

Precut pCas-Guide Cloning kit and pCas-Guide Plasmid

Application Guide

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I. pCas-Guide cloning kit (SKU GE100001)

Package contents

The following components are included:

- One (1) vial of precut pCas-Guide plasmid DNA (SKU GE100001V), lyophilized ready for ligation (10 RXNs). Reconstitute in 10 μ L dH₂O, final concentration 10 ng/ μ L.
- One (1) vial of forward sequencing primer, CF3 (SKU GE100008) to sequence the targeting sequence cloned into pCas-Guide vector; 100 picomoles dried onto the bottom of screw cap tubes. Reconstitute the primer in 10 μ L dH₂O to make a 10 μ M solution.
- One (1) vial of annealing buffer (SKU GE100007) for oligo annealing (10X), 200 μ L
- Certificate of Analysis
- Application Guide

The cDNA clone is shipped at room temperature, but should be kept at -20°C for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

Nuclease free water

T4 DNA ligase and buffer

Competent *E. coli* cells

LB agar plates with ampicillin, 100 μ g/mL

LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)

DNA purification reagents

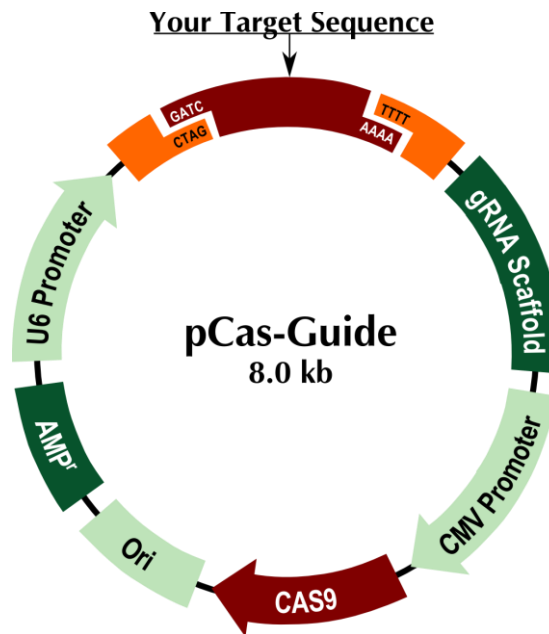
Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund by contacting customer service at custsupport@origene.com.

Production and Quality Assurance

The pre-cut pCas-Guide vector has been tested to successfully religate to annealed oligo DNA fragments. When OriGene experimental protocol is followed (details on page 5-7), 1 μ L of the ligation reaction generated with this pre-cut pCas-Guide vector can produce 100 colonies when transformed into 10^6 cfu/ μ g competent cells. The self-ligation background (vector religating to itself without an insert) is less than 5% of transformants. The amount of digested DNA provided in the kit is sufficient for ten ligation reactions.

