華中農業大學 作物遗传改良全国重点实验室
National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University
农业农村部农业基因组学重点实验室（武汉）

基因编辑
操作手册

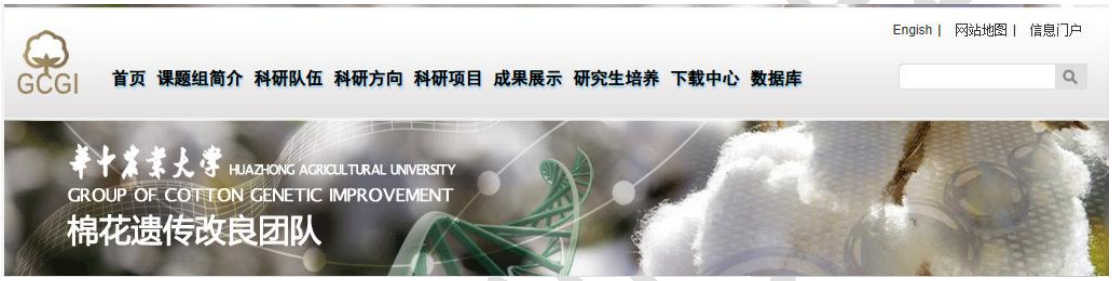
华中农业大学作物遗传改良全国重点实验室
湖北洪山实验室
棉花遗传改良团队

二〇二五年六月·武汉

基因编辑平台
GENOME EDITING PLATFORM

目录

棉花遗传改良团队：https://cotton.hzau.edu.cn/



湖北洪山实验室：https://hbhs.hzau.edu.cn/

基因编辑平台：http://jinlab.hzau.edu.cn/GenomeEditingPlatform/

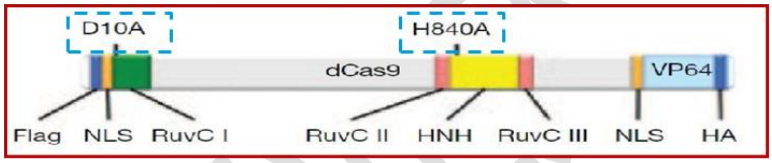
CRISPR/dCas9-TV protocol（中文版）

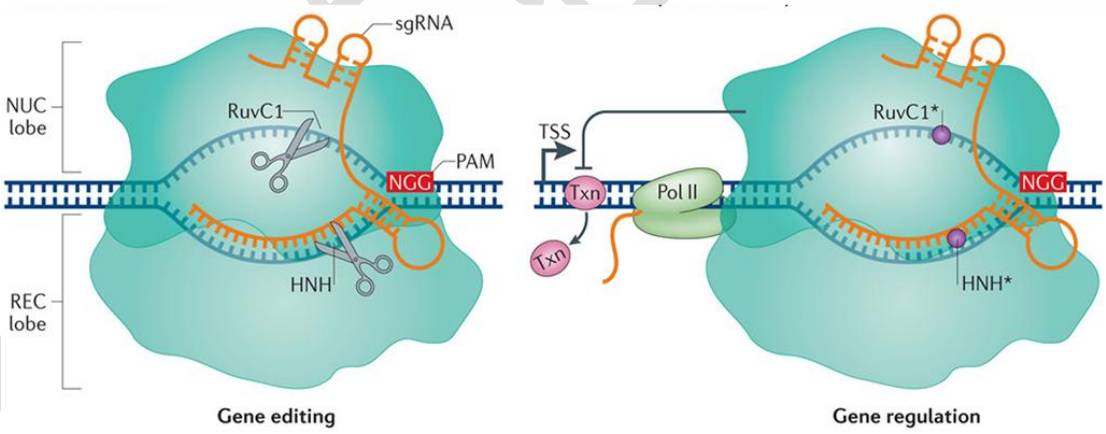
——以 CLA 为例

1.基本原理

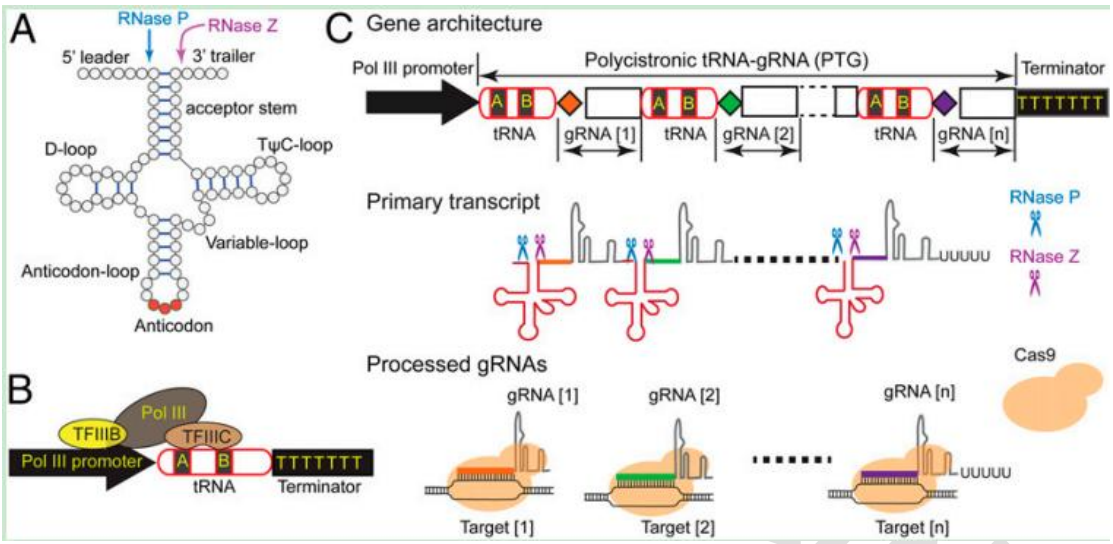
通过 CRISPR 系统将靶标切断后，生物个体会启动自身的修复机制，在修复后产生突变，导致目标基因功能沉默（如下图）。Cas9/gRNA 可以靶向任何含 5'-N20-NGG-3'（N＝A,T,G,C）或 5'-CCN-N20-3'的 DNA 位点，其中 NGG 为 Cas9 识别所需的 PAM。Cas9/gRNA 会在 PAM 前 3bp 左右的位置精确的切割靶 DNA 双链。

