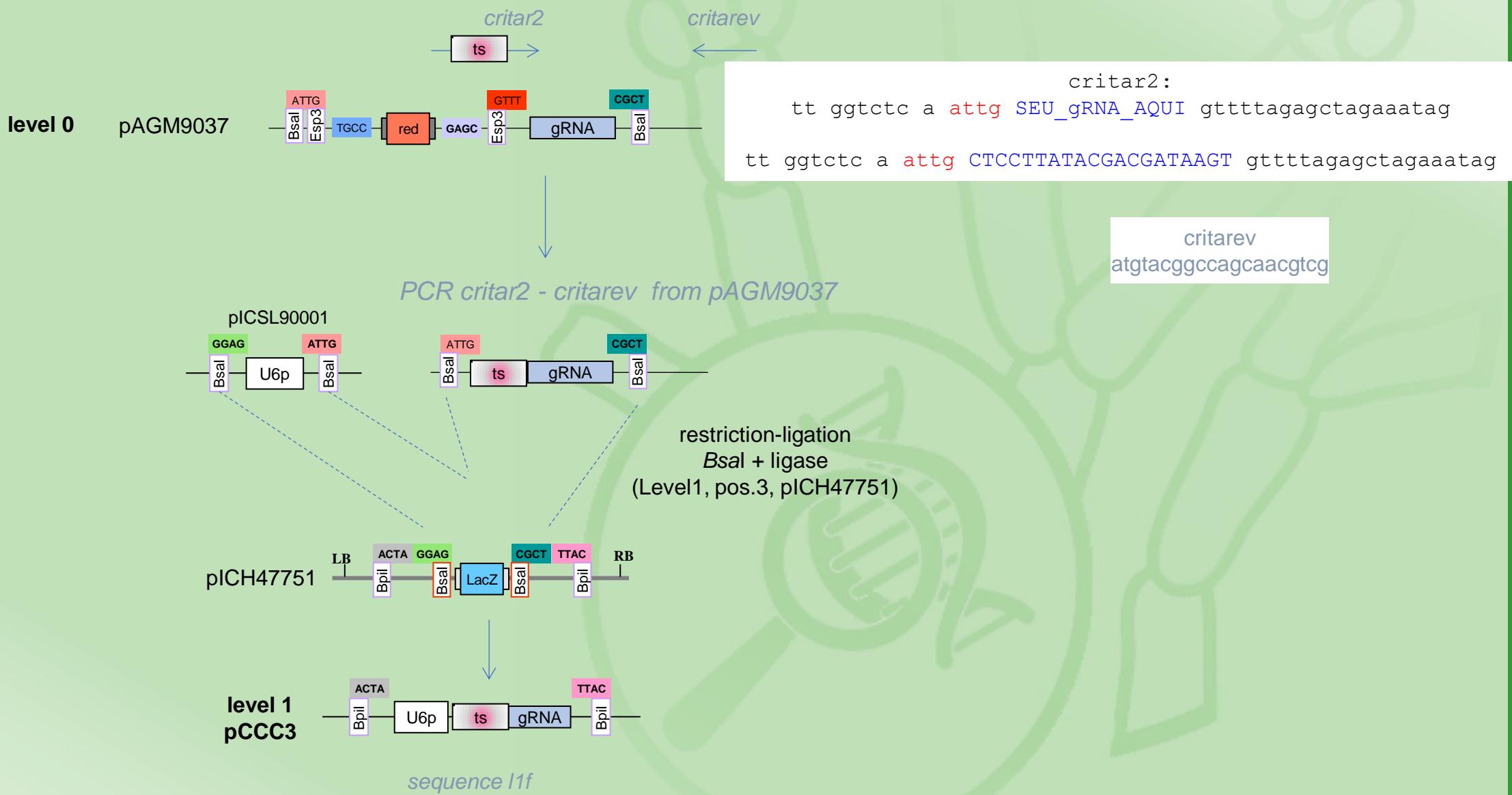
**PAM**  
TGG



tt ggtctc a **attg** CTCCTTATACGACGATAAGT gtttttagagctagaaatag

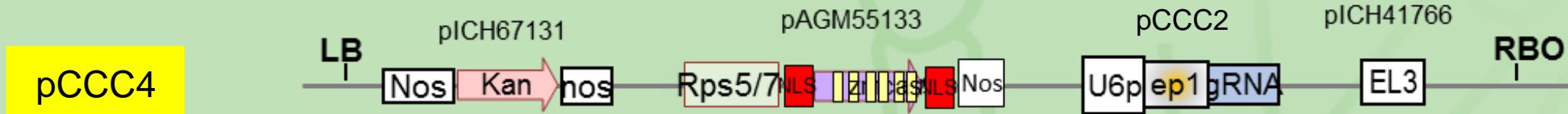
*citar2*

## Cloning of guide RNA, level 1



Primer l1f – GTGGTGTAACAAATTGACGC

# Overview of Level 2 (kan), [Clone Bp1](#) in pAGM35831



# Protocolo de transformação em Agro

- Add 1-5 µL (500-250 ng/µL) of the plasmid in 200 µL competent *Agrobacterium tumefaciens* cells
- Incubate in ice for 5 min
- Incubate in liquid nitrogen for 5 min
- Incubate at 37 °C for 5 min
- Add 1 mL of YEB medium and incubate at 28 °C (200 rpm) for 1-3 hours
- Distribute about 100 µL in each Petri dish with antibiotic (Rif/kan)
- Incubate it at 28 °C for 2 days

# Transformação Mediada por *Agrobacterium tumefaciens*



2 dias

3-6 meses

# Protocolo de transformação em *S. tuberosum*

## Potato leaf disk transformation

### Material:

4 weeks old potato plants *desiree* (sterile tissue culture), cultivated on 2MS media

20ml of agrobacteria suspension cultivated 2 days at 28°C

-Transfer the agrobacteria suspension into 50ml Falcons (don't forget to make glycerol stocks)

-centrifuge bacteria 10min 5000 rpm

-resuspend the pellet in 20ml sterile 10mM MgSO<sub>4</sub> (filtrated or autoclaved)

-centrifuge 10min 5000rpm

-resuspend pellet in 10ml media 3MS

-potato laminar box: prepare sterile petri dishes with 10ml 3MS media

-cut leaves of potato plants: ca 15 leaves per petri dish

-cut the leaves with a sharp blade on a wet sterile filter paper

-put the leaves (surface down) into the petri dishes

-to every petri dish add 100µl bacteria suspension, don't wrap dishes with parafilm!

-wrap with alufoil and incubate in Phytochamber2 for 2days

-after 2 days: dry leaves on sterile filter paper

-put leaves on 76 MS Media (Auxin based)

-every 2 weeks change to a new 75k medium until get new plants



Feltkamp, D., Baumann, E., Schmalenbach, W., Masterson, R. & Rosahl, S. Expression of the mannopine synthase promoter in roots is dependent on the mas elements and correlates with high transcript levels of mas-binding factor. *Plant Sci* **109**, 57–65 (1995).

