

Construction of a Single Transcriptional Unit for Expression of Cas9 and Single-guide RNAs for Genome Editing in Plants

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[Abstract] The CRISPR (clustered regularly interspaced short palindromic repeats)-associated protein9 (Cas9) is a simple and efficient tool for genome editing in many organisms including plant and crop species. The sgRNAs of the CRISPR/Cas9 system are typically expressed from RNA polymerase III promoters, such as U6 and U3. In many transformation events, more nucleotides will increase the difficulties in plasmid construction and the risk of wrong integration in genome such as base-pair or fragment missing (Gheysen *et al.*, 1990). And also, in many organisms, Pol III promoters have not been well characterized, and heterologous Pol III promoters often perform poorly (Sun *et al.*, 2015). Thus, we have developed a method using single transcriptional unit (STU) CRISPR-Cas9 system to drive the expression of both Cas9 and sgRNAs from a single RNA polymerase II promoter to achieve effective genome editing in plants.

Keywords: CRISPR, Cas9, Genome editing, Single transcriptional unit (STU), Ribozyme

[Background] The sgRNA of the CRISPR-Cas9 system is mainly promoted by the small nuclear RNA promoters such as U6 and U3. Although it has been tested with prospered efficiency in many cases, it also has some limitations: (1) it is hard to achieve coordinated and/or inducible expression of Cas9 and the sgRNAs; (2) manipulating multiple sgRNAs for multiplexed gene editing can be tedious, requiring multiple Pol III promoters. The traditional RNA polymerase II promoter can't be used in driving sgRNA expression, extra nucleotides will be added to the 5'- and 3'-ends of gRNA by RNA polymerase II and may interrupt the normal gRNA function. Additionally, RNAs transcribed by RNA polymerase II are exported rapidly into the cytoplasm while nuclear localization is required for the CRISPR-Cas9/gRNA duplex to access the genome editing (Lei *et al.*, 2001). To overcome these obstacles, we use the ribozyme's self-catalyzed cleavage to release the precise processing mature sgRNA under a RNA polymerase II promoter which drives expression of both Cas9 and sgRNA (named STU CRISPR-Cas9 system, Figure 1). Compared to the traditional small nuclear RNA promoters used in sgRNA expression, our STU CRISPR-Cas9 system has some advantages: (1) it's shorter and easier in vector construction, and it will increase the transformation efficiency under some circumstances; (2) it only needs extra ribozyme flanking sequence (shorter than any RNA polymerase III promoter we are currently using) for multiple sgRNAs expression;(3) it has shown higher deletion efficiency induced by double sgRNAs. Thus, the STU CRISPR-Cas9 system driven by a single RNA polymerase II promoter can replace the

traditional CRISPR-Cas9 system now we are using whether *in vivo* or *in vitro* if appropriate promoters are chosen.

