Takara Bio USA, Inc.

Guide-it™ Recombinant Cas9 (10 µg/µl) User Manual

Cat. Nos. 632678, 632679
(121620)

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I. Introduction

A. Summary

The CRISPR/Cas9 system has emerged as a powerful tool for gene editing because of its high targeting specificity, editing efficiency, and ease-of-use in virtually any organism. CRISPR/Cas9 technology consists of two key components that form a complex: Cas9 endonuclease and a single guide RNA (sgRNA) that directs Cas9 to cleave genomic DNA in a sequence-specific manner (Jinek et al. 2012). This RNA-programmable method exploits the error-prone nature of the non-homologous end joining (NHEJ) DNA repair pathway to generate gene knockouts (via insertion/deletion). The method can also be used to generate knockins via the homology-directed repair (HDR) pathway.

CRISPR/Cas9 system components have been delivered successfully into target cells through a variety of approaches, including vector-based expression systems, transfection of RNA, and more recently, introduction of Cas9-sgRNA ribonucleoprotein (RNP) complexes. Delivery of Cas9-sgRNA RNPs provides a fast turnaround for gene editing experiments while minimizing the likelihood of off-target effects compared to vector-based approaches (Sander and Joung 2014), and this approach has been optimized for various cell types using microinjection, electroporation, and lipid-mediated transfection (Liang et al. 2015).

Guide-it Recombinant Cas9 (10 µg/µl) is a recombinant wild-type *Streptococcus pyogenes* Cas9 nuclease expressed with a C-terminal nuclear-localization signal (NLS) and purified from *E. coli* for use in CRISPR/Cas9-mediated gene editing experiments. The Cas9 protein solution has been verified to be sterile and well-tolerated by mammalian cells when electroporated as a ribonucleoprotein complex (RNP) with a single guide RNA (sgRNA) for knockout experiments, or as an RNP with a donor repair template for knockin experiments.

II. List of Components

Guide-it Recombinant Cas9 (10 µg/µl) (Cat. No. 632678)

- 20 µl (200 µg) Guide-it Recombinant Cas9 (10 µg/µl) in 50% glycerol

Guide-it Recombinant Cas9 (10 µg/µl) (Cat. No. 632679)

- 50 µl (500 µg) Guide-it Recombinant Cas9 (10 µg/µl) in 50% glycerol

- Store Guide-it Recombinant Cas9 (10 µg/µl) at –20°C.

III. Additional Materials Required

The following reagents/materials are required but not included.

A. Electroporation Supplies

Use of this product requires an electroporator, electroporation chamber (typically cuvettes or tips), and an electroporation buffer that is suitable for your target cells. Here we provide separate guidelines for the Neon Transfection System (Thermo Fisher Scientific, Cat. No. MPK5000) and the 4D-Nucleofector System (Lonza, Cat. No. AAF-1002B).

B. Mammalian Cell Culture Supplies

- Culture medium, supplies, and additives specific to your target cells
- Cell culture plates
- PBS without Ca^{2+} or Mg^{2+}
- Trypsin/EDTA or equivalent
- Humidified incubator (set at 37º C, 5% CO_2)

C. General Supplies

- Single-channel pipettes
- Nuclease-free thin-wall PCR tubes or strips

D. sgRNA Development and Production

CRISPR/Cas9 gene editing requires a custom sgRNA with a user-designed targeting sequence that is homologous to the target gene or genomic region of interest. Selecting an appropriate DNA sequence at the target region is critical for maximizing the potential for efficient cleavage at the target site and for minimizing the likelihood of non-specific cleavage events. There are several freely available online tools that can be helpful for determining suitable sgRNA target sequences for a given organism and genomic target. For a list of these tools, please refer to: http://www.takarabio.com/US/Products/Genome_Editing/CRISPR_Cas9/Resources/Online_tools_for_guide_RNA_design.