将 Cas9 进行定点突变后获得的 dCas9 仅保留靶向活性，打靶到基因的启动子序列后，其融合的转录激活结构域即可实现基因的定向上调。

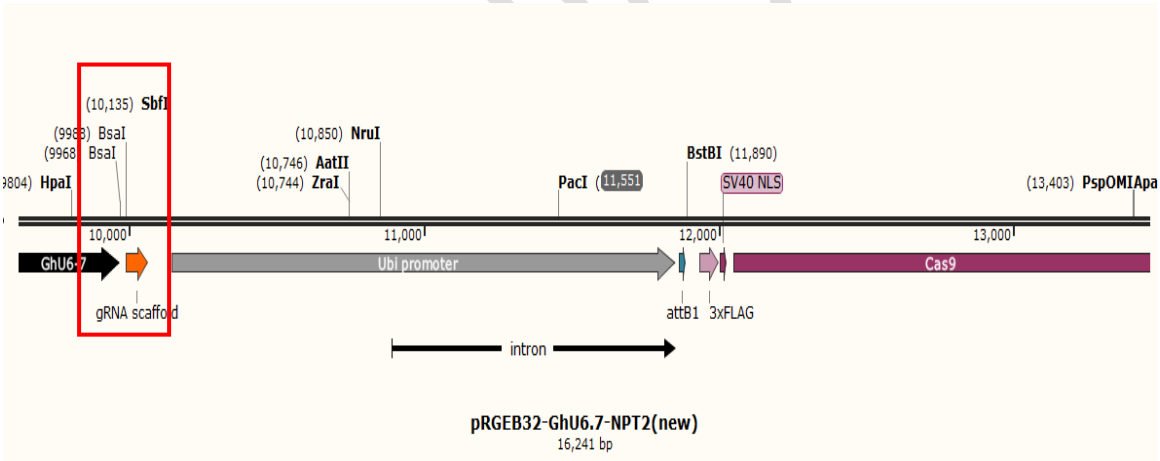




我们采用的载体[1]可以串联多个 tRNA+靶标+gRNA（下图 C），从而提高突变效率，植物体自身的 RNase P 和 RNase Z 分别切割 tRNA 的 5'端和 3'端（如下图 A），可以将多个靶标（引导序列）的 gRNA 释放，与目标序列互补后，dCas9/gRNA 会在精准靶向靶 DNA 双链。



目前我们实验室在 PRGEB32 载体基础上，将真核中抗性标记潮霉素替换为卡纳抗性，以及引导 gRNA 转录的棉花内源 U6 启动子（编号为 U6-7、U6-9），但驱动 Cas9 表达的依然是水稻 Ubiquitin2 启动子，原核中的抗性均为 kan。以下主要以 U6-7 启动子载体连接两个靶标位点，针对同一基因为例介绍载体构建过程。



2.搜索靶标以及设计引物

获取 CLA 启动子序列并靶标

可参考 CRISPR-P 网站 http://cbi.hzau.edu.cn/crispr/进行搜索靶标，选择分数最高序列即可，但是此网站只有雷蒙德氏棉的基因组数据，所以仅供参考。可以进行手动搜索，Blast 后，具有特异性的序列可以作为靶标。

[-500]-[+5]

TTATATGTTTAGTAACATTTTTAGTTCAGTCTAACTAACTAATATAGTATAGTATCTAAATATTAATTTGATA
AGAATAAATTTAATAATTGCAATATTTAAATATAAATAATATCTAAATATTAATTAAGTAAAAATAAACTTAA
TAATTAAAATATCTAATACAAATTTCATAATATTTTGACGATGAAAACATCTCAGGTAAGCCGAGTATACT
GTAACCATGTTTATTTTATATAATATCCAAACACCCCCCACCCCCCAATTAACATTTCTAGCTGACCTTCGA
TACCAGCCCGACCCCCAAGTTAAATAATCCATTTCACATCAACAGCATAAAGTAACGTAAGCCCACAATG
ATCAGATTGAGAACGATAACAGACAACAACCTTCCCTCAAGAAATAGGAACAAAAGGAAAGAAAAAA
AAGGTACCTTCTTCTAACGCCGTAATCTATCCATTTGTTTTTTCCATTGACGTGGTCCAATCAGAGAACG
ACCTGCCACT

插入目标序列连接在载体图方框所示两个 Bsa I 酶切位点之间，最终连接方法为一步克隆法。

*aagcatcagatgggca**AACAAA*GCACCAGTGGTCTAGTGGTAGAATAGTACCCTGC
CACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCA*CATAGGCCCACCGA*
*TTTGCT**GTTTTAGAGCTAGAAATA***GCAAGTTAAAATAAGGCTAGTCCGTTA
TCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGT
CTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCG
GCTGGTGCA*GTGAAGTTCGATCCGGCAAC**gttttagagctagaa*****……（载体 gRNA）**

注：小写部分是载体序列；下划线部分是 tRNA;加粗部分是 gRNA；斜体部分是靶标位置。

需要用到的引物：

CLA1 as: 5’AGCAAATCGGTGGGCCTATGtgcaccagccgggaat3’（靶标 1 互补序列）
CLA 2 s:5’CATAGGCCCACCGATTTGCTgttttagagctagaaata 3’（靶标 1 序列）
CLA 2 as: 5’GTTGCCGGATCGAACTTCACtgcaccagccgggaat3’（靶标 2 互补序列）
inf CLA as: 5’ ttctagctctaaaacGTTGCCGGATCGAACTTCAC3’（靶标 2 互补序列）
pRGEB32-7 s: 5’ AAGCATCAGATGGGCAAACAAAGCACCAGTGGTCTAG3’
inf pRGEB32-7 s: 5’ AAGCATCAGATGGGCAAACAAA 3’

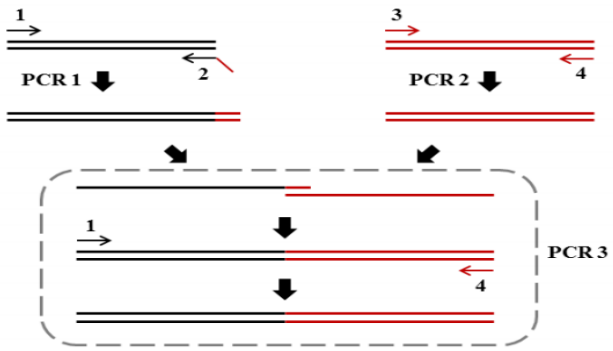
小写部分是固定序列，引物设计只需修改引物中的下划线标示的靶标序列即可。

其中，**inf pRGEB32-7 s、pRGEB32-7 s、u6-7** 为通用引物无须更改。pRGEB32-7 s 含有载体一部分序列，方便 In-fusion 连接。因插入序列中有多个 tRNA，inf pRGEB32-7 s 为增强扩增特异性，序列长度比 pRGEB32-7 s 短。 Inf CLA as 含有载体序列接头，方便后续 In-fusion 连接。