Mediums:

- **2MS (Potato cultivation):**

20g saccharose

4.41g MS-Salt

Add H<sub>2</sub>O to 800 mL

Ajust pH to 5.8 with KOH(10N)

6g agar

Add H<sub>2</sub>O to 1L

- **GMS (Transformation medium):**

Same as 2MS, but instead of 20 g saccharose, use 16g of Glucose

- **3MS (Transformation)**

Same as 2MS, but instead of 20 g of saccharose, use 30 g, no agar.

- **76MS (Transformation)**

1 L of GMS

1 mL of Cefataxime/carbenicillin (against the agrobacterium – 250 mg/mL)

100 µL of Cytokinin (BAP – 1 mg/mL)

5 mL Auxin (NAA - 1 mg/mL)

500 µL Kanamycin (selection for plants - 100 mg/mL)

- **75K (selection of plants)**

1 L of GMS

1 mL 1 mL of Cefataxime/carbenicillin (against the agrobacterium – 250 mg/mL)

2 mL ZR (Zearinriboside – 1 mg/mL)

20 µL Auxin (NAA - 1 mg/mL)

20 µL GA3 (giberellic acid – 1 mg/mL)

500 µL Kanamycin (selection for plants - 100 mg/mL)

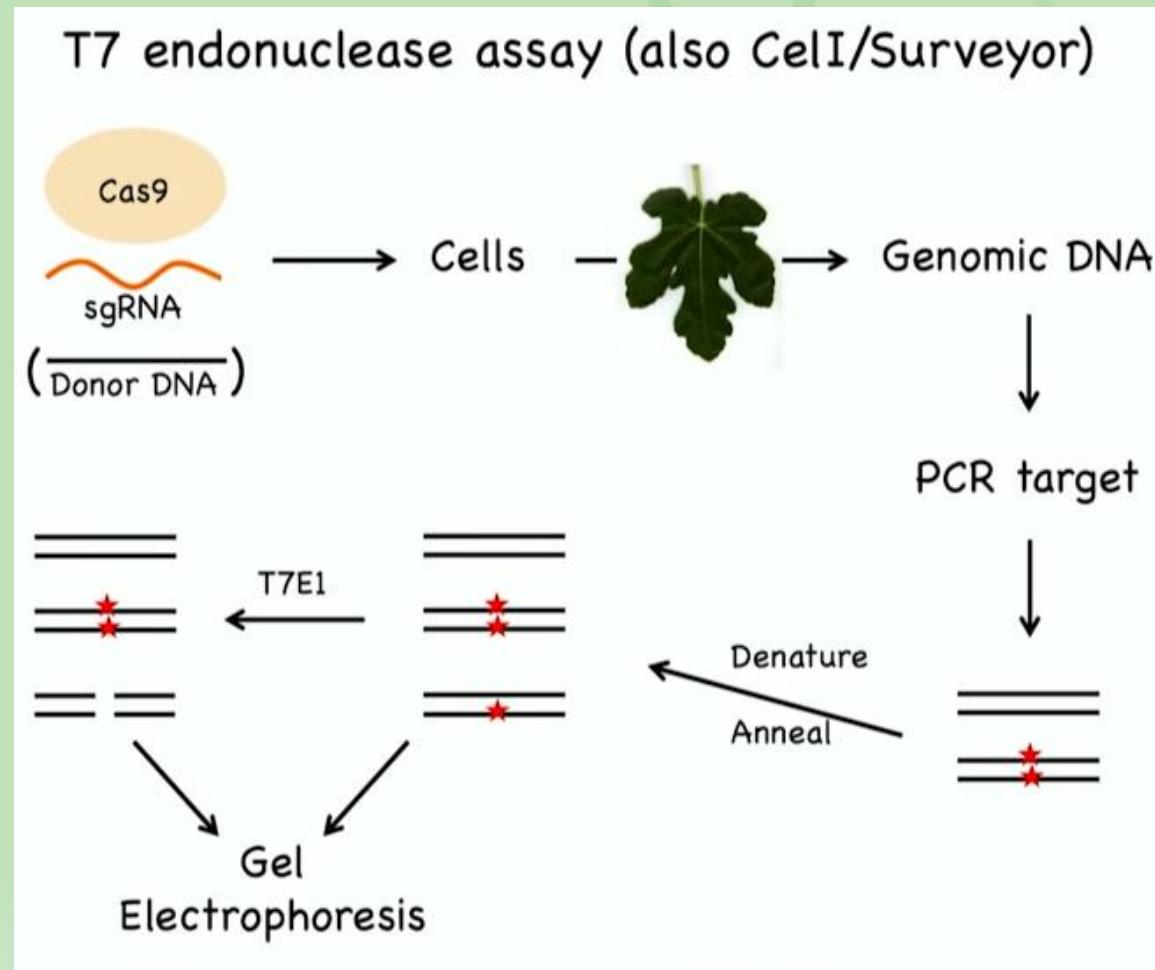
# Seleção dos organismos editados

- Gene de resistência
- Fenótipo
- PCR
- SouthernBlot
- WesternBlot
- NorthernBlot
- RT-qPCR



