CRISPR/Cas9 Genome Editing

Application Guide

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I. pCas-Guide precut 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 µL dH_2O, final concentration 10 ng/µL.
- 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 µL dH_2O to make a 10 µM solution.
- One (1) vial of annealing buffer (SKU GE100007) for oligo annealing (10X), 200 µL
- Certificate of Analysis
- Application Guide

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit).*

The cDNA clone is shipped at room temperature, but should be kept at -20℃ 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 µg/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

Related OriGene Products

- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes (custom service)
  - GFP-CMV-LoxP-Puro-LoxP
  - RFP-CMV-LoxP-BSD-LoxP
  - Luciferase-CMV-LoxP-Puro-LoxP
- TrueClone™ FL cDNA clones
- HuSH™ shRNA Plasmids
- Validated Antibodies
- Purified Proteins
- Over-expression lysates
- Transfection Reagents
- Anti-tag Antibodies

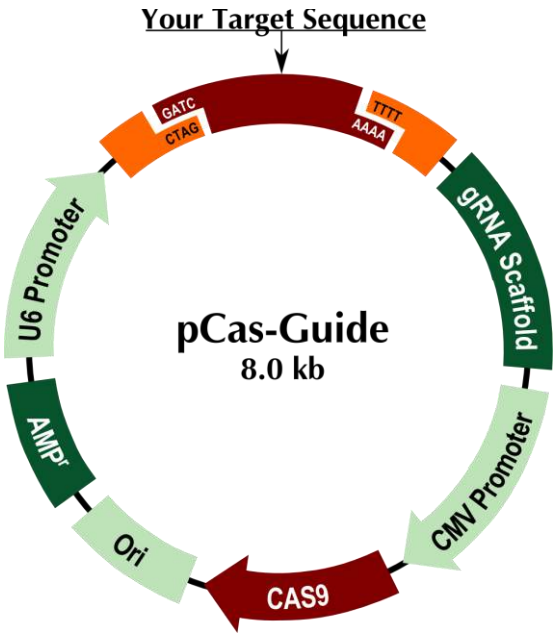
Notice to purchaser

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 precut pCas-Guide vector has been tested to successfully religate to annealed oligo DNA fragments. When OriGene experimental protocol is followed (details on page 6-8), 1 μL of the ligation reaction generated with this precut pCas-Guide vector can produce 100 colonies when transformed into 10^6 cfu/μg 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.

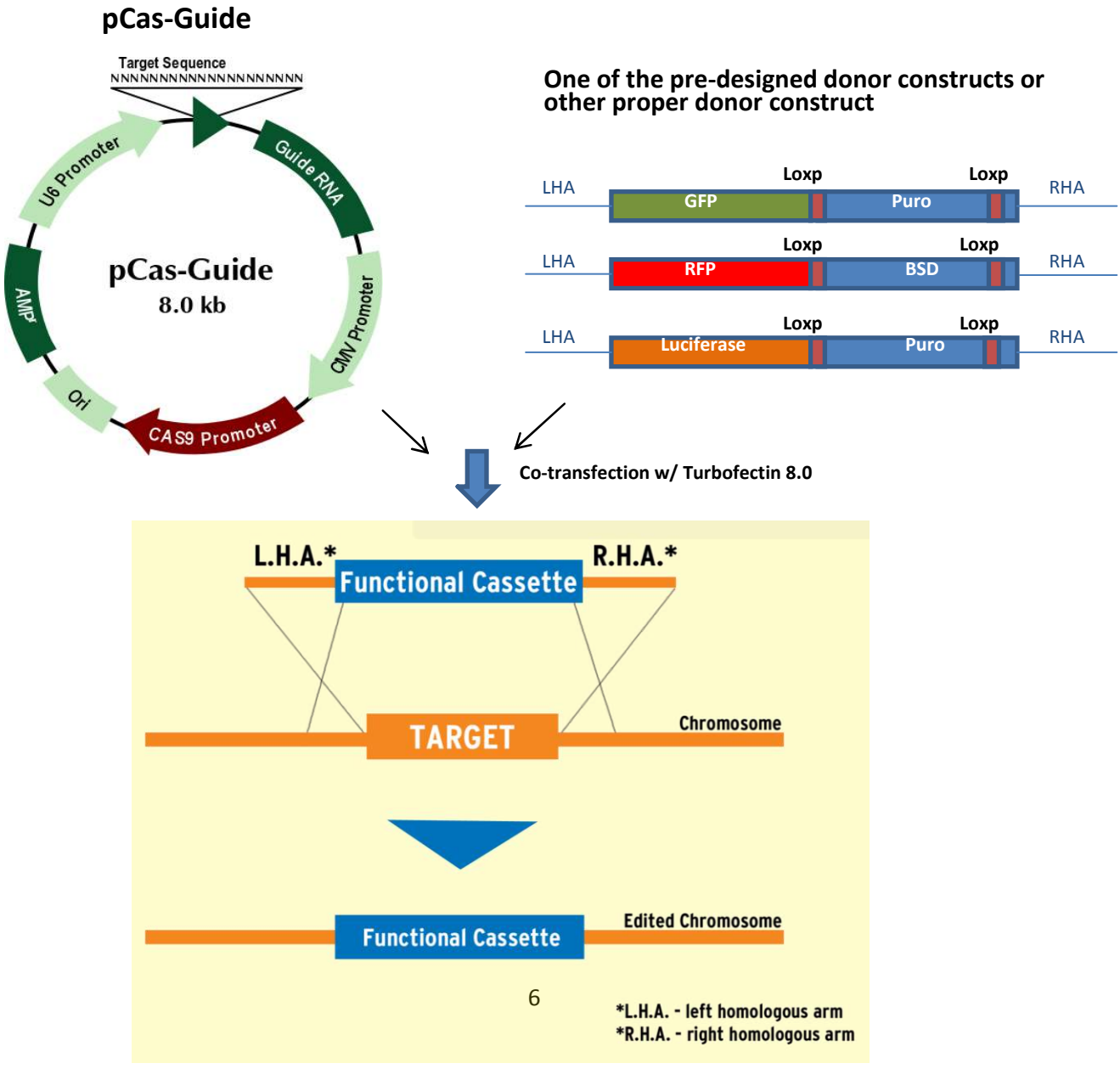
Figure 1. The vector map of pre-cut pCas-Guide.