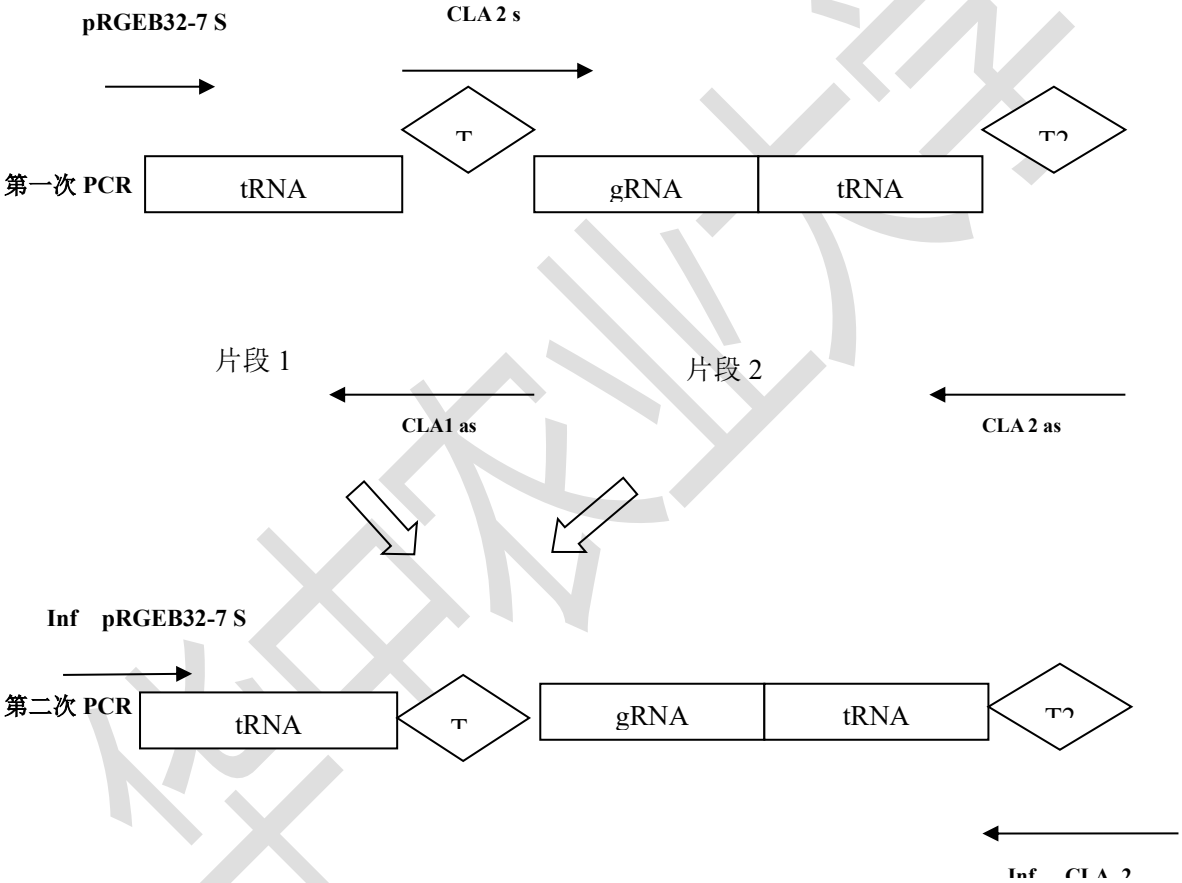
3. 第一次 PCR 扩增

重叠延伸 PCR：

PCR 循环中，扩增产生的新链是引物和延伸产物共同组成的，而且新链可以作为下一循环的模版。如果在引物的 5‘端加入接头，下一轮循环过后产生的链便会带有接头互补的序列。如此循环，最后的产物中，几乎所有的产物都含有接头序列。如果将接头序列设计得和待融合片段的引物互补，那么两个独立 PCR 的产物便可以借助这一区域产生配对，经 Taq 酶延伸成为融合片段。



重叠延伸示意图



本试验过程示意图

gRNA+tRNA 组合序列可自行合成序列连接到公共载体（如用 T 载体），第一次 PCR:1、2 两个片段分别从含 gRNA+tRNA 载体的菌液或者质粒中扩增。（相关序列可见附件）

20μl 体系：

	1	2
ddH_2O	**16.1**	**16.1**
Buffer	**2**	**2**
dNTP	**0.3**	**0.3**

S primer	**pRGEB32-7 s**	**0.2**	**CLA 2 s**	**0.2**
AS primer	**CLA1 as**	**0.2**	**CLA 2 as**	**0.2**
Taq	**0.2**		**0.2**	
模板	**1**		**1**	

PCR 条件：

预变性：95℃ 4min

变性：95℃ 30S

退火：55℃ 30S

延伸：72℃ 20S

循环：3C

变性：95℃ 30S

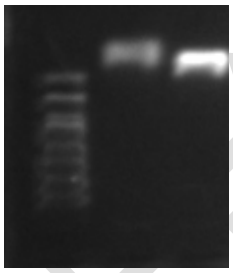
退火：60℃ 30S

延伸：72℃ 20S

循环：27C

最后延伸 72℃ 5min

电泳检测



5K 1

4.第二次 PCR 及纯化:

使用重叠延伸 PCR 将两个小片段进行连接，

		100μl 体系
ddH$_2$O		**83.5μl**
Buffer		**10μl**
dNTP		**1.5μl**
S primer	**Inf pRGEB32-7 s**	**1μl**
AS primer	**Inf CLA as**	**1μl**
Taq		**1μl**
片段 1		**1μl**
片段 2		**1μl**

PCR 条件：

预变性：95℃ 4min

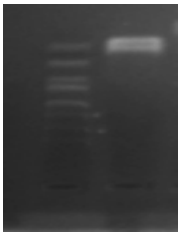
变性：95℃ 30S

退火：59℃ 30S

延伸：72℃ 20S

循环：28C

最后延伸 72℃ 5min



5k 拼接

电泳检测

使用凝胶回收的试剂盒对 PCR 产物进行纯化、测浓度

5.载体 酶切：

	100μl 体系:
质粒（**Cas9 载体，作为中间载体**）	10μg
10X cut smart Buffer	10μl
BSA1	4μl
ddH_2O	up to 100μl

37℃ 酶切 5.5h

可**与第四步同时做**，电泳检测酶切效果、使用凝胶回收的试剂盒对酶切产物进行纯化、测浓度。

6.In-fusion 连接:

目的片段	100ng
线性化表达载体（**Cas9 载体，作为中间载体**）	100ng
Exnase	0.5μl
CE Buffer	1μl

37℃水浴 30min，冰上放置 5min，可-20℃保存备用

7.转化感受态

连接产物与感受态混合，冰上放置 30min，42℃热激 90S，冰上静置 5min。取 300μlSOC 加入新的 2ml 离心管，将热激产物加入 SOC 中，37℃震荡 45min，涂于 KAN 皿，37℃培养过夜，挑单克隆于 500μl kan 抗性液体 LB,37℃震荡 3h，使用 Inf pRGEB32-7 s Inf CLA as 引物阳性检测，电泳跑胶，挑阳性送测序（**GC rich**）.测序正确后保菌，提质粒。