NOTE: For many applications, it is advisable to design and test several variant sgRNAs against the same genomic target region.

Candidate sgRNAs must ultimately be produced in sufficient quantity for the generation of functional Cas9-sgRNA RNPs. For development and production of user-designed sgRNAs, we recommend either of the following kits:

- For constructing and purifying sgRNAs: Guide-it sgRNA *In Vitro* Transcription Kit (Takara Bio, Cat. No. 632635).
- For constructing and purifying sgRNAs, and testing target cleavage efficiencies *in vitro*: Guide-it Complete sgRNA Screening System (Takara Bio, Cat. No. 632636).

E. Detection and Characterization of Gene Editing

These items from Takara Bio are recommended for determining the efficiency of gene editing and the nature of the edits:

Cat. No.	Product	Size
631443	Guide-it Mutation Detection Kit	100 rxns
631448	Guide-it Mutation Detection Kit	25 rxns
632611	Guide-it Genotype Confirmation Kit	100 rxns
631444	Guide-it Indel Identification Kit	10 rxns

IV. Protocol Overview

Please read each relevant protocol completely before starting. Successful results depend on understanding and performing the following steps correctly.

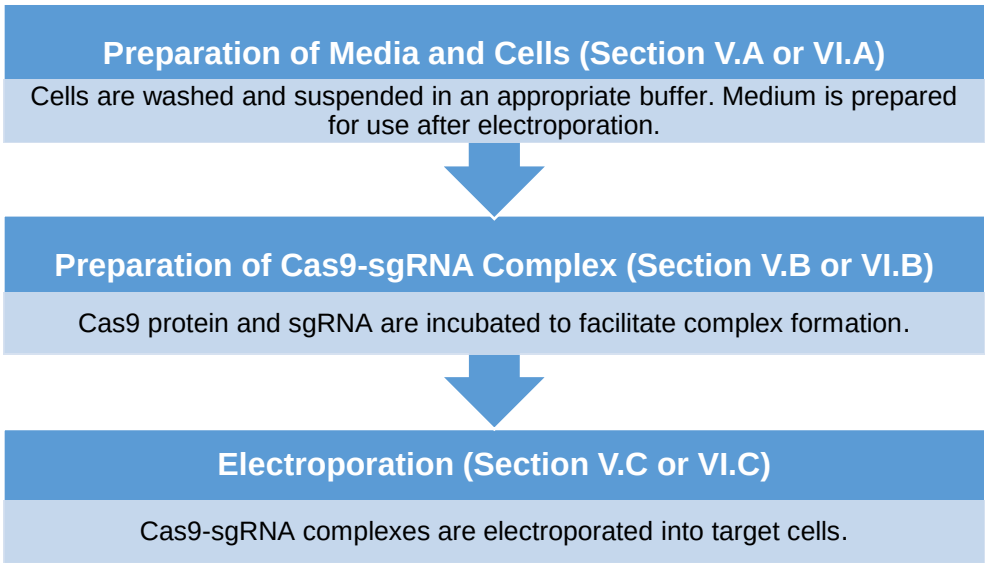

Figure 1. Protocol overview for Guide-it Recombinant Cas9 (10 µg/µl).

V. Electroporation Protocol for Neon Transfection System

Here we provide protocols for performing knockout and knockin experiments in hiPS cells and CD34-positive stem cells using the Neon Transfection System. While these protocols may serve as a helpful starting point for electroporation of other cell types as well, further optimization will be required. Please refer to the Neon Transfection System User Manual and manufacturer's website for detailed operating instructions for the Neon Transfection System.

A. Protocol: Preparation of Cells and Media

Cultured target cells are harvested, washed, and resuspended in the appropriate buffer.