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.



Product Description

The pCas-Guide vector is designed for cloning a guide RNA insert for genome editing purpose. The vector also has 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. The vector is supplied as a precut vector, ready for insert ligation. This system has been successfully validated in multiple cases of genome editing.

Experimental Protocols

I. Design target sequence

OriGene has developed a proprietary Cas9 guide RNA designing tool which is free to use, http://www.blueheronbio.com/. Follow the instructions below to design your guide RNA:

1. Select your desired Cas9 cutting site from your genomic region of interest.
2. Copy around 100 bp of genomic sequence flanking the cutting site (-50 to +50). Paste the sequence to the sequence box and click the Search button.
3. The program will return all possible targeting sequences with location and GC content obtained from searching both the plus and minus strands. If there is no target returned, expand your genomic region of interest (-100 to +100) and search again until there is a positive return.
4. Select a few target sequences to Blast against the genomic DNA database to check sequence specificity.
5. Select 2 to 3 target sequences to clone into pCas-Guide vector.

II. Addition of extra bases to the ends of the target sequence

To facilitate cloning of the 20-bp target sequence, extra bases need to be added to the ends.

1. Select a desired 20-bp sequence as a target. The following is an example sequence:

   Forward sequence: 5′ ATGGGAGGTGGTATGGGAGG 3′
   Reverse complement sequence: 5′ CCTCCCATACCACCTCCCAT 3′

2. Add ‘gatcg’ to the 5’ end of the forward sequence and ‘g’ to its 3’ end. The final sense oligo in this example will be

   5’ gatcgATGGGAGGTGGTATGGGAGGg 3’

3. Add ‘aaaac’ to the 5’ end of reverse complementary sequence and ‘c’ to its 3’ end. The final reverse complementary sequence is

   5’ aaaacCCTCCCATACCACCTCCCATc 3’

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

```
5′gatcgxxxxxxxxxxxxxxxxxxxg 3′
    3′cxxxxxxxxxxxxxxxxxxxxcaaaa 5′
```

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 µL Forward oligo (100 µM stock)
   2 µL Reverse oligo (100 µM stock)
   4 µL 10X annealing buffer
   32 µL dH_2O
   Mix the solution and follow the steps to anneal the oligos in a PCR machine:
   94^0C for 4min
   75^0C for 5 min
   65^0C for 15 min
   25^0C for 20 min
   After annealing, transfer the solution to a 1.5 mL tube and add 360 µL of dH_2O.
   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 µL
Precut pCAS-Guide vector (10 ng/ µL)	1 µL
Annealed double-stranded oligos (diluted from step 1)	1 µL
Ligase (0.5 u/ µL, Weiss unit)	0.5 µL
dH_2O	6.5 µL
Total Volume	10 µL

   B. Mix the solution and incubate the tube at 22 to 37^0C or room temperature for two hours according to the manufacturer's recommendation.

   C. Add 1 µL of the ligation mixture to 10 µL of competent cells (efficiency rated > 10^6 cfu/µg 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 42^0C.

F. Put the tube on ice for 2 minutes, then add 500 µL LB or SOC medium.

G. Rock the tube gently at 37^{0}C for 1 hour.

H. Spread 50 µL 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 µL 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 37^{0}C 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 PowerPrep mini-prep kit. Sequence the purified DNA and analyze the sequencing data to identify a correct clone for proper insert identification and orientation.

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 ng/ µL.
- 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

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit).*

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

Related Optional Reagents

- Nuclease free water
- Oligo annealing buffer, SKU GE100007
- BamH I and BsmB I
- T4 DNA ligase and buffer
- Competent *E. coli* cells
- LB agar plates with ampicillin, 100 μg/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

Related OriGene Products

- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes (custom service).

  GFP-CMV-LoxP-Puro-LoxP
  RFP-CMV-LoxP-BSD-LoxP
  Luciferase-CMV-LoxP-Puro-LoxP

- TrueClone™ FL cDNA clones
- HuSH™ shRNA Plasmids
- Validated Antibodies
- Purified Proteins
- Over-expression lysates
- Transfection Reagents
- Anti-tag Antibodies

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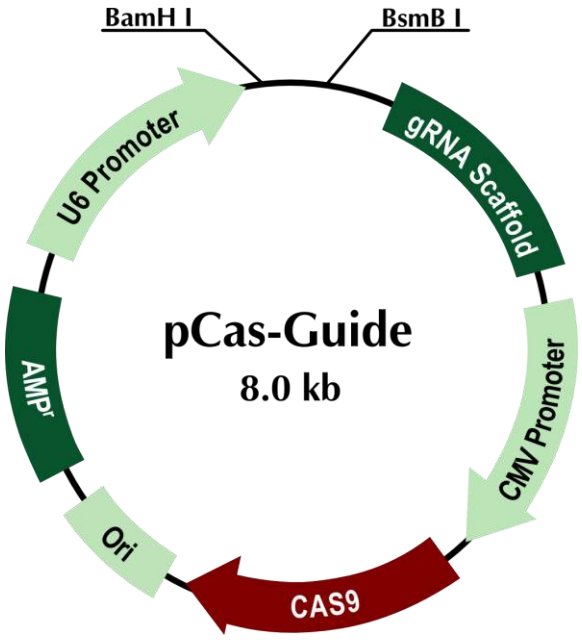
Product Description

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 BamH I and BsmB I sites. The vector also expresses a CMV-driven codon-optimized Cas9. When co-transfected with a proper donor DNA, your desired 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.

Component	Volume
10X restriction buffer	3 µL
BamH I	0.8 µL
BsmB I	0.8 µL
Nuclease free water	15.4 µL
Vector DNA	10 µL
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 37ºC 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 designing and cloning into the precut pCas-Guide vector, please follow the detailed protocol from page 7-9 in this manual.

III. pLenti-Cas-Guide precut cloning kit (SKU: GE100009)

Package contents

The following components are included:

- One (1) vial of precut pLenti-Cas-Guide plasmid DNA (SKU GE100009V), lyophilized ready for ligation (10 RXNs). Reconstitute in 10 µL dH_2O, final concentration 10 ng/ µL.
- One (1) vial of forward sequencing primer, CF3 (SKU GE100008) to sequence the targeting sequence cloned into pLenti-Cas-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.
- One (1) vial of annealing buffer (SKU GE100007) for oligo annealing (10X), 200 µL
- Certificate of Analysis
- Application Guide

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit*).