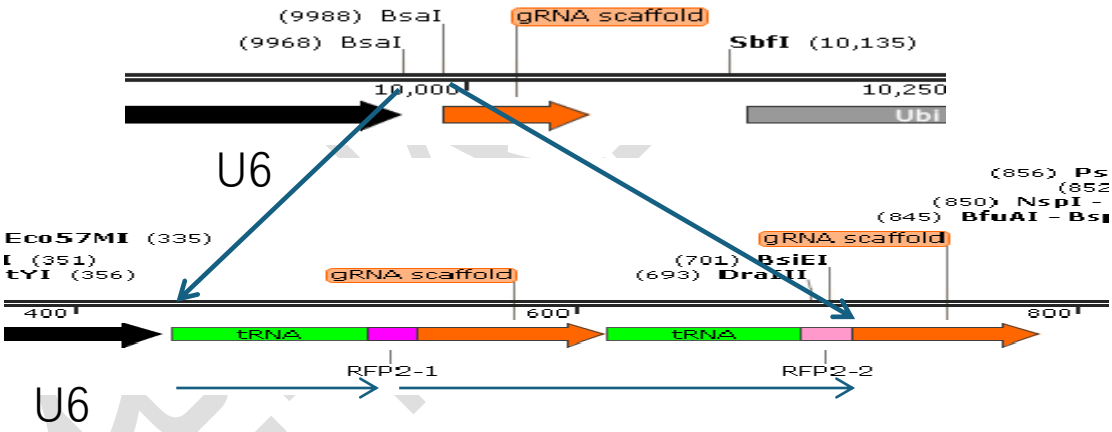
阳性检测引物/测序引物

u6-7 s: TGTGCCACTCCAAAGACATCAG + Inf XX AS

阳性检测电泳图：

5K 阴 阳

构建成功载体图：



8. PCR 扩增目的条带及纯化:

使用 PCR 扩增含有靶标序列的片段进行连接，以酶切位点 Hpa1 和 Sbf1 为两端进行扩增。

	100μl 体系	
ddH_2O	**83.5μl**	
Buffer	**10μl**	
dNTP	**1.5μl**	
S primer	**Inf TV s**	**1μl**
AS primer	**Inf sbf as**	**1μl**
Taq	**1μl**	
cas9 载体	**2μl**	

Inf TV s 接头： 5‘ CTTGCCACGTGTGTTaacctcgttttcatcatgtg 3'

Inf sbf as 接头： 5‘ ATTTGTGGACCTGCAgctaaaaacggactagcctt 3'

PCR 条件：

预变性：95℃ 4min

变性：95℃ 30S

退火：54℃ 30S

延伸：72℃ 40S

循环：28C

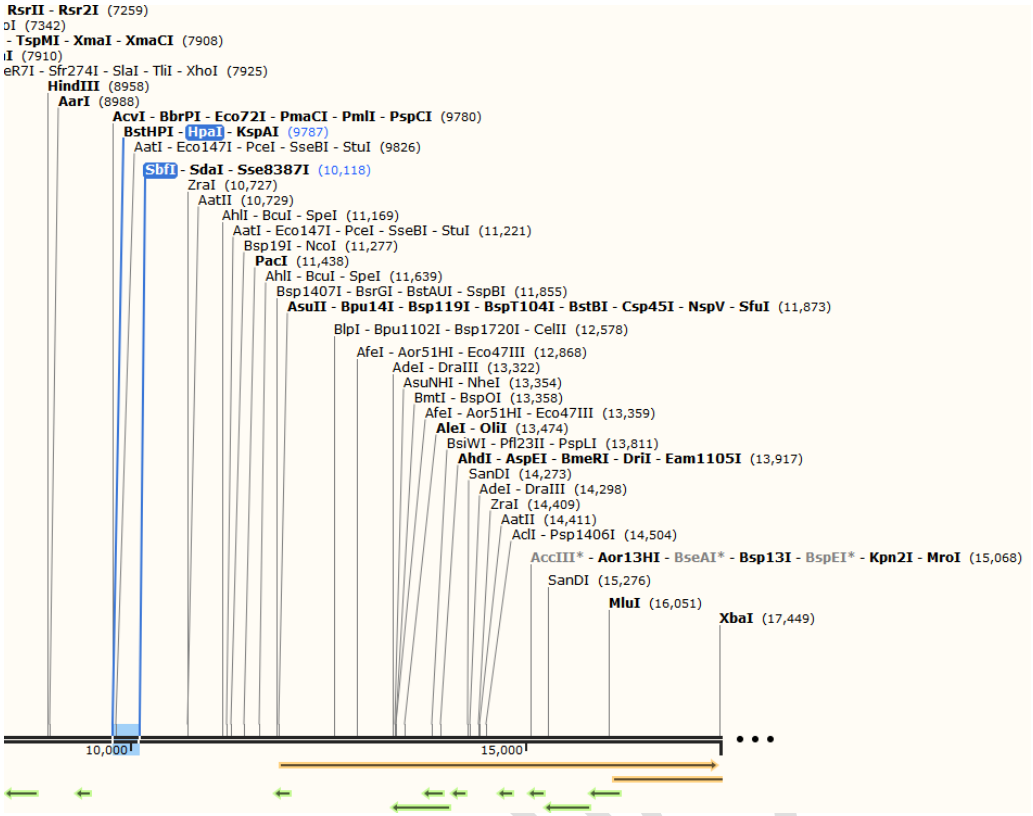
最后延伸 72℃ 5min

电泳检测
使用凝胶回收的试剂盒对 PCR 产物进行纯化、测浓度

9. 载体酶切（dCas9-TV 载体）：

	100μl 体系:
质粒（dCas9-TV 载体）	10μg
10X cut smart Buffer	10μl
Hpa1	2μl
Sbf1	2μl
ddH_2O	up to 100μl

37℃ 酶切 5.5h



电泳检测酶切效果、使用凝胶回收的试剂盒对酶切产物进行纯化、测浓度。

10. In-fusion 连接:

目的片段	100ng
线性化表达载体(dCas9-TV 载体)	100ng
Exnase	0.5μl
CE Buffer	1μl

37℃水浴 30min，冰上放置 5min，可-20℃保存备用

11.转化感受态

连接产物与感受态混合，冰上放置 30min，42℃热激 90S，冰上静置 5min。取 300μlSOC 加入新的 2ml 离心管，将热激产物加入 SOC 中，37℃震荡 45min，涂于 KAN 皿，37℃培养过夜，挑单克隆于 500μl kan 抗性液体 LB,37℃震荡 3h,使用 u6-7s 和 ubi-as 引物阳性检测，电泳跑胶，挑阳性送测序（**GC rich**）.保菌，提质粒。