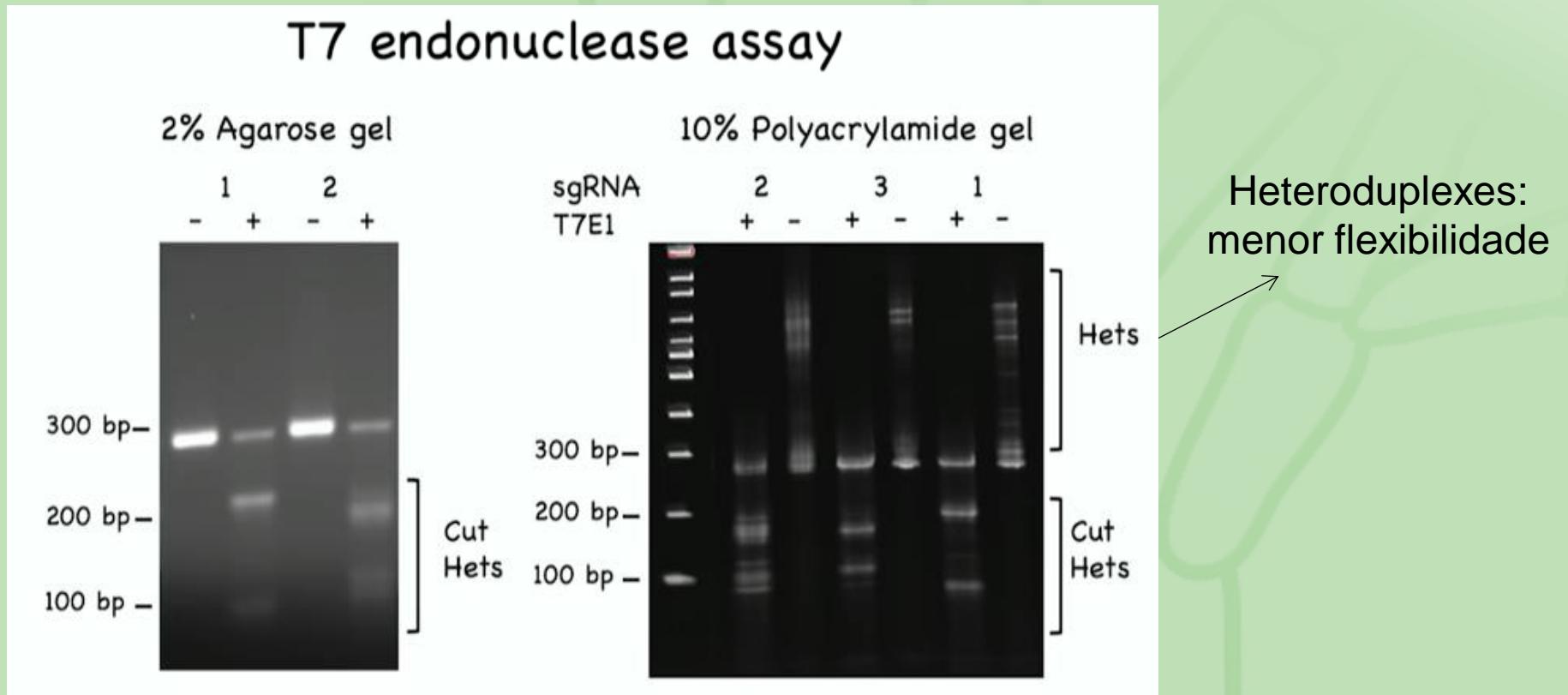
# Seleção dos organismos editados

- Teste com T7-endonuclease



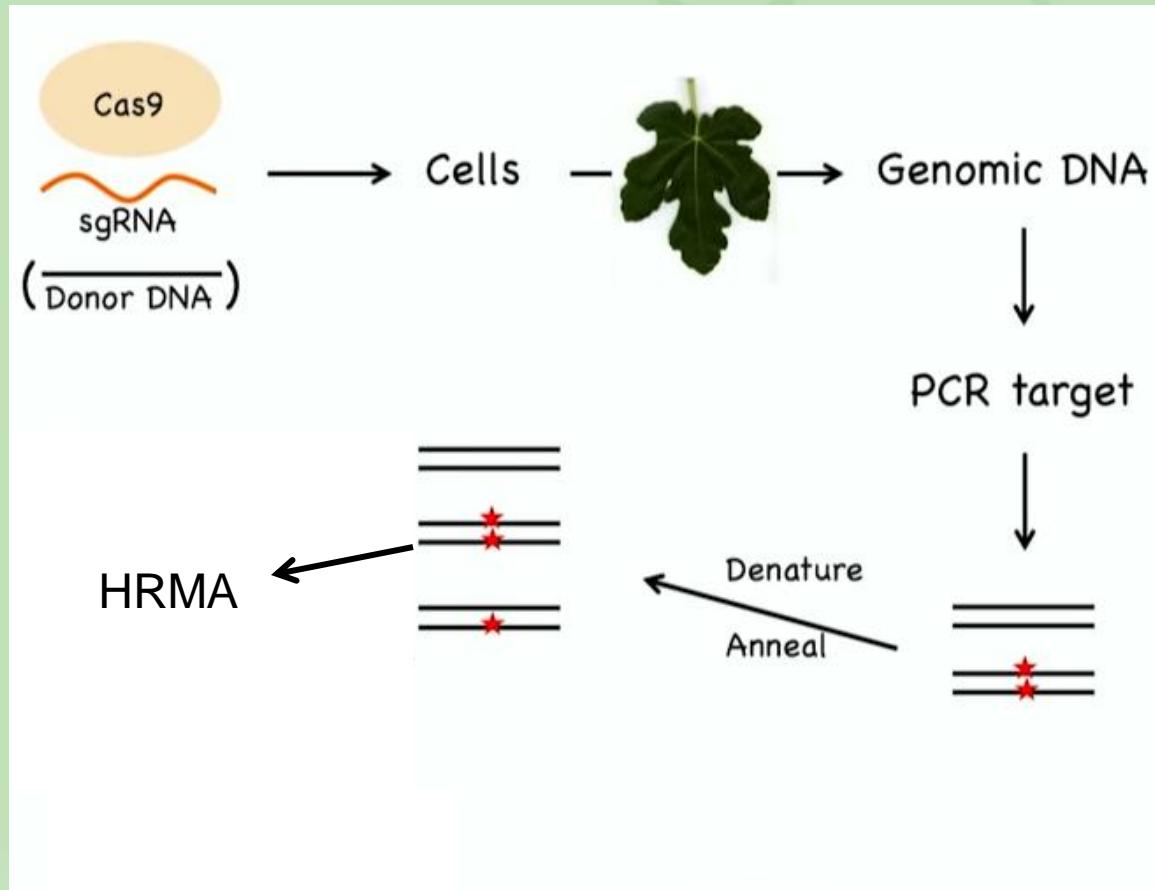
# Seleção de Plantas editadas

- Teste com T7-endonuclease



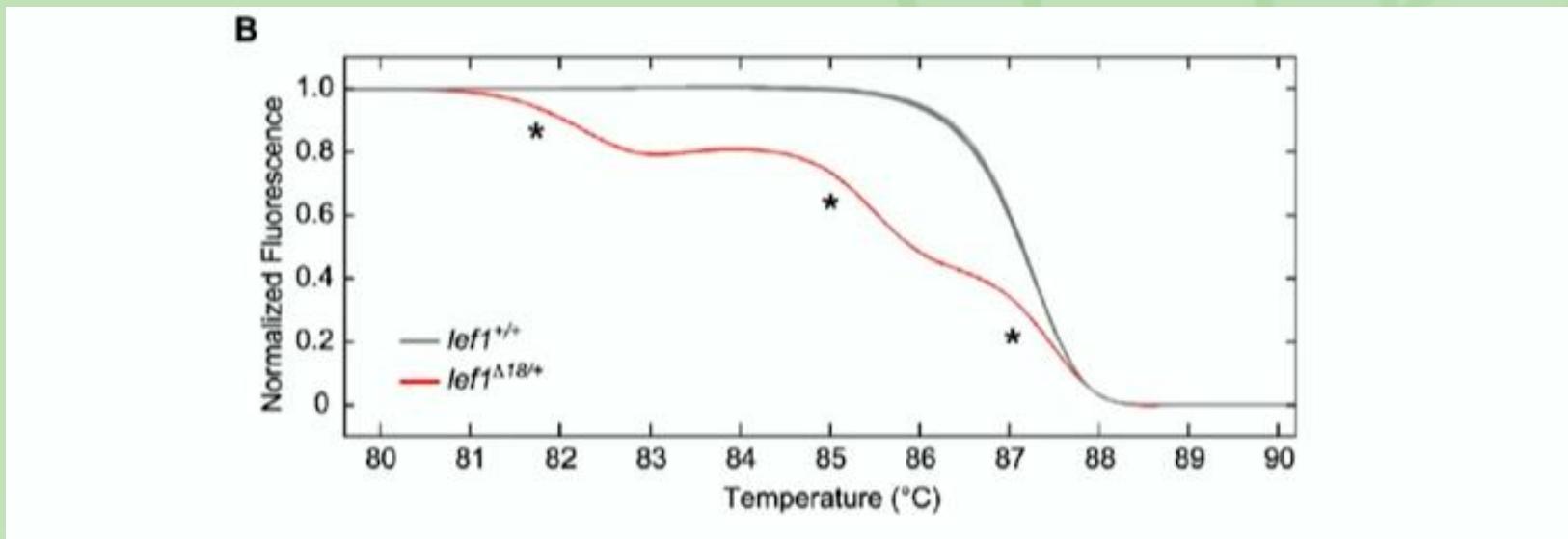
# Seleção de Plantas editadas

- HRMA (High-resolution melting analysis)