The cDNA clone is shipped at room temperature, but should be kept at -20℃ 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 chloramphenicol (34 µg/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

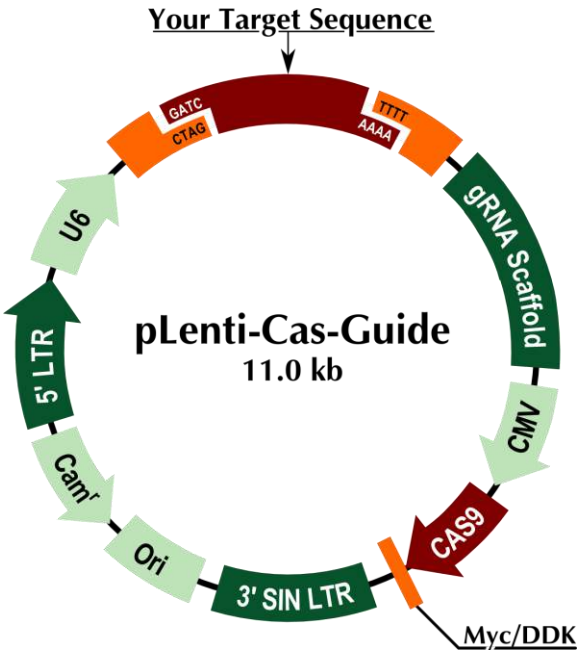
Related OriGene Products

- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes (custom service).
  - GFP-CMV-LoxP-Puro-LoxP
  - RFP-CMV-LoxP-BSD-LoxP
  - Luciferase-CMV-LoxP-Puro-LoxP

Product Description

The pLenti-Cas-Guide vector is designed for cloning a guide RNA insert for genome editing purpose. pLenti-Cas-Guide vector is also a 3rd generation lentiviral vector that you can generate lentiviral particles to infect your hard to transfect cells including primary and stem cells. The target sequence can be cloned into the vector via BamH I and BsmB I sites. The vector also expresses a CMV-driven codon-optimized Cas9, C-terminal Myc-DDK tagged. When co-transfected with a proper donor DNA, your desired targeted genome editing can be achieved. The vector retains the chloramphenical resistance gene for the selection of *E. coli* transformants.

Figure 4. Vector map of precut pLenti-Cas-Guide



Experimental protocol

1. Target sequence designing and cloning into the precut pLenti-Cas-Guide vector, please follow the detailed protocol from page 7-9 in this manual.

2. pLenti-Cas-Guide vector with the target sequence cloned can be used as a regular expression plasmid for transient transfection. You can also make lentiviral particles using a lentiviral packaging mix (cat. TR30022); you can then infect your hard to transfect cells including primary cells and stem cells, etc.

Lenti-based protocols:

NOTE: Performing Lentiviral experiments REQUIRES special laboratory conditions and/or permissions (BL2). Follow the guidelines and regulations of your institution. Perform the experiments with due caution to avoid exposure to infectious materials.

A. Production of pseudovirus (10 cm plate format, the production size can be scaled up or down accordingly):

1. Day 1, plate HEK293T cells in a 10 cm dish to approximately 40% confluency the day before transfection. Cells should reach 65-70% confluency within 24 hours.

2. Day 2
    a. Re-suspend the Lenti plasmid in 100 µl dH2O to obtain a final concentration 100 ng/µl. Label a sterile 1.5 mL eppendorf tube and to the tube, add 50 µl of the Lenti plasmid (5 µg) and 50 µl of the packaging plasmids (6 µg) from the Lenti-vpak packaging kit, cat# TR30022.
    b. Add 110 µl Opti-MEM (Invitrogen) to the tube. Mix the DNA by gently vortexing. Add 44 µl of MegaTran 1.0 (or other transfection reagent) to the DNA tube. Mix the solution by gently vortexing.
    c. Set the DNA tube inside a cell culture hood for 20 mins.
    d. Transfer the DNA:transfection solution to the 10 cm plate prepared the day before by gentle and even dropping. Gently rock the plate back-and-forth and from side-to-side to achieve even distribution of the transfection complex. Incubate the plate in a CO2 incubator.

3. Day 3, change the growth medium and continue to incubate the plate for 48 hours.

4. Day 5
    a. After the 48 hour incubation, transfer the cell culture supernatant to a 15 mL centrifuge tube.
    b. Centrifuge the tubes at 3K RPM for 10 mins and filter the supernatant through a syringe filter (0.45 micron) and collect the viral solution to a new sterile tube.

5. The viral particles are ready to be used. They can be stored at 4 ^{o}C for 2 weeks or put at -80 ^{o}C for long-term storage.

B. Transduction of lentivirus to target cells

1. Day 1, plate target cells in three 10 cm plates at a density that will produce approximately 60% confluency in 24 hours. Note: other size formats can also be used depending on the nature of your experiment. Adjust the reagent amount accordingly.

2. Day 2, Remove the growth media from the plates prepared the day before. To plate 1, add 4.5 mL of fresh growth medium and 0.5 mL of Lentiviral particles; To plate 2, add 4.0 mL of growth medium and 1 mL of Lentiviral particles; To plate 3, add 2.5 mL of

growth medium and 2.5 mL of Lentiviral particles (for a low titer viral preparation, the amount of virus added can be increased to 5 mL). Mix the solution by gentle swirling.

3. Add 5 µl polybrene (1,000x, 8 mg/mL) to each plate. Mix by gentle swirling.

4. Incubate the cells at 37 ^{o}C with 5% CO2 for 4 hours. Remove the transduction medium and add 10 mL of fresh growth medium. Incubate the cells for three more days.

The transduced cells are ready for downstream analyses such as RNA and protein detection.

IV. pLenti-Cas-Guide plasmid (SKU: GE100010)

Package contents

- One (1) vial of circular pLenti-Cas-Guide plasmid DNA, 10 μg (SKU GE100010V), lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL.
- One (1) vial of CF3 sequencing primer (SKU GE100008) to sequence the targeting sequence cloned into pLenti-Cas-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

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit*).