阳性检测引物/测序引物

u6-7 s: TGTGCCACTCCAAAGACATCAG

Ubi-as: TGTTGGTCGCCGTTAGGA

12. 电转

测序成功后，电转农杆菌
阳性检测引物
u6-7 s: TGTGCCACTCCAAAGACATCAG
Ubi-as: TGTTGGTCGCCGTTAGGA

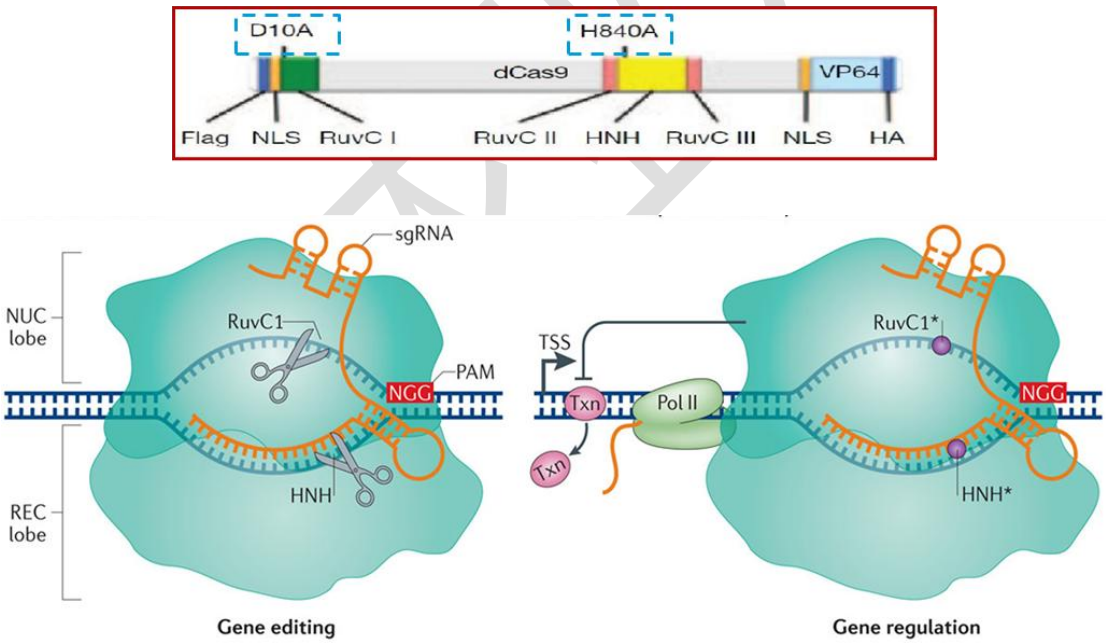
CRISPR/Cas9 protocol（英文版）

——Using CLA as an Example

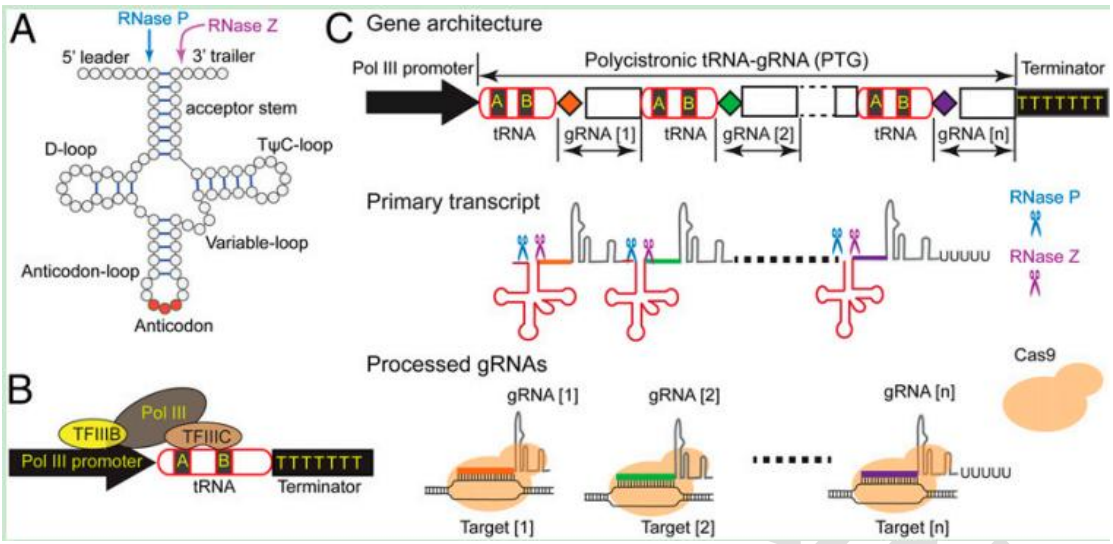
1. Basic Principles

After the target sequence is cleaved by the CRISPR system, the organism initiates its own repair mechanisms, leading to mutations and silencing of the target gene function (as shown below). The Cas9/gRNA complex can target any DNA site containing the motifs **5'-N_{20}-NGG-3'** or **5'-CCN-N_{20}-3'** (where NGG is the PAM required for Cas9 recognition). Cas9/gRNA precisely cleaves the target DNA double strand approximately 3 bp upstream of the PAM.

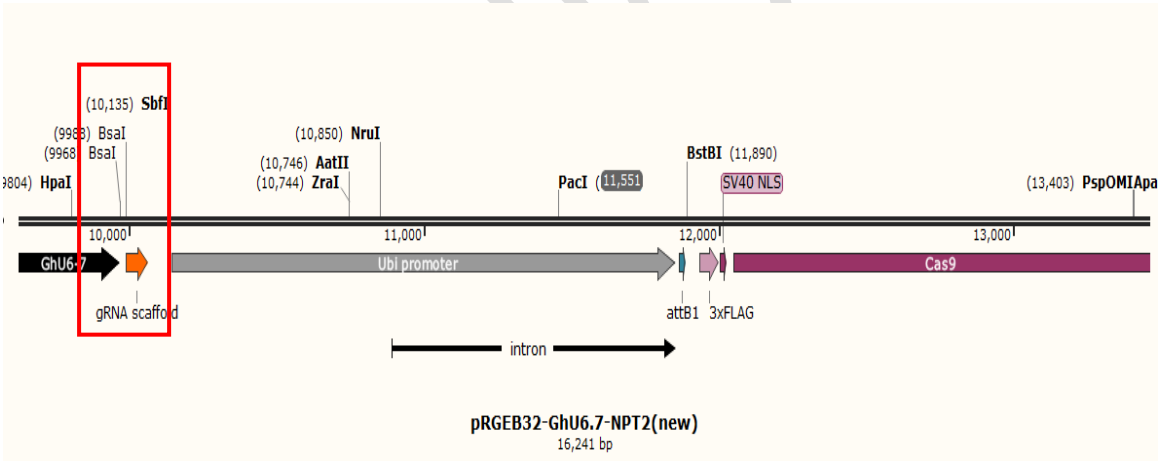
The dCas9, obtained through the targeted mutation of Cas9, retains only its targeting activity. When directed to the promoter sequence of a gene, its fused transcriptional activation domain can facilitate the targeted upregulation of that gene.