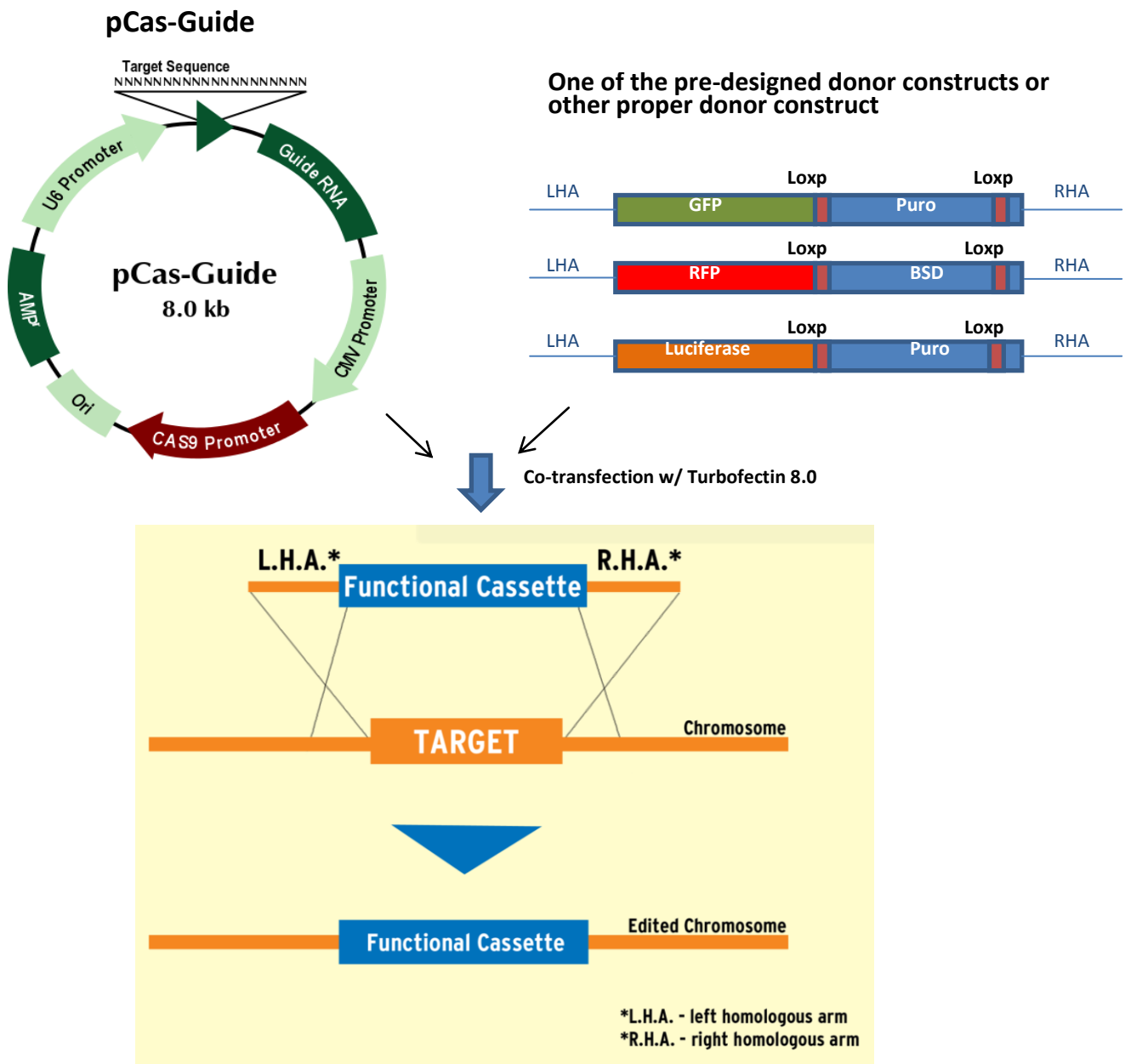
Figure 1. The vector map of pre-cut pCas-Guide.



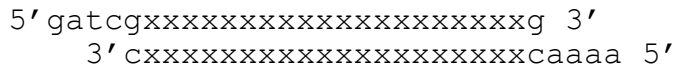
Introduction

Cas9 based genome editing has become a popular tool for targeted genome manipulation because of its simplicity and high cutting efficiency. This system requires a functional cas9 protein and a guide RNA for effective double-stranded breakage at a desired site. OriGene has developed the pCas-Guide system, a dual-function vector with both guide RNA and Cas9 expression. OriGene also designed a set of donor cassettes for construction of donor vectors. These include Luciferase-Loxp-Puro-Loxp, tGFP-Loxp-Puro-Loxp and tRFP-Loxp-BSD-Loxp.

Figure 2. Flow chart of genome editing using Cas9/CRISPR.



The two oligos should anneal to form the following double strand:



4. Order the two final oligos from a commercial oligo provider, such as IDT.

III. Cloning the double-stranded oligos into the pCas-Guide vector

1. Anneal the oligos to form double-stranded duplexes

In a PCR tube, add the following:

- 2 μ L Forward oligo (100 μ M stock)
- 2 μ L Reverse oligo (100 μ M stock)
- 4 μ L 10X annealing buffer
- 32 μ L dH₂O

Mix the solution and follow the steps to anneal the oligos in a PCR machine:

- 94°C for 4min
- 75°C for 5 min
- 65°C for 15 min
- 25°C for 20 min

After annealing, transfer the solution to a 1.5 mL tube and add 360 μ L of dH₂O.
The double-stranded oligo DNA is ready for ligation.