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

Related Optional Reagents

- Nuclease free water
- Oligo annealing buffer, SKU GE100007
- BamH I and BsmB I
- T4 DNA ligase and buffer
- Competent *E. coli* cells,
- LB agar plates with LB_Chloramphenicol (34 µg/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

Related OriGene Products

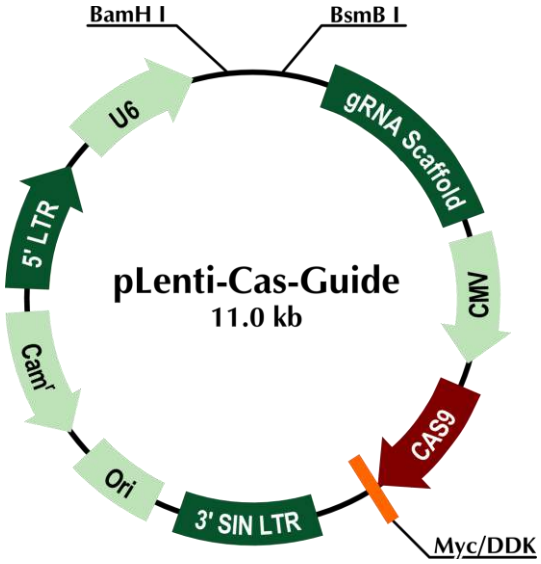
- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes (custom service).

  GFP-CMV-LoxP-Puro-LoxP
  RFP-CMV-LoxP-BSD-LoxP
  Luciferase-CMV-LoxP-Puro-LoxP

Product Description

The pLenti-Cas-Guide vector is designed for cloning a guide RNA insert for genome editing purpose. Target sequence can be cloned into the vector via BamH I and BsmB I sites. The vector also expresses a CMV-driven codon-optimized Cas9, C-terminal Myc-DDK tagged. When co-transfected with a proper donor DNA, your desired targeted genome editing can be achieved. The vector retains the chloramphenicol resistance gene for the selection of *E. coli* transformants.

Figure 5. Plasmid map of pLenti-Cas-Guide



Experimental Protocol

1. Digest pLenti-Cas-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.

Component	Volume
10X restriction buffer	3 µL
BamH I	0.8 µL
BsmB I	0.8 µL
Nuclease free water	15.4 µL
Vector DNA	10 µL
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 37ºC 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 designing and cloning into the precut pLenti-Cas-Guide vector, please follow the detailed protocol from page 7-9 in this manual. Regarding lenti-based protocol, please refer to page 13-15 in this manual.

V. pCas-Guide-GFP precut cloning kit (SKU: GE100011)

Package contents

The following components are included:

- One (1) vial of precut pCas-Guide-GFP plasmid DNA (SKU: GE100011V), lyophilized ready for ligation (10 RXNs). Reconstitute in 10 µL dH_2O, final concentration 10 ng/ µL.
- One (1) vial of forward sequencing primer, CF3 (SKU GE100008) to sequence the targeting sequence cloned into pCas-Guide-GFP 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.
- One (1) vial of annealing buffer (SKU GE100007) for oligo annealing (10X), 200 µL
- Certificate of Analysis
- Application Guide

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit*).

The cDNA clone is shipped at room temperature, but should be kept at -20℃ 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 µg/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

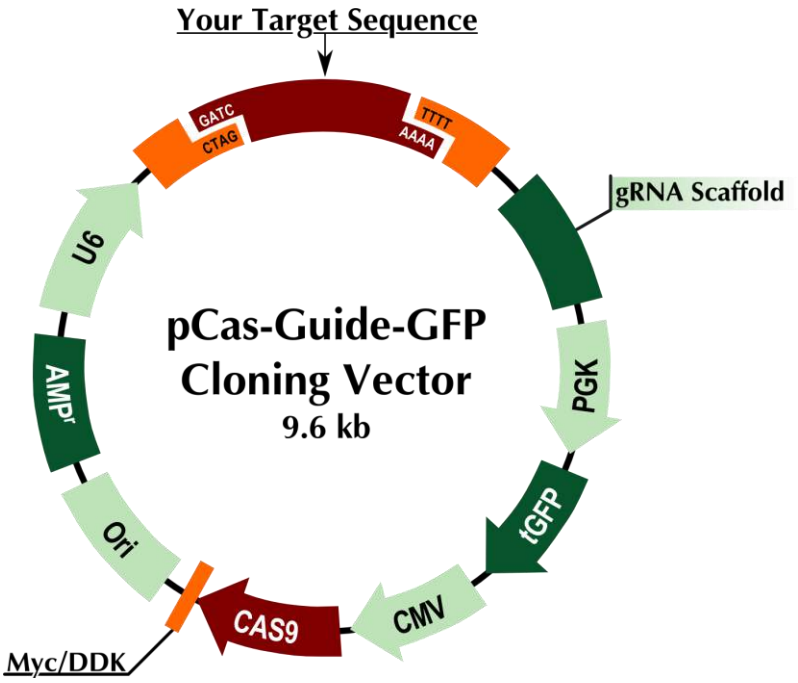
Related OriGene Products

- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes (custom service).
  - GFP-CMV-LoxP-Puro-LoxP
  - RFP-CMV-LoxP-BSD-LoxP
  - Luciferase-CMV-LoxP-Puro-LoxP

Product Description

The pCas-Guide-GFP vector is designed for cloning a guide RNA insert for genome editing purpose. The vector also expresses a CMV-driven codon-optimized Cas9, C-terminal Myc-DDK tagged. In addition, pCas-Guide-GFP vector also expresses PGK promoter driven GFP to monitor transfected cells. When co-transfected with a proper donor DNA, your desired targeted genome editing can be achieved. The vector retains the ampicillin resistance gene for the selection of *E. coli* transformants.

Figure 6. Vector map of precut pCas-Guide-GFP



Experimental protocol

Target sequence designing and cloning into the precut pCas-Guide-GFP vector is the same as the precut pCas-Guide vector; please follow the detailed protocol from page 7-9 in this manual.