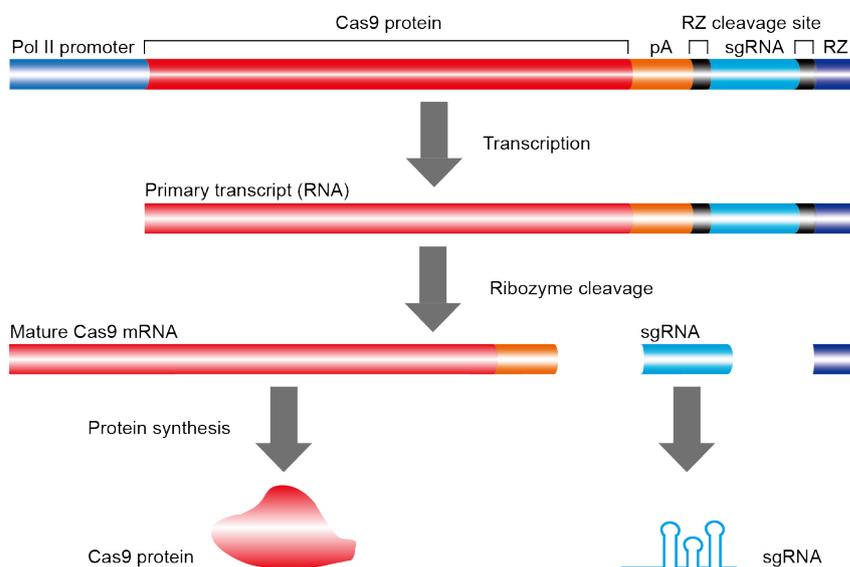


Figure 1. Schematic illustration of the single transcription unit (STU) CRISPR-Cas9 system. Once transcribed by a Pol II promoter, the STU CRISPR-Cas9 primary transcripts will undergo self-cleavage by hammerhead ribozyme (RZ) to release the mature Cas9 mRNA and sgRNA. The Cas9 mRNA is terminated with a synthetic polyA (pA) sequence to facilitate translation, The RZ sequence (in blue) and its target sequence (in black) are illustrated.

Materials and Reagents

1. 0.2 ml PCR tubes (Biosharp, catalog number: BS-02-P)
2. 1.5 ml Eppendorf tubes (Biosharp, catalog number: BS-15-M)
3. Pipette tips (Biosharp, catalog numbers: BS-10-T, BS-200-T, BS-1000-T)
4. Competent *E. coli* DH5 α cells (Homemade)
5. pTX171 plasmids (Addgene, catalog number: 89258)
6. pTX172 plasmids (Addgene, catalog number: 89259)
7. *Bsal* (New England Biolabs, catalog number: R0535L)
8. Deionized water (sterile)
9. Agarose (Biowest, catalog number: 111860)
10. Ethidium bromide (Solarbio Life Scientific, catalog number: E1020)
11. AxyPrep™ DNA Gel Extraction Kit (Corning, Axygen®, catalog number: AP-GX-250)
12. T4 DNA ligase (New England Biolabs, catalog number: M0202L)
13. dNTPs mixture (Tiagen Biotech, catalog number: CD117-11)
14. Taq DNA polymerase (Tiagen Biotech, catalog number: ET101-01-02)
15. Q5® High-Fidelity DNA polymerase (New England Biolabs, catalog number: M0491L)
16. AxyPrep™ Plasmid Miniprep Kit (Corning, Axygen®, catalog number: AP-MN-P-250)

17. Kanamycin (Solarbio Life Scientific, catalog number: K8020)
18. TAE electrophoresis buffer (see Recipes)
 - Tris (Solarbio Life Scientific, catalog number: T8060)
 - Acetic acid (Kelong)
 - 0.5 M EDTA (Solarbio Life Scientific, catalog number: E1170)
19. LB medium (see Recipes)
 - Tryptone (Oxoid, catalog number: LP0042)
 - Yeast extract (Oxoid, catalog number: LP0021)
 - Sodium chloride (NaCl) (Kelong)

Equipment

1. Pipettes (Dragon-Lab)
2. Heating block (Hangzhou Allsheng Instruments, model: MK-20)
3. Thermal cycler (Thermo Fisher Scientific, Thermo Scientific™, model: Arktik™ Thermal Cycler)
4. Water bath (Yongguangming, model: DZKW-S-4)
5. Microcentrifuge (Eppendorf, model: 5424)
6. DNA electrophoresis apparatus (Bio-Rad Laboratories, model: Mini-Sub® Cell GT Systems)
7. NanoDrop (Thermo Fisher Scientific, Thermo Scientific™, model: NanoDrop™ 2000)

Procedure

A. Design sgRNAs to target the genes of interest

1. Select appropriate sgRNA targets for the genes of interest using the online sgRNA design tools such as CRISPR-P v2.0 (<http://cbi.hzau.edu.cn/CRISPR2/>); CRISPR RGEN tools (<http://www.rgenome.net/cas-offinder/>); E-CRISP (<http://www.e-crisp.org/E-CRISP/designcrispr.html>). These are several web-based tools available for sgRNA design. They have mainly the same functions in sgRNA design and Off-target prediction. The main difference is the algorithm of the scoring system.

Notes: sgRNA targets containing a restriction enzyme site at the Cas9 cleavage site would contribute to identify mutant using polymerase chain reaction-restriction endonuclease digestion assay.