Our vector system allows tandem integration of multiple **tRNA + target + gRNA** units (Fig. C), enhancing mutation efficiency. Plant endonucleases RNase P and RNase Z cleave the 5' and 3' ends of tRNA (Fig. A), releasing gRNAs that guide Cas9/gRNA to cleave the target DNA at the specified site.



In our lab, we modified the pRGEB32 vector by replacing the kanamycin resistance marker with hygromycin and incorporating the cotton endogenous U6 promoters (U6-7, U6-9) for gRNA transcription. The protocol below focuses on constructing a vector with two target sites under the U6-7 promoter using rice Ubiquitin2 as the Cas9 promtor.



2. Target Search and Primer Design

CLA Sequence and Targets: Use the CRISPR-P website (http://cbi.hzau.edu.cn/crispr/) to search for targets (limited to *Gossypium raimondii* data). Alternatively, manually BLAST sequences for specificity. Prefer targets with unique restriction sites near the PAM (3 bp upstream) for downstream validation.

[-500]-[+5]

TTATATGTTTAGTAACATTTTTAGTTCAGTCTAACTAACTAATATAGTATAGTATCTAAATATTAATTTGATA
AGAATAAATTTAATAATTGCAATATTTAAATATAAATAATATCTAAATATTAATTAAGTAAAAATAAACTTAA
TAATTAAAATATCTAATACAAATTTCATAATATTTTGACGATGAAAACATCTCAGGTAAGCCGAGTATACT
GTAACCATGTTTATTTTATATAATATCCAAACACCCCCCACCCCCCAATTAACATTTCTAGCTGACCTTCGA
TACCAGCCCGACCCCCAAGTTAAATAATCCATTTCACATCAACAGCATAAAGTAACGTAAGCCCACAATG
ATCAGATTGAGAACGATAACAGACAACAACCTTCCCTCAAGAAATAGGAACAAAAGGAAAGAAAAAA
AAGGTACCTTCTTCTAACGCCGTAATCTATCCATTTGTTTTTTCCATTGACGTGGTCCAATCAGAGAACG
ACCTGCCACT

Insert the target sequence and ligate between the two Bsa I digestion sites shown in the box of the vector diagram. The final ligation method is the one-step cloning method.

*aagcatcagatgggca**AACAAA*GCACCAGTGGTCTAGTGGTAGAATAGTACCCTGC
CACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCA*CATAGGCCCACCGA*
*TTTGCT**GTTTTAGAGCTAGAAATA***GCAAGTTAAAATAAGGCTAGTCCGTTA
TCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGT
CTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCG
GCTGGTGCA*GTGAAGTTCGATCCGGCAAC**gttttagagctagaa*****……（载体 gRNA）**

notes：Note: Lowercase = vector backbone; underscores = tRNA; bold = gRNA; italics = target site.

Primers:：

CLA1 as: 5’AGCAAATCGGTGGGCCTATGtgcaccagccgggaat3’（靶标 1 互补序列）
CLA 2 s:5’ CATAGGCCCACCGATTTGCTgttttagagctagaaata 3’（靶标 1 序列）
CLA 2 as: 5’GTTGCCGGATCGAACTTCACtgcaccagccgggaat3’（靶标 2 互补序列）
inf CLA as: 5’ ttctagctctaaaacGTTGCCGGATCGAACTTCAC3’（靶标 2 互补序列）
pRGEB32-7 s: 5’ AAGCATCAGATGGGCAAACAAAGCACCAGTGGTCTAG3’
inf pRGEB32-7 s: 5’ AAGCATCAGATGGGCAAACAAA 3’

（**U6-9**
pRGEB32-9 s: 5’ CAGCACATAACTGGCAAACAAAGCACCAGTGGTCTAG3’
inf pRGEB32-9 s: 5’ CAGCACATAACTGGCAAACAAA 3’）

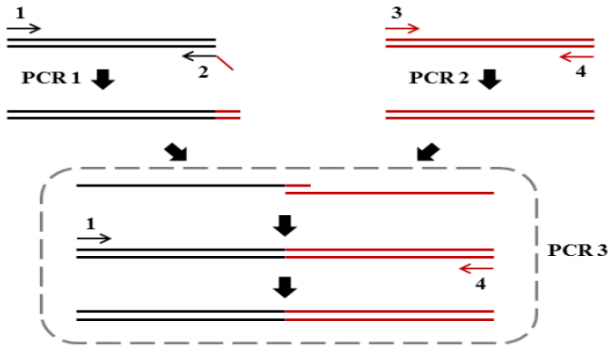
The lowercase part is a fixed sequence. Primer design only requires modifying the target sequence indicated by the underline in the primer.

Among them, inf pRGEB32-7 s, pRGEB32-7 s, pRGEB32-9 s, inf pRGEB32-9 s, u6-7 s, and u6-9 s are universal primers and do not need to be changed. pRGEB32-7s and pRGEB32-9s contain a part of the vector sequence, facilitating In-fusion ligation. Because there are multiple trnas in the inserted sequence, inf pRGEB32-7 s and inf pRGEB32-9 s, in order to enhance amplification specificity, have shorter sequence lengths than pRGEB32-7 s and pRGEB32-9 s. Inf CLA as contains carrier sequence connectors for convenient subsequent In-fusion connections.