VI. pCas-Guide-GFP plasmid (SKU: GE100012)

Package contents

- One (1) vial of circular pCas-Guide-GFP plasmid DNA, 10 μg (SKU: GE100012V), lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL.
- One (1) vial of CF3 sequencing primer (SKU GE100008) to sequence the targeting sequence cloned into pCas-Guide-GFP 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

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit).*

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

Related Optional Reagents

- Nuclease free water
- Oligo annealing buffer, SKU GE100007
- BamH I and BsmB I
- T4 DNA ligase and buffer
- Competent *E. coli* cells
- LB agar plates with ampicillin, 100 μg/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)
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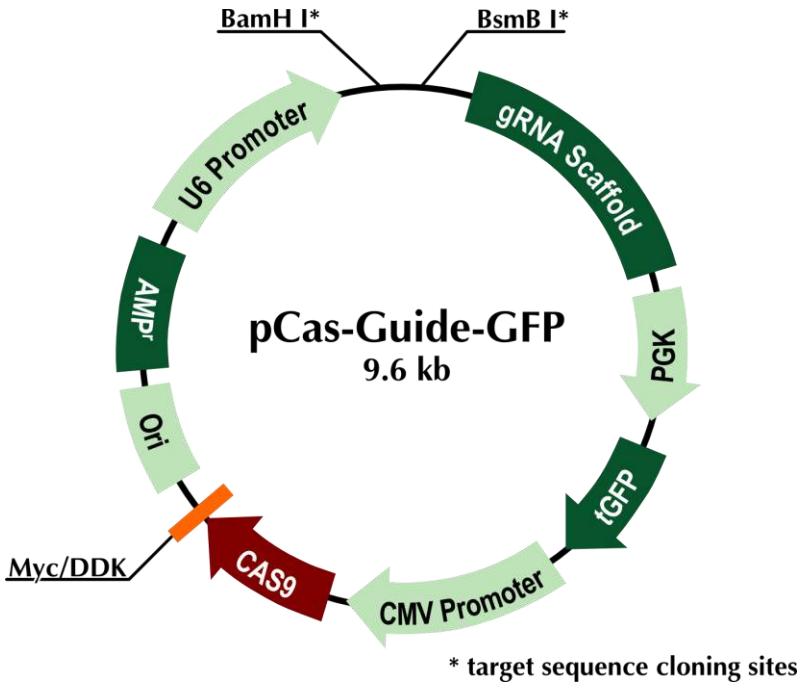
Figure 7. Plasmid map of pCas-Guide-GFP



Experimental Protocol

1. Digest pCas-Guide-GFP 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.

Component	Volume
10X restriction buffer	3 µL
BamH I	0.8 µL
BsmB I	0.8 µL
Nuclease free water	15.4 µL
Vector DNA	10 µL
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 37ºC 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 designing and cloning into the precut pCas-Guide-GFP vector, please follow the detailed protocol from page 7-9 in this manual.

VII. T7 driven CRISPR/Cas system-pT7-Guide (SKU: GE100013) and pT7-Cas9 (SKU: GE100014)

Package contents

- One (1) vial of circular pT7-Guide (SKU: GE100013) plasmid DNA or pT7-Cas9 (SKU: GE100014), 10 μg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL.
- Certificate of Analysis
- Application Guide

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit*).

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

Related Optional Reagents

- Nuclease free water
- Oligo annealing buffer, SKU GE100007
- BamH I and BsmB I
- T4 DNA ligase and buffer
- Competent *E. coli* cells
- LB agar plates with ampicillin, 100 μg/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
- T7 *In vitro* transcription kits

Related OriGene Products

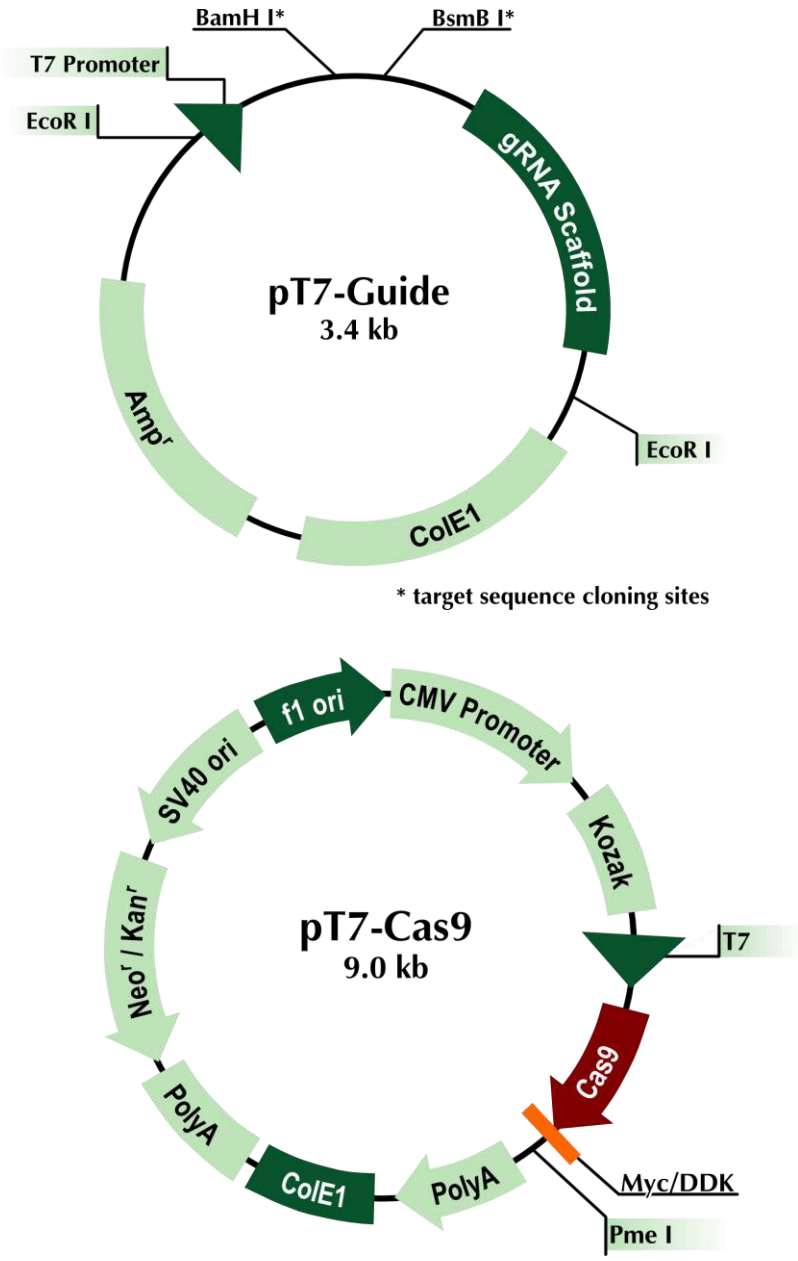
- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes (custom service).