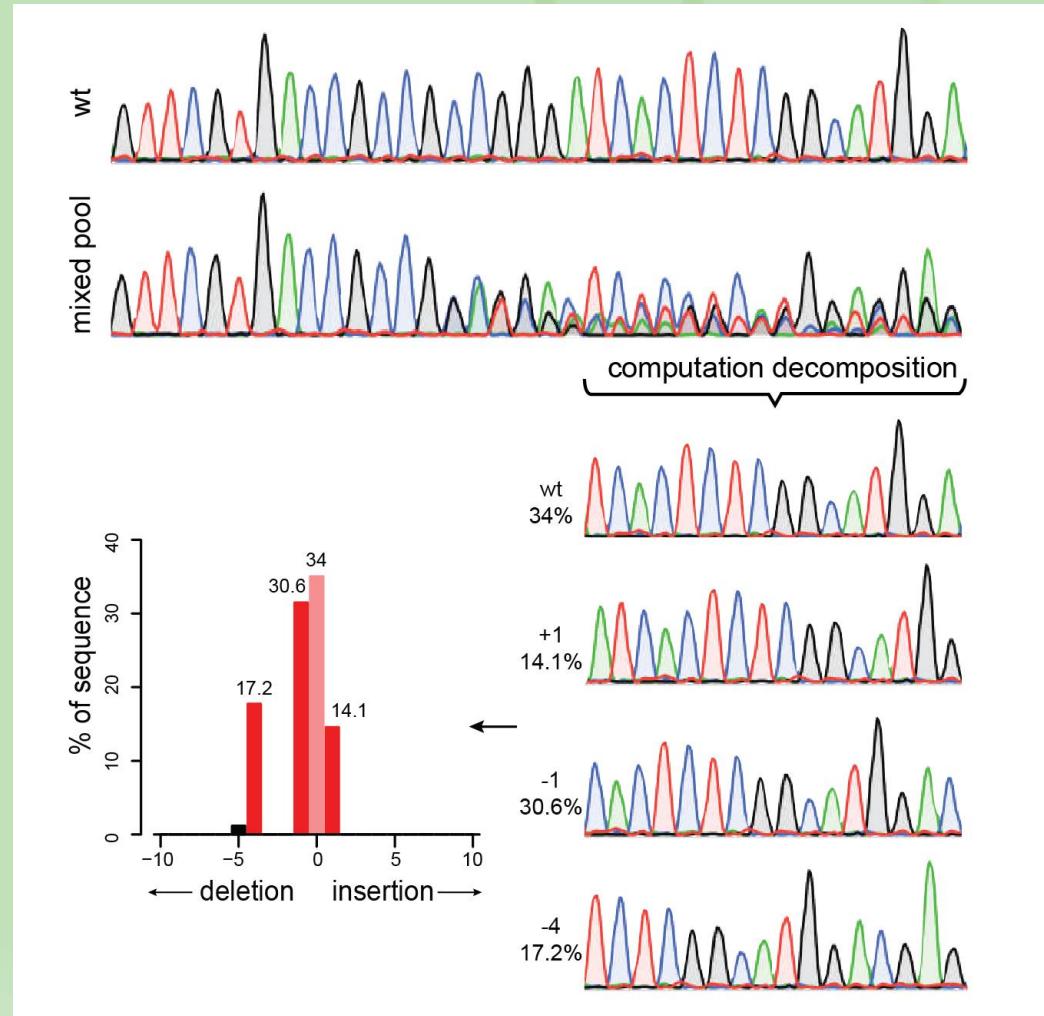
# Seleção de Plantas editadas

- HRMA (High-resolution melting analysis)



