Clontech Laboratories, Inc.

Guide-it™ CRISPR/Cas9 Systems User Manual

Cat. No(s). 632601, 632602
(033015)

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I. Introduction

The Guide-it CRISPR/Cas9 System (Green, Cat. No. 632601; Red, Cat. No. 632602) is a complete system for the cloning and expression of a target single guide RNA (sgRNA) for mammalian gene modification studies using CRISPR/Cas9 technology. The included vector is used to simultaneously express Cas9, a target-specific sgRNA, and an exceptionally bright fluorescent protein (either ZsGreen1 or tdTomato) for monitoring transfection efficiency and/or for further enriching/isolating transfected cells by flow cytometry. The vector is pre-linearized for simple insertion of a target sequence for sgRNA expression from the human U6 promoter; Cas9 nuclease and either ZsGreen1 or tdTomato are co-expressed from a bidirectional CMV promoter.

Sufficient reagents are provided in this kit for construction of 10 different target (sgRNA) expression plasmids. To construct the vector, a pair of user-provided oligos corresponding to the target genomic sequence of interest are annealed to form a duplex. Then, the duplexed DNA is cloned into the pre-linearized vector using the included high-efficiency ligation mix. This kit also contains necessary controls and Stellar™ Competent Cells.

II. List of Components

- **Guide-it CRISPR/Cas9 System (Green) (Cat. No. 632601)**
  - pGuide-it-ZsGreen1 Vector (Linear) (Cat. No. 632603; Not sold separately)
    - 20 µl pGuide-it-ZsGreen1 Vector (Linear) (7.5 ng/µl)
  - Guide-it Ligation Components (Cat. No. 632605; Not sold separately)
    - 50 µl DNA Ligation Mighty Mix*
    - 1.5 ml Guide-it Oligo Annealing Buffer
    - 10 µl Guide-it Control Annealed Oligos (100 fmol/µl)
    - 10 µl Guide-it Sequencing Primer 1 (100 pmol/µl)
    - 1 ml PCR-Grade Water
  - Stellar Competent Cells (Cat. No. 636763)
    - 10 tubes Stellar Competent Cells (100 µl/tube)
    - 10 tubes SOC Medium (1 ml/tube)
    - 10 µl pUC19 Vector (0.1 ng/µl)

- **Guide-it CRISPR/Cas9 System (Red) (Cat. No. 632602)**
  - pGuide-it-tdTomato Vector (Linear) (Cat. No. 632604; Not sold separately)
    - 20 µl pGuide-it-tdTomato Vector (Linear) (7.5 ng/µl)
  - Guide-it Ligation Components (Cat. No. 632605; Not sold separately)
    - 50 µl DNA Ligation Mighty Mix*
    - 1.5 ml Guide-it Oligo Annealing Buffer
    - 10 µl Guide-it Control Annealed Oligos (100 fmol/µl)
    - 10 µl Guide-it Sequencing Primer 1 (100 pmol/µl)
    - 1 ml PCR-Grade Water
  - Stellar Competent Cells (Cat. No. 636763)
    - 10 tubes Stellar Competent Cells (100 µl/tube)
    - 10 tubes SOC Medium (1 ml/tube)
    - 10 µl pUC19 Vector (0.1 ng/µl)

* The DNA Ligation Mighty Mix component is available separately (100 rxns, Cat. No. 6023)

III. General Considerations

A. Storage

- Store the Guide-it Vector and Guide-it Ligation Components at –20°C upon receipt
- Store Stellar Competent Cells at –80°C upon receipt

B. Additional Materials Required

The following materials are required but not supplied:

- Target-specific oligos (see Section IV-A.)
- TE buffer or molecular biology grade, nuclease-free water
- PCR reaction tubes
- Micropipette tips (with hydrophobic filters)
- 1.5-ml Eppendorf tubes
- Thermal cycler
- 42°C heat block
- 37°C incubator/shaker
- LB plates containing ampicillin (100 µg/ml)
- Bacteria spreader

IV. Oligo Design

The CRISPR/Cas9 system requires a custom sgRNA that contains a targeting sequence and a Cas9 nuclease-recruiting sequence. The targeting sequence is homologous to your target gene or genomic region of interest and will direct Cas9 nuclease activity. Selecting the appropriate DNA sequence at the target region is critical for maximizing the potential for efficient cleavage at the target site and for minimizing non-specific cleavage events. There are several online tools (e.g., http://crispr.mit.edu/ or https://chopchop.rc.fas.harvard.edu/) that can be helpful for determining the appropriate target sequence.

Customizing the targeting sequence of the sgRNA with the Guide-it CRISPR/Cas9 System involves designing a pair of oligos that correspond to the target genomic sequence. When designing these oligos, use the following guidelines:

1. The sense oligo (Oligo 1) should contain a 20-nucleotide sequence that is immediately adjacent to an NGG Proto-spacer Adjacent Motif (PAM) sequence at the 3’ end of the target sequence (do not include the PAM sequence). The antisense oligo (Oligo 2) is the complementary sequence of Oligo 1.
    - To minimize off-target cleavage, the entire target sequence including the PAM site should have at least three base mismatches with any other genomic sequence (1, 2); it is particularly useful if the mismatches are in the PAM site or adjacent to the PAM site.
    - For gene knockouts, it is recommended to choose a sequence that corresponds to the N-terminus of the protein.
2. For cloning, additional sequences should be included at the 5’ ends of the oligos. The sense oligo should include the 5’ overhang sequence, **ccgg**; the antisense oligo should include the 5’ overhang sequence, **aaac**.