  GFP-CMV-LoxP-Puro-LoxP
  RFP-CMV-LoxP-BSD-LoxP
  Luciferase-CMV-LoxP-Puro-LoxP

Product Description

To make gene knockout out animals, Cas9 mRNA and gRNA are often injected into the embryos; the T7 driven CRISP/Cas system will serve the purpose. pT7-Guide vector can be used to clone the target genomic sequence; the gRNA expression is under T7 promoter. Therefore gRNA can be produced using T7 *in vitro* transcription system. In pT7-Cas9 vector, Cas9 gene is under T7 promoter; therefore Cas9 mRNA can be produced using T7 *in vitro* transcription system.

Figure 8. Plasmid maps of pT7-Guide and pT7-Cas9



Experimental protocol

I. Design genomic target sequence and cloning into pT7-Guide vector

1. Digest pT7-Guide vector with BamH I and BsmB I restriction enzymes

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.

Component	Volume
10X restriction buffer	3 µL
BamH I	0.8 µL
BsmB I	0.8 µL
Nuclease free water	15.4 µL
Vector DNA	10 µL
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 37ºC 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 designing and cloning into the precut pT7-Guide vector, please follow the detailed protocol from page 7-9 in this manual.

3. Sequencing the cloned target sequence in pT7-Guide can be done by the common M13 forward primer: 5' CGCCAGGGTTTTCCCAGTCACGAC 3'

II. Producing gRNA and Cas9 mRNA using T7 in vitro transcription kits

To make gRNA using pT7-Guide after the genomic target sequence cloned, we recommend using MeGAshortscript T7 kit (Life Technologies) and follow the manufacturer's protocol. pT7-Guide vector can be linearized using EcoR I. EcoR I will cut T7 and gRNA out; but you don't need to purify the fragment. You only need to clean it using a PCR purification column. Then follow the MeGAshortscript T7 kit protocol to produce gRNA. To make Cas9 mRNA using pT7-CAs9, we recommend using mMESSAGE Mmachine T7 ULTRA kit (Life Technologies) and follow the manufacturer's protocol. pT7-Cas9 vector can be linearized using Pme I which is 3' end of Cas9 sequence. You can then clean up the Pme I digested reaction using a PCR purification column; then follow the mMESSAGE Mmachine T7 ULTRA kit protocol to produce capped and polyadenylated Cas9 mRNA.

VIII. Genome-Wide knockout kit using CRISPR

Package contents

- One (1) vial of gRNA vector 1 in pCas-Guide (SKU KN2xxxxxG1), lyophilized. Reconstitute in 10 µL dH_2O, final concentration 10 ng/ µL.
- One (1) vial of gRNA vector 2 in pCas-Guide (SKU KN2xxxxxG2), lyophilized. Reconstitute in 10 µL dH_2O, final concentration 10 ng/ µL.
- One (1) vial of donor vector containing left and right homologous arms and a GFP-puro functional cassette (SKU KN2xxxxxD), lyophilized. Reconstitute in 10 µL dH_2O, final concentration 10 ng/ µL.
- One (1) vial of negative scramble control vector (SKU GE100003), lyophilized. Reconstitute in 10 µL dH_2O, final concentration 10 ng/ µL.
- Certificate of Analysis
- Application Guide

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit).*

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

Related Optional Reagents

- LB agar plates with ampicillin, 100 µg/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)

Related OriGene Products

- Transfection reagents
- Reagents and supplies for immunoblots: user preferred. OriGene has a selection of antibodies and detection reagents.
- DNA purification reagents
- qPCR reagents
- CRISPR/Cas9 products

all are available at www.amsbio.com

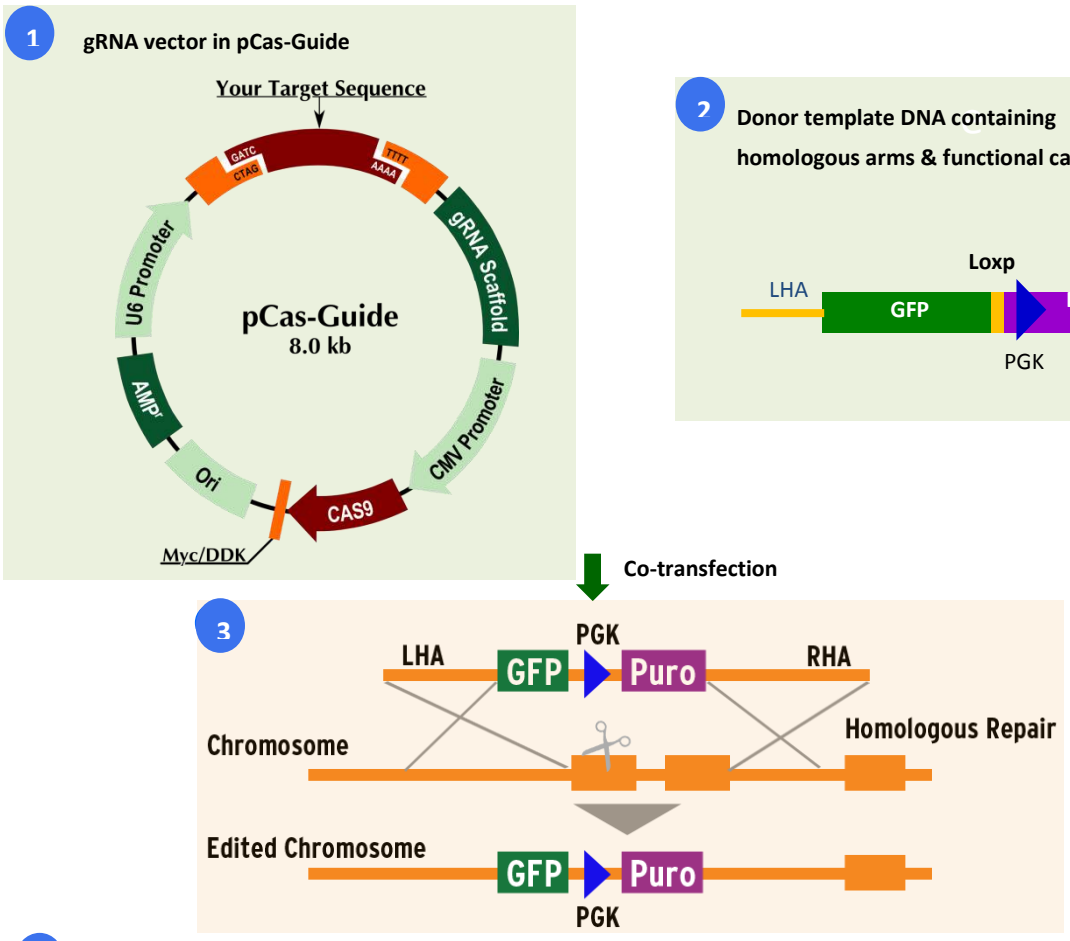
Notice to purchaser

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.