2. Design and order forward and reverse oligonucleotides for cloning sgRNA into the STU CRISPR-Cas9 expression vector. (1) the forward sgRNA oligonucleotide contains a 'CGGA' sequence at the 5' end followed by 20 bases of sgRNA targets without PAM sites (N₂₀); (2) the reverse sgRNA oligonucleotide contains an 'AAAC' at the 5' end followed by the reverse complement of N₂₀.

For example, if the target site is GTTGGTCTTTGCTCCTGCAGAGG (AGG is PAM), the forward and reverse oligonucleotides should be:

Forward oligonucleotide: 5'-CGGAGTTGGTCTTTGCTCCTGCAG-3'

Reverse oligonucleotide: 5'-AAACCTGCAGGAGCAAAGACCAAC-3'

B. Annealing of sgRNA oligos

1. Mix 10 µl of the forward and reverse oligos (100 µM) of each sgRNA in separate microtubes.
2. Incubate the microtubes at 95 °C for 5 min in a heating block or thermal cycler.
3. Allow the microtubes to slowly cool down to room temperature.
4. Make a 1:200 dilution of the annealed mixture with deionized water.

C. Vector cloning

Two methods could be used to clone sgRNAs into the STU CRISPR-Cas9 expression vector: Cut and ligation or Golden Gate method (Figure 2). These two methods are the same in procedure including *BsaI* digestion and T4 DNA ligase ligation. The Golden Gate reaction is much easier because the digestion and ligation will process in one PCR tube, and it may save some time.