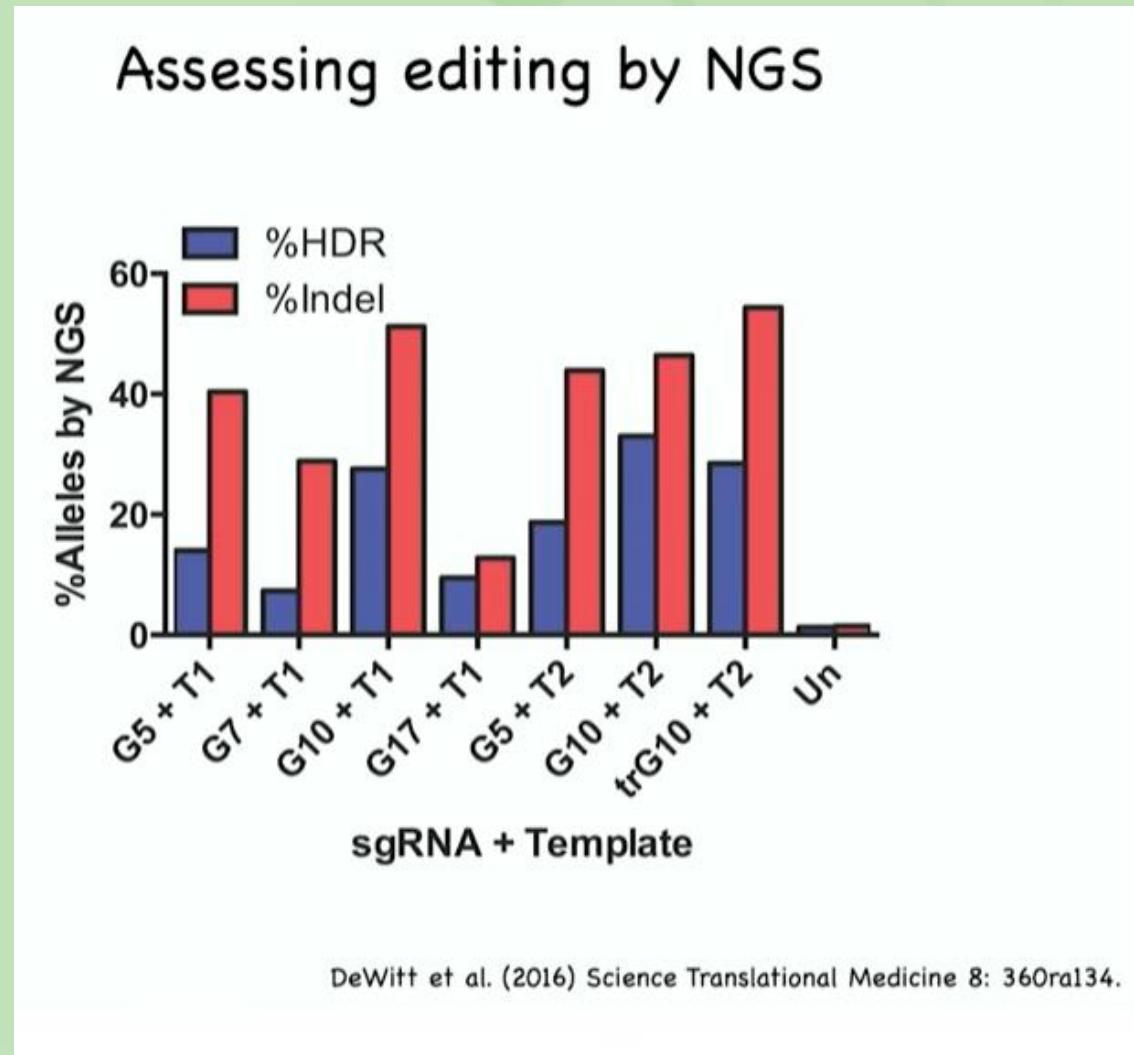
# Seleção de Plantas editadas

- TIDE (<https://tide.nki.nl/>)



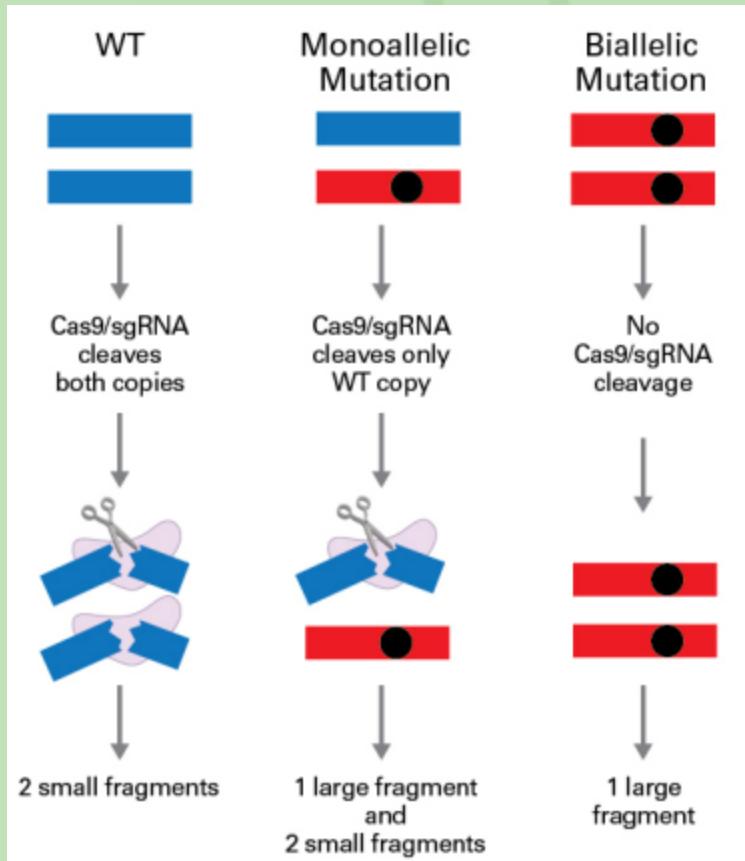
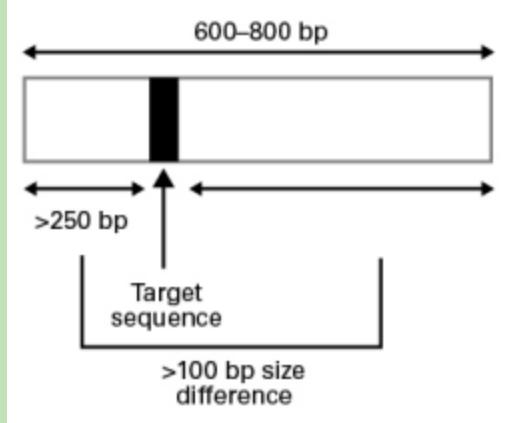
# Seleção de Plantas editadas