Product Description

Genome-editing knockout kit via CRISPR is a complete kit to knockout any human loci and knock in a functional cassette containing GFP and puromycin resistant gene. GFP will be under the native promoter after genome integration; puromycin resistant gene is under PGK promoter. gRNA vectors are provided in pCas-Guide vector with target sequence cloned. Both of the target sequences are at the 5' end of the ORF; therefore gRNA vectors will make a precise cleavage 5' end of the ORF in the genome. A negative scramble gRNA control is also provided.

Fig. 9. Scheme of genome-editing knockout kit



1 CRISPR/Cas cuts the double-stranded DNA at the targeting site

2 Donor template DNA provides the template for the homologous repair.

3 The functional cassette is incorporated into the genome when 1 + 2 are cotransfected.

Experimental Protocol

Each kit contains two gRNA vectors, one scramble negative control and one donor vector. Two gRNA constructs will increase the probability of achieving a high knockdown efficiency. A scrambled vector (negative) control will allow a convenient comparison between experiment group and control group.

A sample protocol listed below is for conducting a knockout experiment in a 6-well plate and using TurboFectin (cat# TF81001) as transfection reagent. If your experiments require other culture plates, just scale up or down the reagents accordingly based on the relative surface area of your plate (see Table 1 for details). Different cells may need different transfection reagents; if a different transfection reagent is used, please follow the manufacturer's protocol.

1. Approximately 18-24 hours before transfection, plate ~3 X 10^5 adherent cells in 2 ml culture medium into each well of a 6-well plate or ~5x10^5 suspension cells per well to obtain 50-70% confluence on the following day. The amount of cells may be different depending on the size of your cells.

2. Transfection in a complete culture medium.
   In a small sterile tube, combine the following reagents in the prescribed order. The order of reagent addition is important to achieve the optimal results.

   a. Dilute 1 µg of one of the gRNA vectors (or scramble control) in 250 uL of Opti-MEM I (Life Technologies). Vortex gently. Then add 1 µg of the donor DNA into the same 250 µL of Opti-MEM I. Vortex gently.
   b. Add 6 µL of Turbofectin 8.0 to the diluted DNA (not the reverse order) and pipette gently to mix completely.
   c. Incubate for 15 minutes at room temperature.

   Note: We recommend starting with the ratios of Turbofectin 8.0 and DNA listed in table 1; however, subsequent optimization may further increase the transfection efficiency.

   d. Add the mixture above drop-wise to the cells. Gently rock the plate back-and-forth and from side-to-side to distribute the complex evenly.
   e. Incubate the cells in a 5% CO_2 incubator.

3. 48hr post transfection, split cells 1:10, grow for additional 3 days; then split the cells again 1:5, grow for another 2 days.

4. Apply puromycin selection. Split cells 1:10, then grow cells directly in the puromycin containing complete media. The dose of puromycin needs to be

determined with a kill curve to find out the lowest dose to kill your cells (4-7 days after applying puromycin to the non-transfected cells; the range of puromycin is 1μg/ml to 10μg/ml). Change the media every 2-3 days.

5. The puromycin resistant cells are ready to be analyzed for genome editing. WB can be used to measure gene knockdown if there is a good protein-specific antibody available. Alternatively, you can do genome PCR to verify the integration of the functional cassette. You can also clone and sequence the amplified genome fragment to verify the integration.

6. When it is necessary for your project, you can isolate individual cell colonies.

Table 1. Recommended starting transfection conditions for Turbofectin 8

Tissue Culture Vessel	Growth area, cm^2/well	μg of DNA	Ratio of Turbofectin:DNA
96-well plate	0.35	0.1-0.3	3:1
24-well plate	2	0.25-1.25	3:1
12-well plate	4	0.5-2.5	3:1
6-well plate	9.5	1-5	3:1
35 mm plate	8	1-5	3:1
60 mm plate	20	2-10	3:1
100 mm plate	60	5-15	3:1

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 precedes 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.
5'-NNNNNNNNNNNNNNNNNNNN 3'-NGG
Seed-region

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: 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
http://www.amsbio.com/Genome-editing-Cas9.aspx

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, please enquire.

Q: Both of the guide and donor plasmids need to be transfected into cells; So transfection may be a limiting factor.
Answer: You can find a good transfection reagent for your cells. For hard to transfect cells, many researchers use electroporation. You can also try our Magnetofection transfection reagent that is good for hard to transfect cells:

Q: For knocking down a target gene, I guess we don't need a donor plasmid, correct?
Answer: Without donor template DNA, the double stranded break will be repaired by NHEJ; unpredicted indels will be introduced. You will screen the deletions/insertions that cause frame shift.

Q: do you have the cas9 antibody?
Answer: We are making the Cas9 antibody. In our CRISPR/Cas9 vectors, Cas9 has a C-terminal Myc-DDK tag. DDK is the same as Flag. You can try OriGene's anti-DDK antibody (SKU TA50011-100).

Q: If I want to use CRISPR/Cas9 to knock down a certain gene, what kind of negative control should I include as negative control?
Answer: You can use a scramble control, pCas-Scramble, SKU GE100003.

Q: Can CRISPR/Cas9 be used to produce knockout Rat?
Answer: Yes, CRISPR/Cas9 can be used to make knockout rat. You can inject mRNA (gRNA and Cas9 mRNA) or plasmid DNA (target sequence cloned in pCas-Guide) into the zygotes, then transfer the zygotes into pseudo pregnant rat.