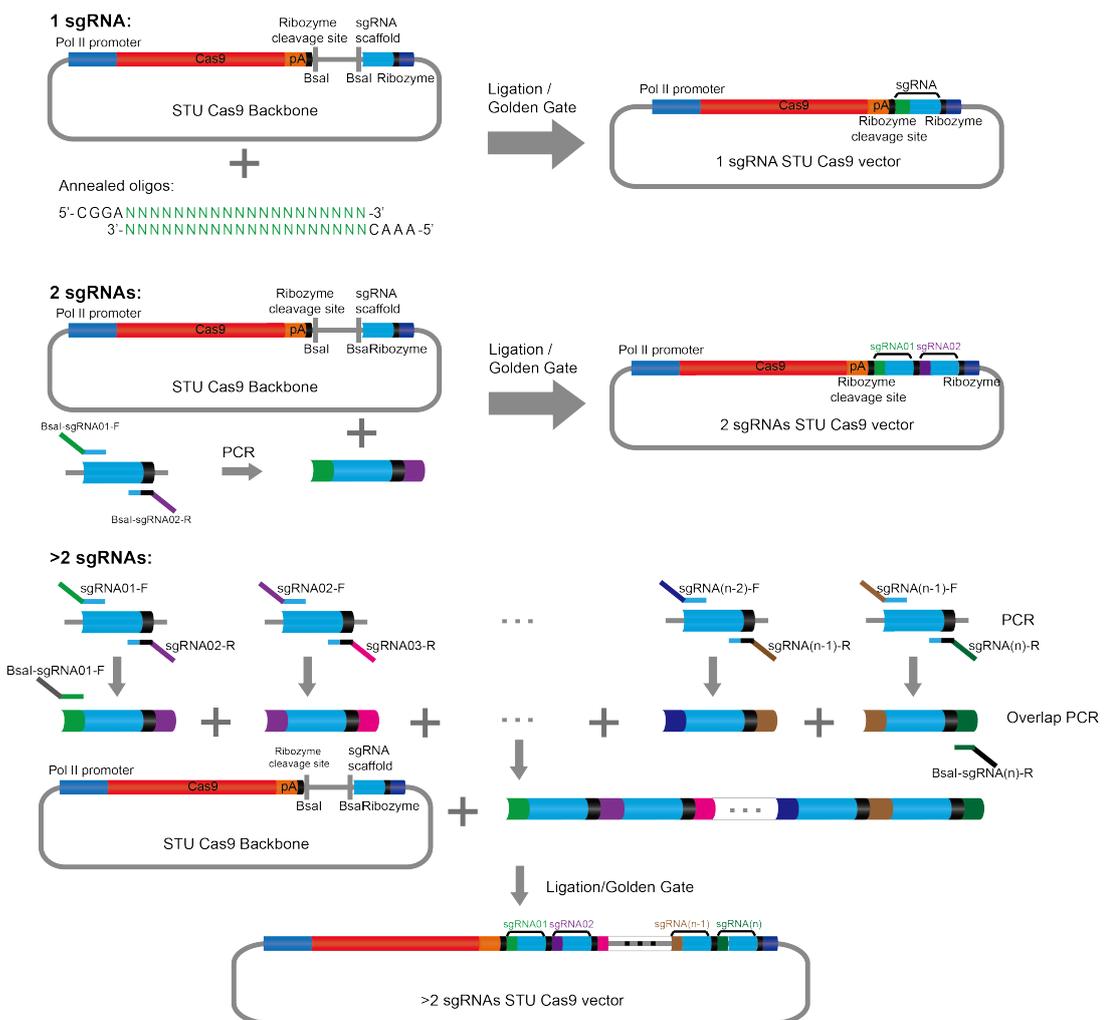


Figure 2. Schematic illustration of the cloning procedure described in the protocol

1. Cut and ligation

- a. Linearize the STU CRISPR-Cas9 plasmid pTX171 or pTX172 with *Bsa*I. Incubate at 37 °C for 2-4 h.

pTX171 or pTX172	2-3 µg
10x Cutsmart buffer	5 µl
<i>Bsa</i> I (10 U/µl)	1 µl
ddH ₂ O	to 50 µl

- b. Load digestion products onto a 1% agarose gel for electrophoresis. Purify the digested vector using the AxyPrep™ DNA Gel Extraction Kit, and quantify DNA concentration using NanoDrop.
- c. Ligate the diluted annealed oligos into linearized STU CRISPR-Cas9 expression vector, incubate at 16 °C overnight or room temperature for 1-2 h.

Linearized pTX171 or pTX172	50-75 ng
Diluted annealed oligos	2 µl
10x T4 DNA ligase buffer	2 µl
T4 DNA ligase (400 U/µl)	1 µl
ddH ₂ O	to 20 µl

- d. Transform 5 µl of the reaction into 50 µl competent DH5α cells, spread the transformed cells on LB plates supplemented with 50 mg/L kanamycin, and then incubate overnight at 37 °C.
- e. Verify the positive clones by colony PCR and Sanger sequencing.
2. Golden Gate method (Make sure annealed sgRNA oligos don't contain *Bsa*I site)

- a. Set up a Golden Gate reaction for cloning sgRNAs into the STU Cas9 expression vector.

pTX171 or pTX172 (100 ng/µl)	1 µl
Diluted annealed oligos	2 µl
10x T4 DNA ligase buffer	2 µl
T4 DNA ligase (400 U/µl)	1 µl
<i>Bsa</i> I (10 U/µl)	1 µl
ddH ₂ O	13 µl

- b. Incubate Golden Gate reactions in a thermal cycler using the following program: 10 cycles of 5 min at 37 °C and 10 min at 16 °C, then heat to 37 °C for another 5 min and 80 °C for 10 min.
- c. Transform 5 µl of the reaction into 50 µl competent DH5α cells, spread the transformed cells on LB plates supplemented with 50 mg/L kanamycin, and then incubate overnight at 37 °C.
- d. Verify the positive clones by colony PCR and Sanger sequencing. Colony PCR can be performed with the forward sgRNA oligonucleotide (e.g., see step A2) and ZY065-RB: (5'-ttctaataaacgctcttttct-3'). The expected product size is approximately 230 bp. ZY065-RB can be used for sequencing.