- NGS



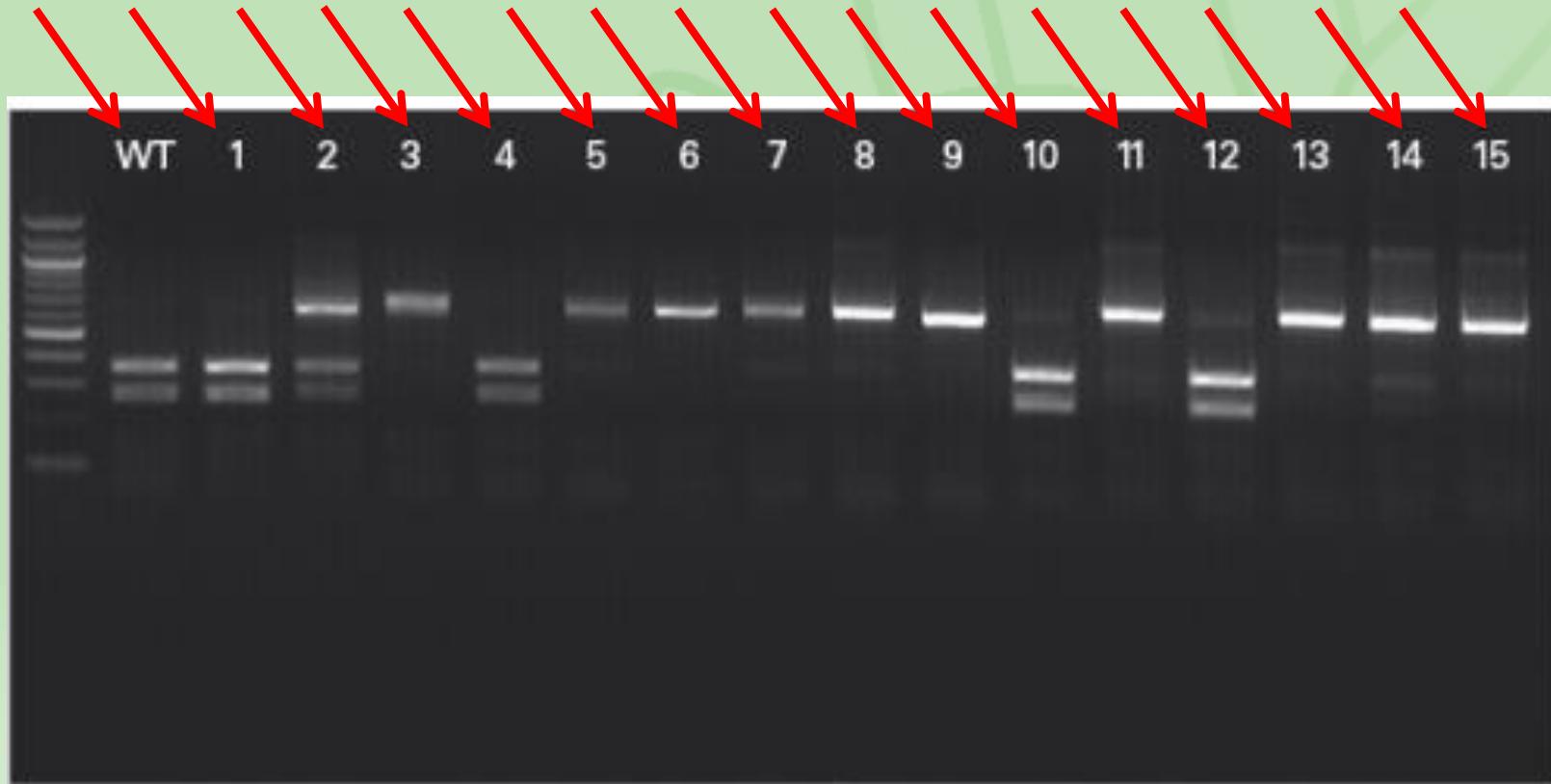
# Seleção de Plantas editadas

- Kits
  - TAKARA Bio (Guide-it Genotype Confirmation Kit)



# Seleção de Plantas editadas

- Kits
  - TAKARA Bio (Guide-it Genotype Confirmation Kit)



# Identification of edited plants

Fully edited

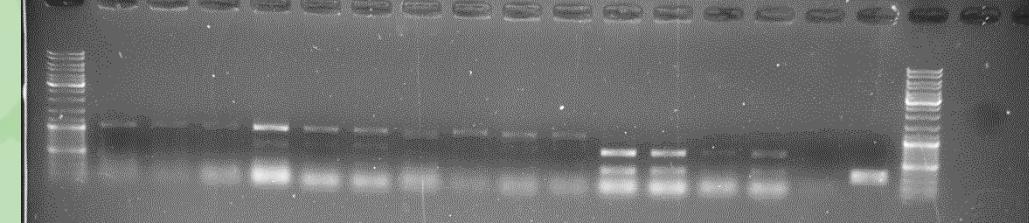
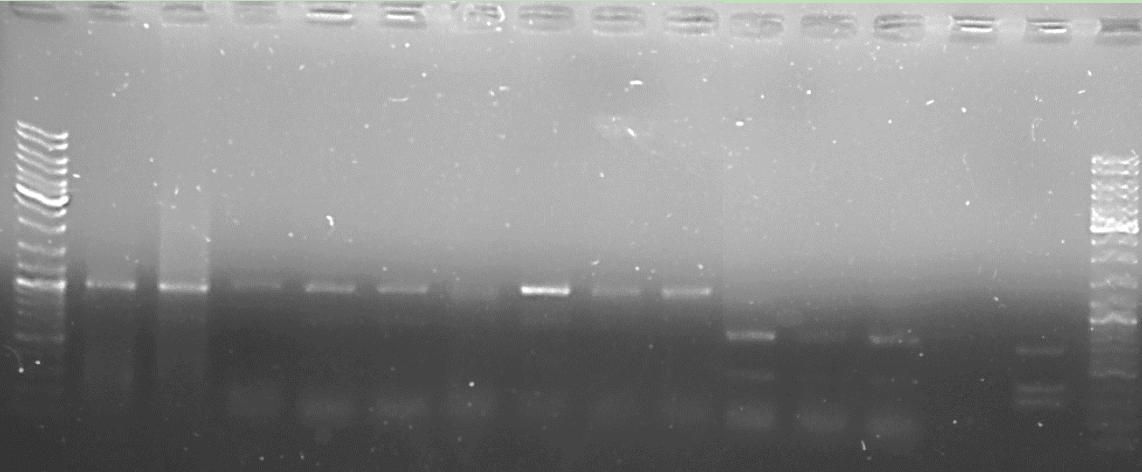
Partially edited

WT allele

951 bp  
585 bp  
366 bp

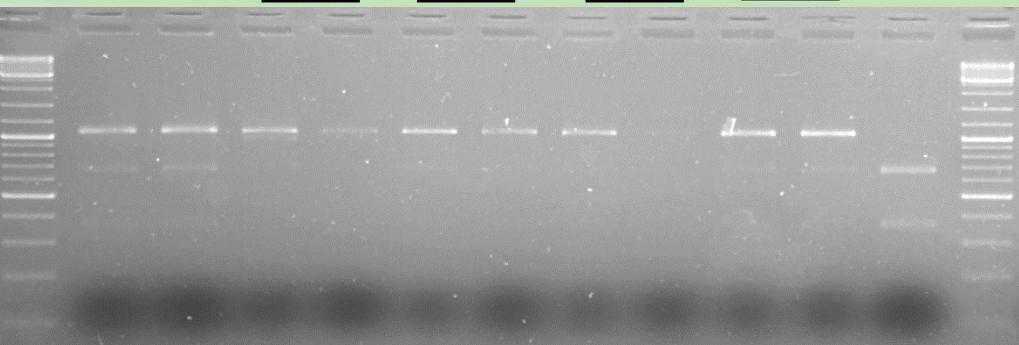
142 142 132 155 155 143 143 149 149 EV UE UE BI C

142 132 132 155 143 149 EV UE B C



DNA from tubers

132 142 143 149 155 UE



Primer 39: GTACAACAAGCCATGGAGATGTCAC  
Primer 40: GTTCATAGGTGACCCACTATAAAC

# *StABCG1* sequencing

Loss of function - CrisprCas-*StABCG1*

Sequence/allele	#132	#142	#143	#149	#155
GGCGAGATAGTCGCCGTCT (WT)					3
GGCGAGATAGTCGCC-TCCT	3	3	6	1	1
GGCGAGATAGTCGCCG-CCT			1	1	6
GGCGAGATAGTCGC--TCCT		1			
GGCGAGATAGTCGCC--CCT	1	2	3	4	
GGCGAGATAGTCGC---CCT		3		2	
GGCGAGATAGTCGCCG---T				2	
GGCGAGATAGTCGCCG----	5				
-----		1			
GGCGAGATAGTCGCCGTCT	1				
<b>TOTAL</b>	<b>10</b>	<b>10</b>	<b>10</b>	<b>10</b>	<b>10</b>

# Seleção de Plantas editadas

Método	Preço total	Preço/rxn
T7-endonuclease (25 rxns)	£ 197,00	£ 7,88 = R\$ 59,59
HRMA (100 rxns)	€ 1554,00	R\$ 102,38
TIDE (1 rxn)	US\$ 7,05	US\$ 7,05 = R\$ 38,48
NGS (1 rxn)	€ 78,83 a > € 641,50	R\$ 519,87 a > R\$4230,56
Guide-it Confirmation Kit (100 rxns) + extras (sgRNA Kit, Clean-up Kit 50 rxns)	€ 524,00 + € 1219,00	~ € 29,62 = R\$ 195,14