2. Ligation and transformation

A. Prepare the ligation according to the following protocol

Component	Volume
10x Ligation buffer	1 μ L
Precut pCAS-Guide vector (10 ng/ μ L)	1 μ L
Annealed double-stranded oligos (diluted from step 1)	1 μ L
Ligase (0.5 u/ μ L, Weiss unit)	0.5 μ L
dH ₂ O	6.5 μ L
Total Volume	10 μL

B. Mix the solution and incubate the tube at 22 to 37°C or room temperature for two hours according to the manufacturer's recommendation.

C. Add 1 μ L of the ligation mixture to 10 μ L of competent cells (efficiency rated > 10⁶ cfu/ μ g DNA) on ice. Do the transformation according to the manufacturer's protocol. For chemically competent cells, follow steps D-E.

D. Mix the tube gently and keep it on ice for 25 minutes.

E. Heat shock the tube for 30 seconds at 42°C.

- F. Put the tube on ice for 2 minutes, then add 500 μ L LB or SOC medium.
- G. Rock the tube gently at 37⁰C for 1 hour.
- H. Spread 50 μ L of the *E. Coli* cells on an LB ampicillin-agar plate.
- I. Centrifuge the remaining *E. Coli* cells at 5K rpm for 5 minutes. Discard the majority of the supernatant (around 50 μ L supernatant left) and resuspend the cell pellet in the remaining liquid. Spread all the *E. Coli* cells on a separate LB ampicillin-agar plate.
- J. Incubate the two plates at 37⁰C for 16 hours to allow colony formation.

3. Screening colonies

In a typical subcloning ligation, at least 95% of the colonies should contain the desired insert. Pick 6 to 10 colonies into 5 mL LB-ampicillin culture each, and culture overnight. Perform DNA purification using a mini-prep kit from OriGene, http://www.origene.com/Other/Plasmid_Purification/. Sequence the purified DNA and analyze the sequencing data to identify a correct clone for proper insert identification and orientation.

Troubleshooting

Problem: Few or no colonies obtained

Cause/Solution: Oligo design error. Please check the sequences of the two oligos. Make sure they can anneal and form the required sticky ends.

Cause/Solution: The ligase may not work properly. Perform the troubleshooting as recommended by the manufacturer of the ligase.

Cause/Solution: Competent cells have lost transformation efficiency. Evaluate the transformation efficiency using the control DNA provided with your kit.

II. pCas-Guide plasmid (SKU GE100002)

Package contents

- One (1) vial of pCas-Guide plasmid DNA, 10 μg (SKU GE100002V), lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 $\text{ng}/\mu\text{L}$.
- One (1) vial of CF3 sequencing primer (SKU GE100008) to sequence the targeting sequence cloned into pCas-Guide vector; 100 picomoles dried onto the bottom of screw cap tubes. Reconstitute the primer in 10 μL dH_2O to make a 10 μM solution.
- Certificate of Analysis
- Application Guide

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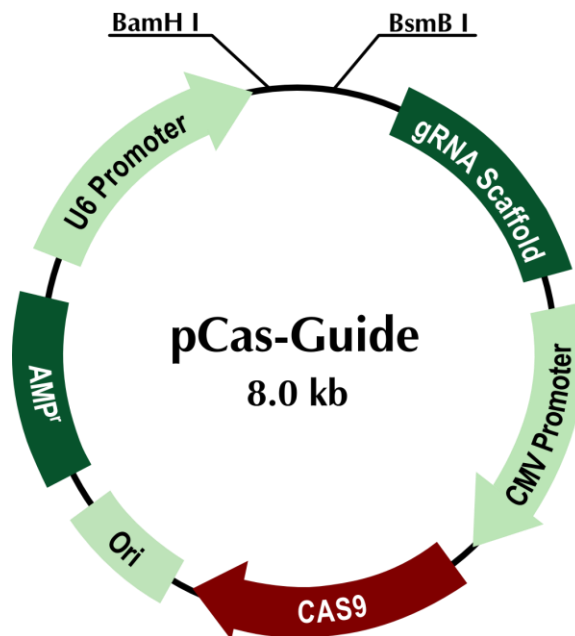
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Product Description