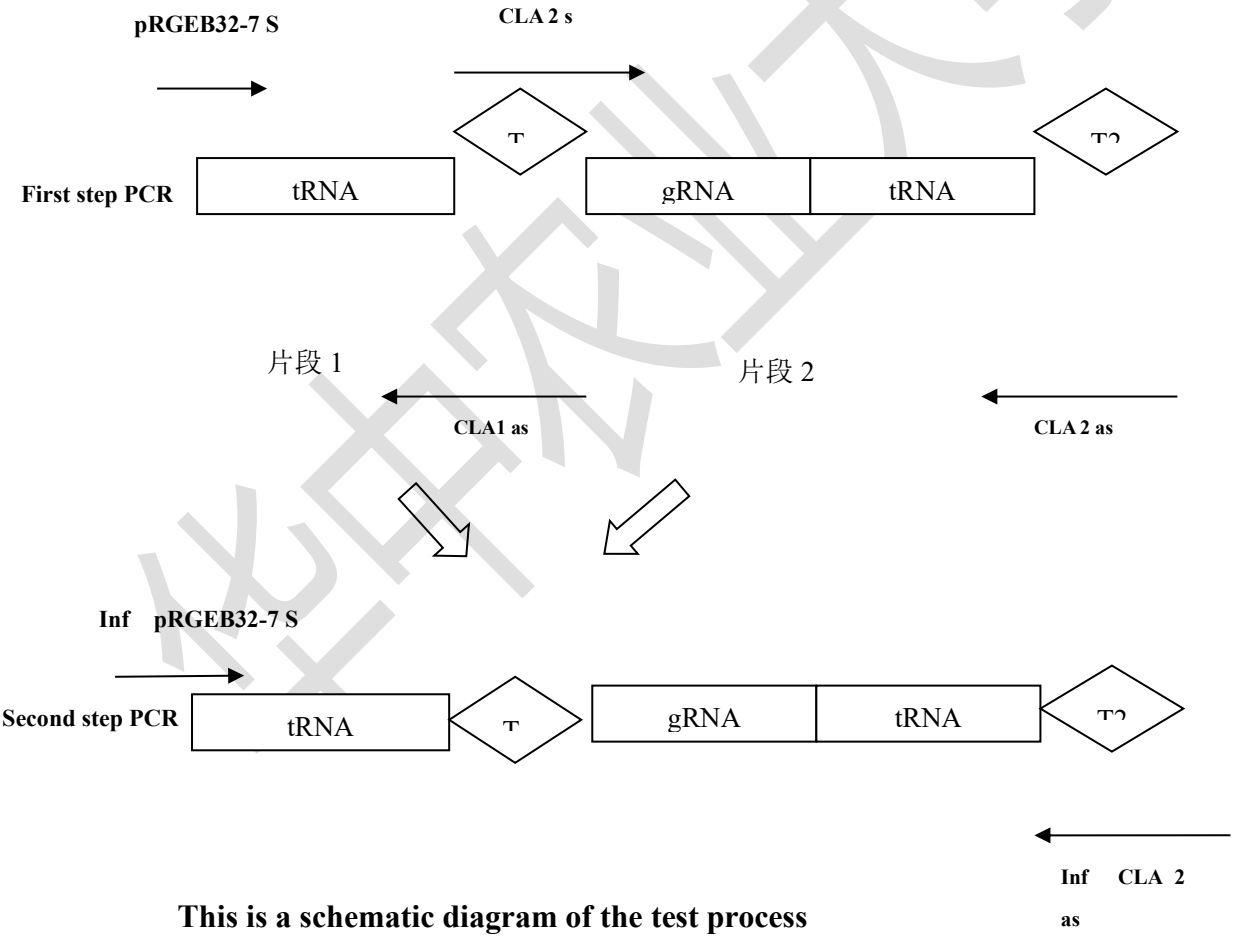
3. First PCR Amplification

Overlap Extension PCR:

This method generates fusion fragments by designing primers with complementary overhangs. Two overlapping PCR products are combined and extended to form a single construct.



Overlapping extension schematic diagram



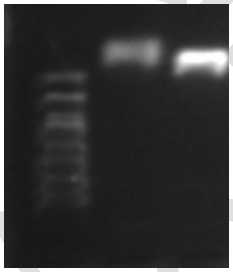
This is a schematic diagram of the test process

The gRNA+tRNA combination sequence can be synthesized by itself and ligated to a common vector (such as using the T vector). In the first PCR: Fragments 1 and 2 were amplified respectively from the bacterial liquid or plasmid containing the gRNA+tRNA vector. (The relevant sequence can be found in the attachment.)

Reaction Mixture (20 μL)：

	1		2	
ddH_2O	**16.1**		**16.1**	
Buffer	**2**		**2**	
dNTP	**0.3**		**0.3**	
S primer	**pRGEB32-7 s**	**0.2**	**CLA 2 s**	**0.2**
AS primer	**CLA1 as**	**0.2**	**CLA 2 as**	**0.2**
Taq	**0.2**		**0.2**	
Template DNA	**1**		**1**	

PCR Conditions:



1. Initial denaturation: 95°C for 4 min
2. Denaturation: 95°C for 30 s
3. Annealing: 55°C for 30 s
4. Extension: 72°C for 20 s
    - Repeat steps 2–4 for 3 cycles
5. Denaturation: 95°C for 30 s
6. Annealing: 60°C for 30 s
7. Extension: 72°C for 20 s
    - Repeat steps 5–7 for 27 cycles
8. Final extension: 72°C for 5 min

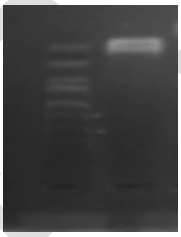
Electrophoresis: Verify amplification.

4. Second PCR and Purification

Combine the two PCR products from Step 3 using overlap extension，

	Reaction Mixture (100 µL)
ddH_2O	**83.5µl**
Buffer	**10µl**
dNTP	**1.5µl**
S primer	**Inf pRGEB32-7 s** **1µl**
AS primer	**Inf CLA as** **1µl**
Taq	**1µl**
Cas9 vector	**2µl**

PCR Conditions:



1. Initial denaturation: 95°C for 4 min
2. Denaturation: 95°C for 30 s
3. Annealing: 59°C for 30 s
4. Extension: 72°C for 20 s
    - Repeat steps 2–4 for 28 cycles
5. Final extension: 72°C for 5 min

Purification: Use a gel extraction kit to purify the product and measure concentration.

5. Vector Digestion

Digest the vector with BsaI（Cas9）.

Reaction Mixture (100 µL):

	Volume (µL):
PCR Product	10 µg
CutSmart Buffer	10 µL

BSA	1 μL
ddH_2O	Up to 100 μL

Digestion: 37°C for 5.5 h.

6. In-Fusion Cloning

Assemble the digested vector and insert using In-Fusion technology.

Components:

- **Insert (100 ng)**
- **Linearized vector (100 ng，Cas9)**
- **Exnase (0.5 μL)**
- **CE Buffer (1 μL)**

Incubation: 37°C for 30 min, then ice for 5 min. Store at -20°C.

7. Transformation
Transform competent cells with the ligation product.

Steps:

Mix with SOC medium and incubate at 37°C for 45 min.

Plate on kanamycin agar plates and incubate overnight.