# Troubleshooting

1. Introns e exons do gene alvo
2. Proteína com isoformas
3. Off-targets
4. Transformação e seleção



MARTIN-LUTHER-UNIVERSITÄT  
HALLE-WITTENBERG



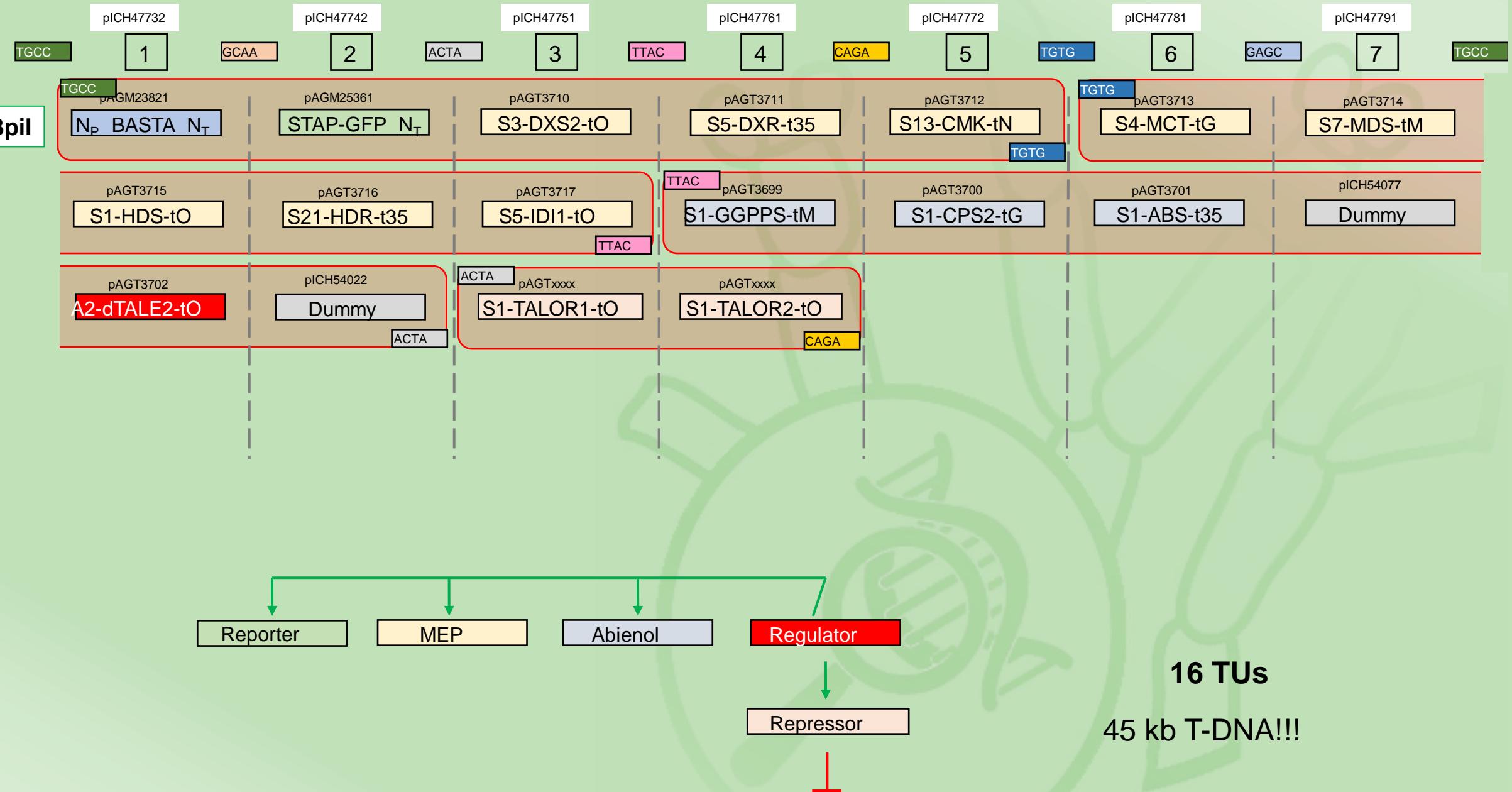
**Hochschule Anhalt**  
Anhalt University of Applied Sciences



Obrigado pela atenção

Contato: eperino@ipb-halle.de

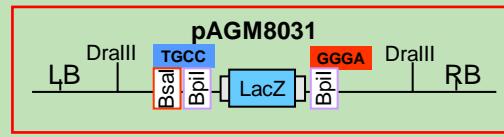




## Level 2 (Level M) cloning vectors

Starting position

1



2



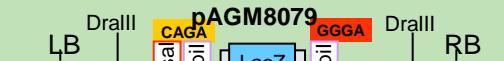
3



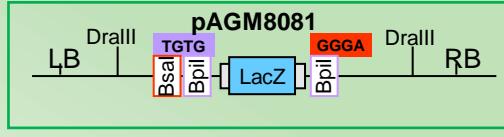
4



5



6



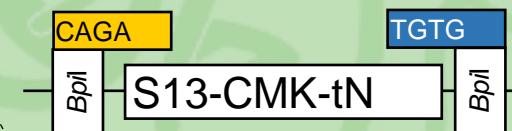
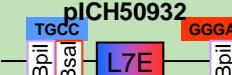
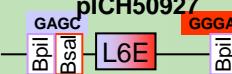
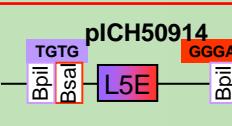
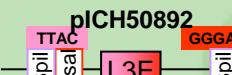
7