D. Two sgRNAs can be cloned into the STU CRISPR-Cas9 expression vector to target two sites simultaneously (Figure 2).

1. Design two primers as follows:

Bsal-sgRNA01-F: 5'-CAGGTCTCACGGA-N20-gttttagagctagaaatagcaagtaa-3'

Bsal-sgRNA02-R: 5'-TCGGTCTCCAAAC-N20-tccggtgacaaaagcaccga-3'

GGTCTC is the *Bsal* recognition sequence;

'N20' is same as the sgRNA01 target-specific sequence;

'N20' is the reverse complement of the sgRNA02 target-specific sequence;

The lowercase letters are complementary with the STU CRISPR-Cas9 expression vector.

Note: PAGE purified oligos are highly recommended, desalted is also OK.

2. Set up a 50 µl PCR reaction to amplify DNA for STU CRISPR-Cas9 expression vector construction.

5x Q5 reaction buffer	10 µl
10 mM dNTPs mixture	1 µl
<i>Bsal</i> -sgRNA01-F (10 µM)	1 µl
<i>Bsal</i> -sgRNA02-R (10 µM)	1 µl
STU CRISPR-Cas9 plasmid (1 ng/µl)	1 µl
Q5 High-Fidelity DNA polymerase (2 U/µl)	0.5 µl
ddH ₂ O	35.5 µl

3. Run PCR in a thermal cycler with the following program:

Step	Temperature	Time
Initial denaturation	98 °C	3 min
35 cycles	98 °C	30 sec
	56 °C	30 sec
	72 °C	30 sec
Final extention	72 °C	3 min
Hold	4-10 °C	

4. Load PCR products onto a 1% agarose gel for electrophoresis. Purify the PCR products using the AxyPrep™ DNA Gel Extraction Kit, and quantify DNA concentration using NanoDrop.

5. Set up a Golden Gate reaction for cloning two sgRNAs into the STU CRISPR-Cas9 expression vector.

pTX171 or pTX172 (100 ng/µl)	1 µl
Purified PCR products (10 ng/µl)	1 µl
10x T4 DNA ligase buffer	2 µl
T4 DNA ligase (400 U/µl)	1 µl
<i>Bsal</i> (10 U/µl)	1 µl
ddH ₂ O	14 µl

6. Incubate Golden Gate reactions in a thermal cycler using the following program: 10 cycles of 5 min at 37 °C and 10 min at 16 °C, then heat to 37 °C for other 5 min and 80 °C for 10 min.

7. Transform 5 µl of the reaction products into 50 µl competent DH5α cells, spread the transformed cells on LB plates supplemented with 50 mg/L kanamycin, and then incubate overnight at 37 °C.

8. Verify the positive clones by colony PCR and Sanger sequencing.

E. Our STU CRISPR-Cas9 system has the potential for multiplex sites genome editing. For more than two sites within one STU CRISPR-Cas9 vector, two-round PCR could be performed to clone different sgRNAs into the expression vector (Figure 2).

1. Design primers as follows:

Bsal-sgRNA01-F: 5'-CAG**GGTCTC**ACGGA-N20-3'

sgRNA01-F: 5'-N20-gttttagagctagaaatagcaagttaa-3'

sgRNA02-F: 5'-N20-gttttagagctagaaatagcaagttaa-3'

sgRNA02-R: 5'-N20-tccggtgacaaaagcaccga-3'

...

sgRNA(n-1)-F: 5'-N20-gttttagagctagaaatagcaagttaa-3'

sgRNA(n-1)-R: 5'-N20-tccggtgacaaaagcaccga-3'

sgRNA(n)-R: 5'-N20-tccggtgacaaaagcaccga-3'

Bsal-sgRNA(n)-R: 5'-TC**GGTCTC**CAAAC-N20-3'

GGTCTC is the *Bsal* recognition sequence;

'N20' is same as the target-specific sequence;

'N20' is the reverse complement of the target-specific sequence;

The different colors represent different target-specific sequences (e.g., N20, N20, N20, N20).

The lowercase letters are complementary with the STU CRISPR-Cas9 expression vector.

2. Set up 1st round PCR reactions to amplify sgRNAs fragments with ribozyme cleavage site flanked using primer pairs (sgRNA01-F/sgRNA02-R, sgRNA02-F/sgRNA03-R...sgRNA(n-1)-F/sgRNA(n)-R).