Pick colonies for PCR validation using primers:

u6-7s: TGTGCCACTCCAAAGACATCAG + Inf XX AS

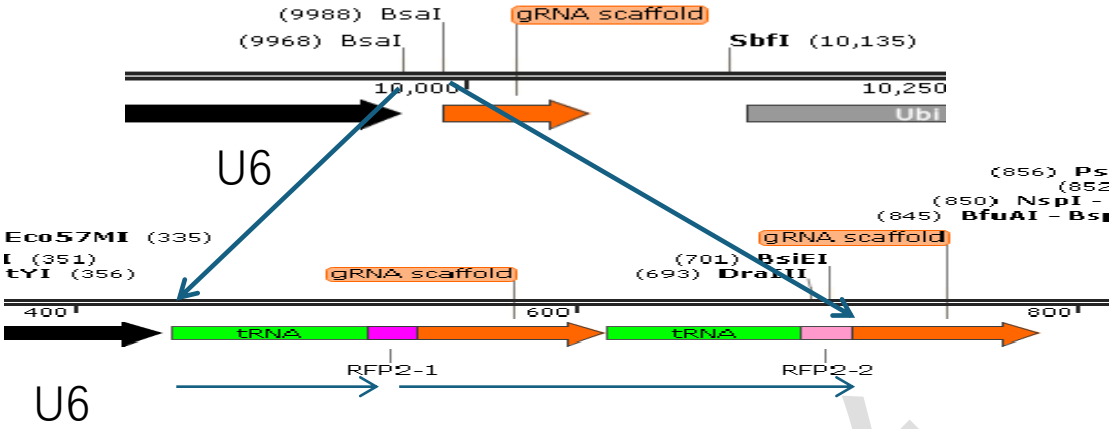
u6-9s: GTCAAAAACTATCCCACATTGCTAA + Inf XX AS

Positive clones are sequenced (GC-rich regions).

Electrophoresis: Confirm positive clones.

Positive detection electrophoresis pattern：

Construct a successful carrier diagram：



8. PCR amplification and purification of target bands:

Fragments containing the target sequence were ligated using PCR amplification with the enzymatic sites Hpa1 and Sbf1 at either end.

	100μl 体系
ddH_2O	**83.5μl**
Buffer	**10μl**
dNTP	**1.5μl**
S primer	**Inf TV s** **1μl**
AS primer	**Inf sbf as** **1μl**
Taq	**1μl**
Last vector	**2μl**

Inf TV s : 5‘ CTTGCCACGTGTGTTaacctcgttttcatcatgtg 3'

Inf sbf as : 5‘ ATTTGTGGACCTGCAgctaaaaacggactagcctt 3

PCR:

Initial denaturation: 95°C for 4 min

Denaturation: 95°C for 30 s

Annealing: 54°C for 30 s

Extension: 72°C for 40 s

Repeat steps 2–4 for 28 cycles

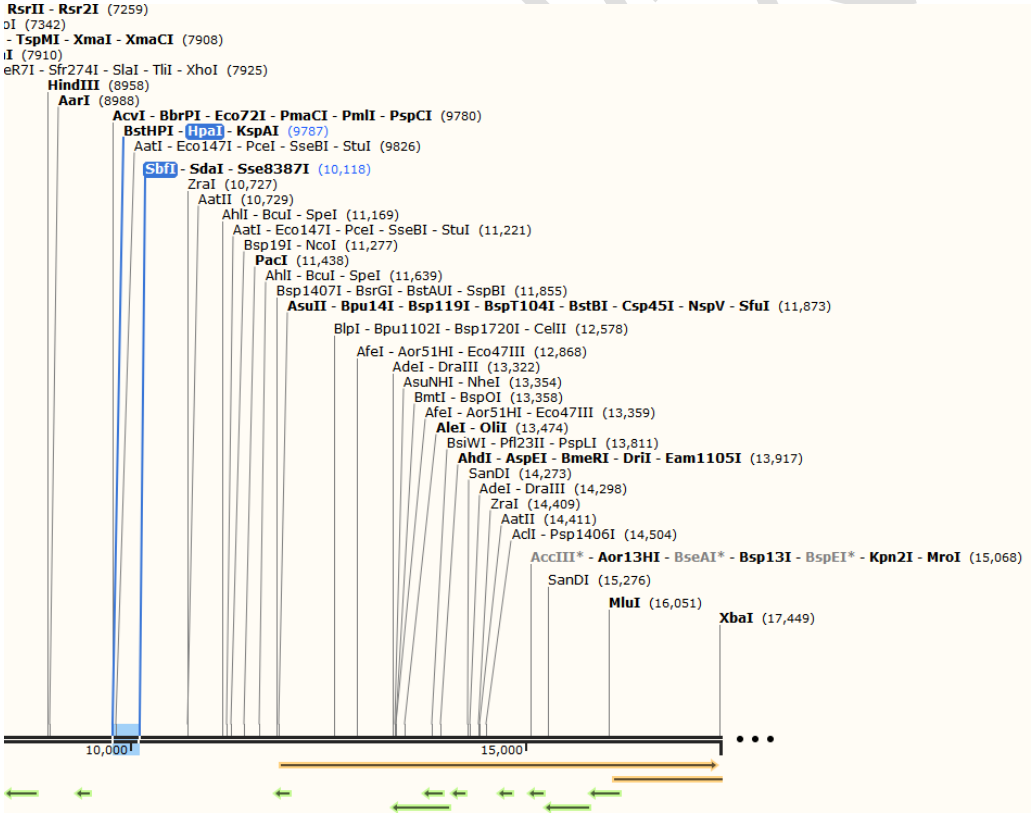
Final extension: 72°C for 5 min

Purification: Use a gel extraction kit to purify the product and measure concentration.

9. Reaction Mixture (100 μL，dCas9-TV):

	Volume (μL):
Vector（dCas9-TV）	10μg
10X cut smart Buffer	10μl
Hpa1	2μl
Sbf1	2μl
ddH_2O	up to 100μl

Digestion: 37°C for 5.5 h.



电泳检测酶切效果、使用凝胶回收的试剂盒对酶切产物进行纯化、测浓度。

10. In-Fusion Cloning

Assemble the digested vector and insert using In-Fusion technology.

Components:

- **Insert (100 ng)**
- **Linearized vector (100 ng，dCas9-TV)**
- **Exnase (0.5 μL)**
- **CE Buffer (1 μL)**

Incubation: 37°C for 30 min, then ice for 5 min. Store at -20°C.

11. Transformation

Transform competent cells with the ligation product.

Steps:

Mix with SOC medium and incubate at 37°C for 45 min.

Plate on kanamycin agar plates and incubate overnight.

Pick colonies for PCR validation using primers:

u6-7s: TGTGCCACTCCAAAGACATCAG + Inf XX AS

u6-9s: GTCAAAAACTATCCCACATTGCTAA + Inf XX AS

Positive clones are sequenced (GC-rich regions).

Electrophoresis: Confirm positive clones.

u6-7 s: TGTGCCACTCCAAAGACATCAG

Ubi-as: TGTTGGTCGCCGTTAGGA

12. Agrobacterium Electroporation

Electroporate the verified construct into *Agrobacterium*.

u6-7 s: TGTGCCACTCCAAAGACATCAG + Inf XX AS
Ubi-as: TGTTGGTCGCCGTTAGGA