**Level M**

vectors  
**Spec<sup>R</sup>**

end-linkers  
**Amp<sup>R</sup>**



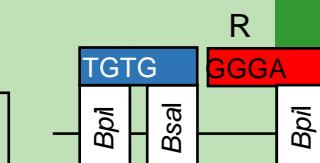
1

2

3

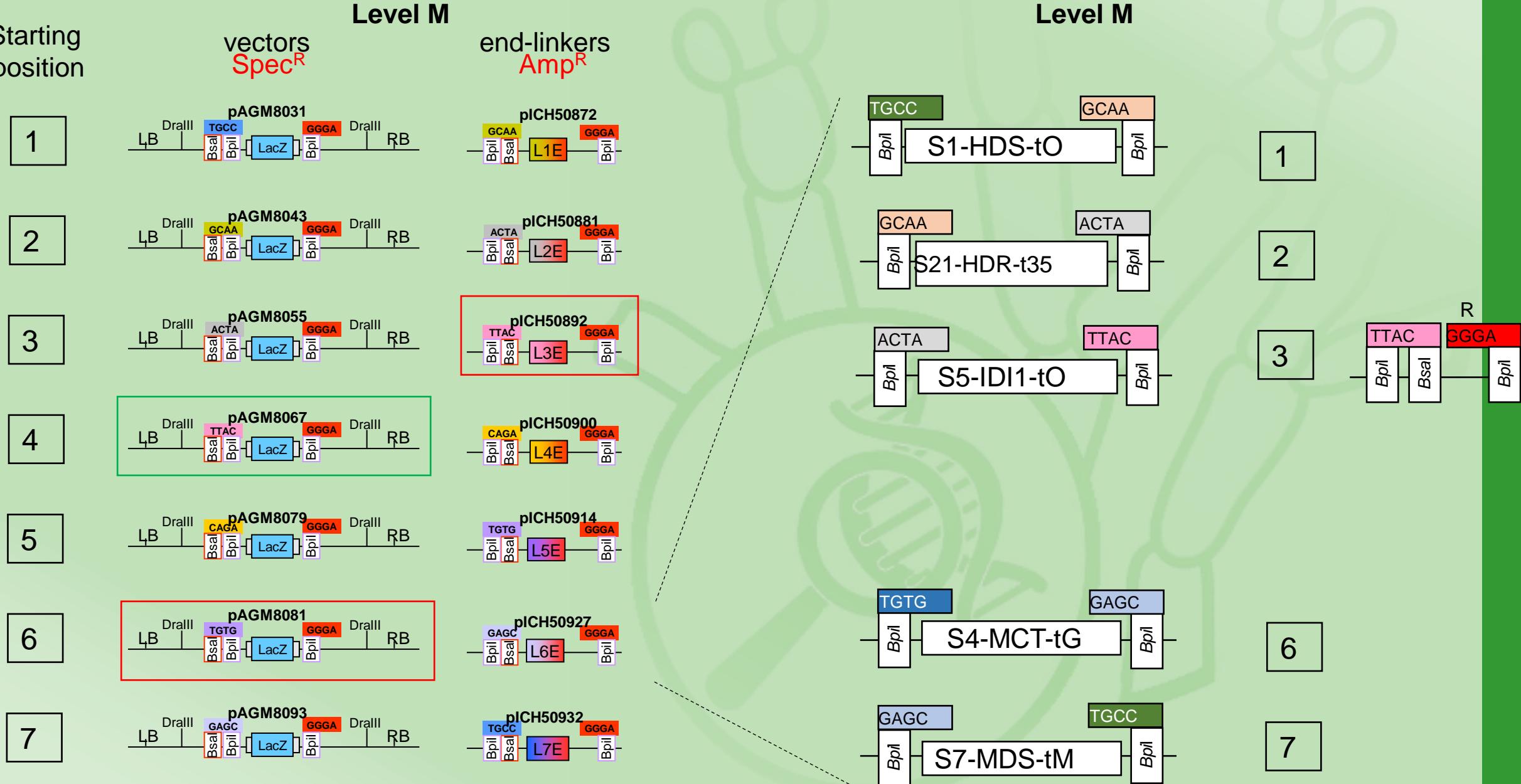
4

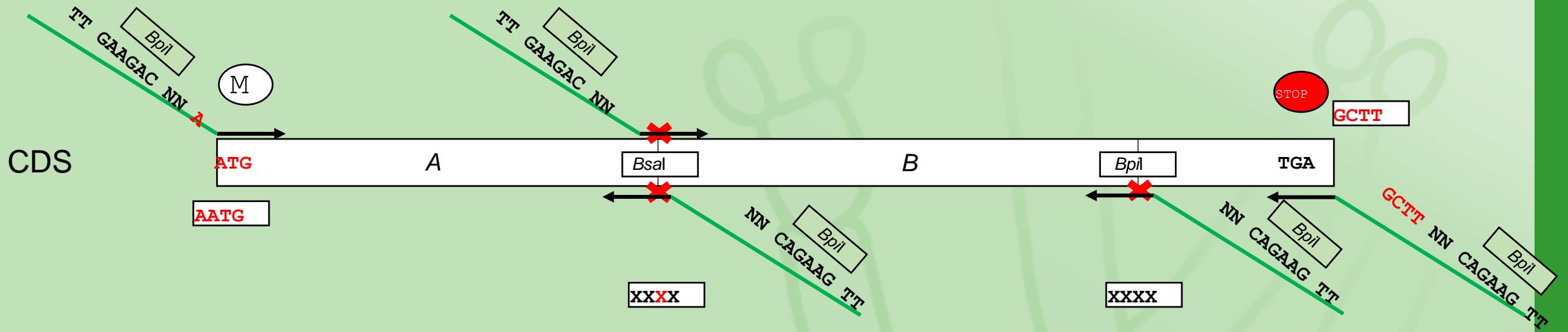
5



## Level 2 (Level M) cloning vectors

Starting position



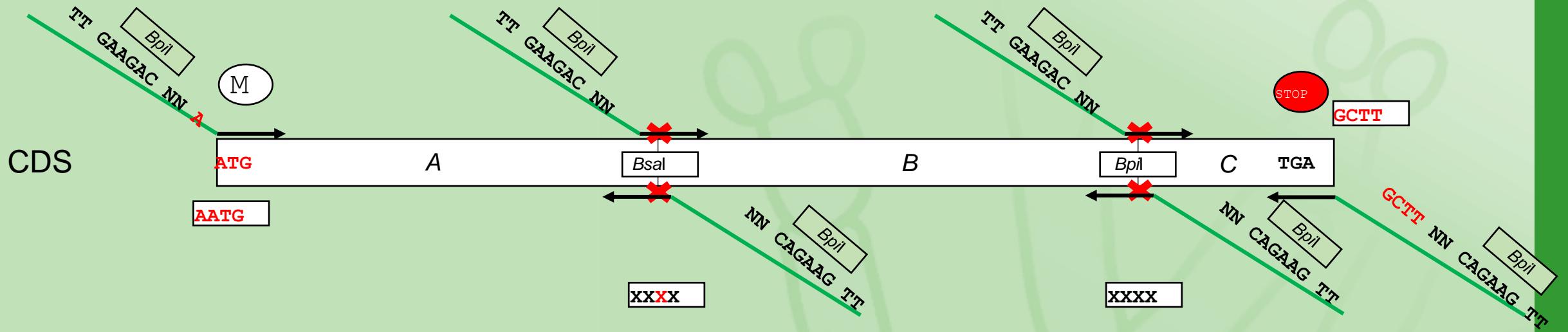


PCR-Products

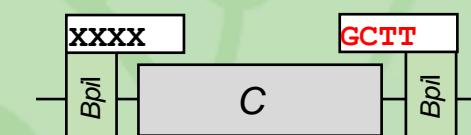
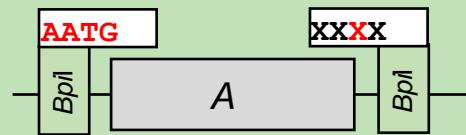


**pAGM4031**  
(Spec<sup>R</sup>)





**PCR-Products**



BpuI, T4 Ligase

**pAGM4031**  
(Spec<sup>R</sup>)