5x Q5 reaction buffer	10 µl
10 mM dNTPs mixture	1 µl
sgRNA01-F (10 µM)	1 µl
sgRNA02-R (10 µM)	1 µl
STU CRISPR-Cas9 plasmid (1 ng/µl)	1 µl
Q5 High-Fidelity DNA polymerase (2 U/µl)	0.5 µl
ddH ₂ O	35.5 µl

3. Run PCR in a thermal cycler with the following program:

Step	Temperature	Time
Initial denaturation	98 °C	3 min
35 cycles	98 °C	30 sec
	56 °C	30 sec
	72 °C	30 sec
	72 °C	3 min
Final extension	72 °C	3 min
Hold	4-10 °C	

4. Load PCR products onto a 1% agarose gel for electrophoresis. Purify the PCR products using the AxyPrep™ DNA Gel Extraction Kit, and quantify DNA concentration using NanoDrop.

5. Set up 2nd round PCR reaction to link the different sgRNAs with ribozyme cleavage site into one fragment.

5x Q5 reaction buffer	10 µl
10 mM dNTPs mixture	1 µl
<i>Bsa</i> I-sgRNA01-F (10 µM)	1 µl
<i>Bsa</i> I-sgRNA(n)-R (10 µM)	1 µl
Fragment 1 (with sgRNA01 and sgRNA02) (20 ng/µl)	1 µl
Fragment 2 (with sgRNA02 and sgRNA03) (20 ng/µl)	1 µl
...	...
Fragment n (with sgRNA n-1 and sgRNA n) (20 ng/µl)	1 µl
Q5 High-Fidelity DNA polymerase (2 U/µl)	0.5 µl
ddH ₂ O	Up to 50µl

6. Run PCR in a thermal cycler with the following program:

Step	Temperature	Time
Initial denaturation	98 °C	3 min
35 cycles	98 °C	30 sec
	56 °C	30 sec
	72 °C	30 sec
Final extention	72 °C	3 min
Hold	4-10 °C	

7. Load PCR products onto a 1% agarose gel for electrophoresis. Purify the PCR products using the AxyPrep™ DNA Gel Extraction Kit, and quantify DNA concentration using NanoDrop.
8. Set up a Golden Gate reaction for cloning multiplex sgRNA Fragment into the STU CRISPR-Cas9 expression vector.

pTX171 or pTX172 (100 ng/µl)	1 µl
Purified PCR products (10 ng/µl)	1 µl
10x T4 DNA ligase buffer	2 µl
T4 DNA ligase (400 U/µl)	1 µl
<i>Bsa</i> I (10 U/µl)	1 µl
ddH ₂ O	14 µl

9. Incubate Golden Gate reactions in a thermal cycler using the following program: 10 cycles of 5 min at 37 °C and 10 min at 16 °C, then heat to 37 °C for other 5 min and 80 °C for 10 min.
10. Transform 5 µl of the reaction products into 50 µl competent DH5α cells, spread the transformed cells on LB plates supplemented with 50 mg/L kanamycin, and then incubate overnight at 37 °C.
11. Verify the positive clones by colony PCR and Sanger sequencing.

Data analysis

Examples of STU CRISPR-Cas9 system application including gene editing and gene deletion with sequencing data in rice, tobacco and *Arabidopsis* can be found in the original paper (Tang *et al.*, 2016; [Link to paper](#)). Additionally, diagrams of the procedure, as well as examples of genome editing and sgRNA multiplex construction, can also be found in the original research paper (Tang *et al.*, 2016).

Notes

1. If failed to get colony using Golden Gate method, increase the number of cycles to 15-20 times.
2. We tested the STU CRISPR-Cas9 system in several organisms (including rice, tobacco and *Arabidopsis*) and successfully achieved efficient genome editing.

Recipes

1. 50x TAE electrophoresis buffer
242 g/L Tris
57.1 ml/L acetic acid
100 ml/L 0.5 M EDTA (pH 8.0)
2. LB medium
10 g/L tryptone
10 g/L NaCl
5 g/L yeast extract

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