Q: How to avoid off target issue using CRISPR/Cas?
Answer: You can blast your target sequences. If the off-target sequences don't have the PAM (NGG), then they won't be targeted by CRISPR/Cas9. You also want to choose target sequences with mismatches in the 8-14 bp at the 3' end of the target sequences. This way, the off-target issue can be decreased dramatically. For therapeutic purpose, you can use Cas9 nickase which only cuts one strand.

Q: What is the limit to multiple gene disruption?
Answer: You can do multiplexes using CRISPR/Cas9 system. You can do co-transfection or co-injection several guide RNAs into your cells; so you will achieve multiple gene disruption or genome editing. The limit could be transfection efficiency. Regarding how many genes you can do, it is not known yet.

Q: How to screen the edited cells after transfecting the CRISPR/Cas9 vector?
Answer: If it is gene knockout, you can do WB to verify it. You can also do genomic PCR to detect the genomic integration. For mutations, you can amplify the genomic sequence, then clone the PCR fragment and sequence it. If you do gene knockout, the selection marker in the donor template DNA will help the selection. You will need to isolate individual cell colonies for introduction of specific mutations in the genome and other applications.

Q: How do you make sure that Cas9 will not integrate in genome if you use lentivector?
Answer: For screening purpose, for short term, integration of Cas9 into the genome for 2 weeks seems to be ok. There is also a non-integration lenti packaging kit commercially available from Clontech.

Q: Do you need to linearize a donor template before transfection for efficient repair?
Answer: The donor template DNA does not need to be linearized. For short insertion/deletion or mutations, you can use oligos as donor template DNA.

Q: How to select for positive clones if using long oligos as a donor template?
Answer: Isolate single cell colonies, do WB (for gene knockout or tagging) or genomic PCR or sequencing (for mutations) to detect the genome editing depending on the nature of the editing.

Q: can we buy predesigned donor vectors?
Answer: Yes, please enquire.

Q: Does Crispr/Cas system work for non-dividing cells?
Answer: NHEJ repair works in non-dividing cells; HDR is not active in non-dividing cells. Therefore, you can do gene disruption using CRISPR/Cas9 system without donor template DNA.

Q: For gene targeting in mice, do you recommend transfecting ES cells or pronuclei?
Answer: Either method works. You can inject mRNA (gRNA and Cas9 mRNA) or plasmid DNA (target sequence cloned pCas-Guide) into the zygotes or ES cells.

Q: Can you introduce mutations anywhere in the genome, including in promoters or enhancers?
Answer: Yes. The 20 bp target sequences only need to precede NGG.

Q: How long should LHA and RHA be?
Answer: 500-1000 bp should be enough for the left or right homologous arms for HDR mediated repair using CRISPR.

Q: Do you know the specific cleavage site of the Cas:gRNA complex in terms of where in the targeting sequence the cleaving occurs?
Answer: Cas9 cleaves at 3 bp away from the 3' end of the target sequence in the genome.

Q: Is there a method for cloning knockout cell lines from the engineered pool of cells?
Answer: Isolate individual cell colonies.

Q: Do you see variability in success with different cell lines?
Answer: Yes, depending on the cell line and target sequences.

Q: For fluorescently labeling a gene of interest, is it necessary to serial dilute transfected cells for clonal analysis?
Answer: If in the donor construct the fluorescent protein does not have a mammalian expression promoter, then you can sort the fluorescent cells out; you might not need to get individual cell colonies. If the fluorescent protein in the donor template DNA does contain a mammalian expression promoter, you will need to pass the transfected cells several generations to dilute the cells containing the donor construct expressing the fluorescent protein.

Q: What is the known efficiency relative to other genome editing approaches?
Answer: In general, the genome editing efficiency of CRISPR/Cas9 is similar or higher than TALEN. However, CRISPR/Cas9 is much more simple and easy to do. You will need to engineer the protein to recognize new DNA sequence in TALEN system, while CRISPR/Cas9 is RNA based.

Q: why do you need T7-drived vector to express gRNA and cas-9?
Answer: For making gene knockout mice and genome editing in other organisms, such as Drosophila, some researchers do microinjection of gRNA and Cas9 mRNA into cells.

Question: Is there any safety issue with this pLenti vector?
Answer: The pLenti vector is a third generation lentiviral vector and it is the safest lenti-viral vector because both LTRs are truncated. Please contact the biosafety office at your institution prior to use of the pLenti vector for permission and for further institution-specific instructions. BL2/(+) conditions should be used at all times when handling

lentivirus. All decontamination steps should be performed using 70% ethanol/1% SDS. Gloves should be worn at all times when handling lentiviral preparations, transfected cells or the combined transfection reagent and lentiviral DNA.

Question: What is unique about the 3rd generation of Lentiviral vectors?
Answer: The 3rd generation lentiviral vectors are safer than the 2nd generation vectors. The 3rd generation packaging systems express gag and pol from one packaging vector and rev from another. The 3rd generation packaging systems DO NOT express tat (Trans-Activator of Transcription).

Question: What cell line should be used in order to produce lentivirus?
Answer: HEK293T cells are commonly used to produce lentivirus. The HEK293T cell line for producing lentiviral particles can be obtained from ATCC (www.atcc.org).

Can pLenti vectors be used in direct transfections as opposed to making virus?
Answer: OriGene's pLenti vectors can also be used in transient transfections to achieve expression of the transgene. This usually involves lower levels of protein production due to diminished transfection efficiency.

Question: What is the difference between a lentivirus and a retrovirus?
Answer: Lenti viruses are a subtype of retrovirus. The main difference between lentiviruses and standard retroviruses from an experimental standpoint is lentiviruses are capable of infecting both non-dividing and actively dividing cell types whereas standard retroviruses can only infect mitotically active cell types. Both lentiviruses and standard retroviruses use the gag, pol, and env genes for packaging. However, the isoforms of these proteins used by retroviruses and lentiviruses are different and lentiviral vectors may not be efficiently packaged by each other's packaging systems.

Question: Can I use a second generation packaging system with the pLenti vectors?
Answer: Yes, a second generation packaging system should work with OriGene's third generation pLenti vectors although we have not explicitly tested this. You can use OriGene's high efficient third generation lenti-packaging kit (cat# TR30002) for pLenti-vectors.

Question: How can I sequence the target sequenced cloned in pT7-Guide vector?
Answer: M13 forward primer, 5' CGCCAGGGTTTTCCCAGTCACGAC 3'