The pCas-Guide vector is designed for cloning a guide RNA insert for genome editing purpose. Target sequence can be cloned into the vector via BamHI and BsmB I sites. The vector also expresses a CMV-driven codon-optimized Cas9. When co-transfected with a proper donor DNA, targeted genome editing can be achieved. The vector retains the ampicillin resistance gene for the selection of *E. coli* transformants.

Figure 3. The vector map of pCas-Guide plasmid.



Experimental protocol

1. Digest pCas-Guide plasmid with BamH I and BsmB I

Resuspend the 10 µg lyophilized DNA in 100 µL dH₂O, and incubate for at least 30 min before use. Set up a digestion reaction as described below.

<u>Component</u>	<u>Volume</u>
10X restriction buffer	3 µL
BamH I	0.8 µL
BsmB I	0.8 µL
Nuclease free water	15.4 µL
<u>Vector DNA</u>	<u>10 µL</u>
Total volume	30 µL

Incubate the reaction at 37°C for 3 hrs, then add 1 µL antarctic phosphatase (units used according to the manufacturer's protocol), and continue the incubation at 37°C for another 30 min. Dephosphorylation of the digested vector is essential to eliminate self-ligation. Purify the desired vector fragment by running the digestion reaction on an agarose gel, and isolate the appropriate band using a gel purification column. Elute the digested plasmid vector in 40 µL of 10 mM Tris buffer.

2. Target sequence design and cloning into the precut-pCas-Guide vector, please follow the detailed protocol from page 5-7 in this manual.

FAQ

Question: a 20bp target sequence is needed with a NGG PAM seq. Shall the NGG be exactly immediately following the 3' of this 20bp sequence?

Answer: Yes the NGG is located immediately next to the 3' end of the 20bp sequence.

Question: How to design the 20bp target-specific sequence?

Answer: The 20bp target-specific sequence should proceed NGG (PAM). Please BLAST the seed region (12bp PAM-proximal) of the 20bp target sequence to make sure it's unique along the genome to guarantee specificity. Seed-region , 5'-NNNNNNNNNNNN-NGG-3', is specific to the relevant genome. Do not selection the sequence has less than 3bp mismatch at the seed region and follows with NGG or NAG.

Question: How many target RNA sequence should I use for a genome editing project?

Answer: Due to un-predicable nature of gRNA, we recommend 2 and more gRNA targeting sequences to be designed to make sure that at least you will have one targeting sequence that works.

Question: How to analyze genome editing if the donor sequence does not have a fluorescence protein marker or antibiotic selection marker?

Answer: You can use WB if the gene encodes a protein that can be distinguished from the endogenous protein. You can also use junction PCR to detect the donor sequence, one primer in the donor sequence and one primer in the region downstream of the donor sequence.

Question: How to address the off-target effect of pCas9 system?

Answer: When designing the target sequence, make sure to blast the sequence that there is no matching sequences containing less than 3 mismatches proximal PAM. The other sequences should not precede NGG or NAG.

Question: What is the sequence of CF3 sequencing primer?

Answer: 5'-ACGATACAAGGCTGTTAGAGAG-3'

Question: What is your validation data for your pCas-Guide system?

Answer: Please see the downloadable validation data at www.origene.com/Cas9

Question: What is the scrambled sequence in pCas-Scramble?

Answer: 5' GCACTACCAGAGCTAACTCA 3'

Question: Do you provide gRNA cloning service and donor vector service?

Answer: Yes, you can order them through OriGene's gene synthesis company