- Oligo 1: 5'-ccgg XXX XXX XXX XXX XXX XXX XX-3'
- Oligo 2: 5'-aaac YY YYY YYY YYY YYY YYY YYY-3'

NOTE: The first guanine in the "ccgg" sequence of Oligo 1 functions as the transcription initiation site for sgRNA synthesis from the U6 promoter. When the target sequence is designed, it is not necessary to add an extra guanine or choose a guanine at the 5' end of your target sequence.

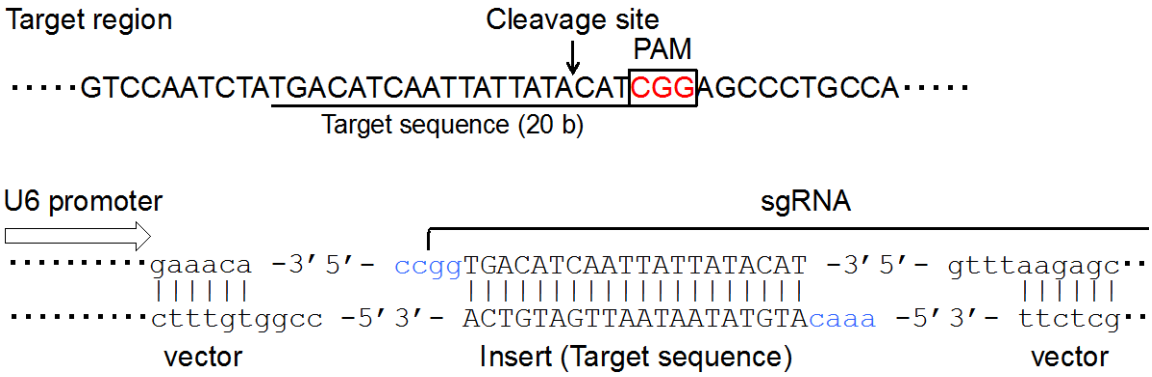

Figure 1. Example of cloning a target sequence using the Guide-it CRISPR/Cas9 System. This particular example corresponds to the sequence of the Guide-it Control Annealed Oligos included in the kit.

V. Plasmid Construction

A. Protocol: Annealing Oligos

1. Resuspend each oligo completely in TE buffer or molecular-biology grade, nuclease-free water such that the concentration is 100 µM.
2. Mix the oligos in a 200-µl PCR tube as follows:

1 µl	Oligo 1 (100 µM)
1 µl	Oligo 2 (100 µM)
8 µl	Guide-it Oligo Annealing Buffer
10 µl	Total Volume

NOTE: The concentration of the oligos is 10 µM.

3. Anneal the oligos by using a thermal cycler to denature at 95°C, and then reanneal the oligos by slowly reducing the temperature.
   Program your thermal cycler with the following cycling conditions:

   95°C, 2 min
   10 min slope from 85°C to 30°C
   25°C, hold

4. Mix 1 µl of the annealed oligo solution with an additional 99 µl of Guide-it Oligo Annealing Buffer to make a 100 nM (fmol/µl) solution.
5. Store the annealed oligos at –20°C until use.

B. Protocol: Cloning the sgRNA targeting sequence into the pGuide-it Vector

1. Thaw the necessary reagents at room temperature and set up the reaction as follows:

2 µl	pGuide-it Vector (Linear) (7.5 ng/µl)
1 µl	Target annealed oligos (100 fmol/µl; from Section V.A, Step 5) or Guide-it Control Annealed Oligos (100 fmol/µl)
2 µl	PCR Grade Water
5 µl	DNA Ligation Mighty Mix
10 µl	Total Volume

2. Incubate the reaction mix at 16°C for 30 min.
3. Meanwhile, thaw one vial of Stellar Competent Cells on ice.
4. Add the entire 10 µl ligation mixture to the competent cells and mix gently by tapping.
5. Allow the mixture to stand on ice for 30 min.
6. Heat shock the cells at 42°C for 45 sec and immediately place on ice. Incubate for 2 min.
7. Add 1 ml of SOC medium and incubate at 37°C for 1 hr with vigorous shaking.
8. Plate an appropriate amount of the culture on pre-warmed (37°C) LB plates containing ampicillin (final concentration 100 µg/ml).
9. Incubate the plates at 37°C overnight.

C. Protocol: Isolate and Analyze Plasmids

1. Pick single colonies and inoculate into an appropriate amount of LB medium containing ampicillin (final concentration 100 µg/ml).
2. Incubate with shaking overnight at 37°C.
3. Purify plasmid DNA from bacteria. We highly recommend NucleoSpin Plasmid (Cat. No. 740588.50) for rapid, high-yield, and high-purity purification. For transfection-grade plasmid preparation, use NucleoBond Xtra Midi (Cat. No. 740410.10) or NucleoBond Xtra Maxi (Cat. No. 740414.10).
4. Determine the plasmid DNA concentration and perform sequencing analysis using the included Guide-it Sequencing Primer 1 and your preferred sequencing protocol.

VI. References

1. Mali, P., *et al*. (2013) *Nat. Biotechnol*. **31:**833–838.
2. Hsu, P.D., *et al*. (2013) *Nat. Biotechnol*. **31:**827–832.

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