1. Prepare a sufficient number of fresh cells for your experiment.
   NOTE: Each electroporation requires 1 x 10^5 cells. However, due to the potential variation of pipette and tip volumes, we recommend preparing 1.5X the necessary volume of cell suspension (i.e., 1.5 x 10^5 cells) for electroporation with a 10-µl Neon Tip to ensure that there is sufficient volume.
2. For hiPS cells (adherent cells), continue to Step 3. For CD34-positive stem cells (suspension cells), skip to Step 5.
3. Aspirate the medium, wash the cell layer once with PBS (without Ca^{2+} and Mg^{2+}), and dissociate the cells using TrypLE Select Enzyme (1X) (Thermo Fisher Scientific, Cat. No. 12563011).
4. Harvest the cells in growth medium.
5. Take an aliquot of the cell suspension and measure the cell density using your preferred method.
6. Harvest the cells by centrifugation at 400*g* for 5 min in a 15-ml conical tube.

7. Wash the cells once with PBS (without Ca^{2+} and Mg^{2+}), and then resuspend hiPS cells in Buffer R and CD34-positive stem cells in Buffer T (included with Neon kits) at a concentration of 2 x 10^7 cells/ml (i.e., 1.5 x 10^5 cells in 7.5 µl).

   NOTE: Use Resuspension Buffer R for established adherent and suspension cells as well as primary adherent cells, and use Resuspension Buffer T for primary blood-derived suspension cells.

8. Keep the cell suspension on ice until use.

B. Protocol: Preparation of Cas9-sgRNA RNP Complex

Cas9 and sgRNA components are combined to form RNP complexes for electroporation.

1. Combine the following components in a 200-µl PCR tube to mix the Cas9 protein and sgRNA at a 5:1 mass ratio. The molar ratio of Cas9 protein to sgRNA will be approximately 1:1 in this mixture, and the total volume will be 7.5 µl. Be sure to use the same buffer that was used to resuspend the cells.

   NOTE: The reaction volume indicated below is 1.5X the required volume.

   Per reaction:

0.45 µl*	sgRNA (e.g., 1 µg/µl)
0.23 µl	Guide-it Recombinant Cas9 (10 µg/µl)
6.82 µl*	Resuspension Buffer R or T
7.5 µl	Total volume

   *The added volume of sgRNA will vary depending on sgRNA concentration, and the added volume of Resuspension Buffer should be adjusted such that the total reaction volume is 7.5 µl. The volumes indicated above are based on an sgRNA concentration of 1 µg/µl.

   NOTE: Make a master mix if you are performing multiple electroporations.
   NOTE: The optimal amount of RNP complex may vary for different cell types.
   NOTE: To maximize electroporation efficiency, the combined volume of the Cas9 and sgRNA solutions should be ≤20% of the total volume of the Cas9-sgRNA RNP complex reaction (e.g., for the 7.5-µl reaction specified above, the combined volume of the sgRNA and Cas9 solutions should be ≤1.5 µl).
   NOTE: If you plan to use donor DNA to induce HDR-mediated knockin, add the DNA after the subsequent incubation step (Step 3). We recommend using ≤1 µg of DNA for knockin experiments. Adjust the volume of Resuspension Buffer R or T included in the reaction such that the final volume upon addition of donor DNA is 7.5 µl.

2. Mix the reaction well by gently pipetting up and down. Incubate using a thermal cycler preheated to 37°C with the following program:

37°C	5 min
4°C	hold

3. OPTIONAL: Add donor DNA and keep on ice until use.

C. Protocol: Electroporation

Cas9-sgRNA RNPs are electroporated into target cells.

1. Fill the Neon Tube with 3 ml of Buffer E (included with Neon kits) and insert the Neon Tube into the Neon Pipette Station.
2. Using the touchscreen on the Neon system, set up the electroporation parameters as follows:

   Pulse voltage / Pulse width / Pulse number = 1100 v / 20 ms / 2 pulses

   NOTE: We have used these parameters successfully for hiPS cells and CD34-positive stem cells using the Neon Transfection System. Optimization of electroporation parameters will be required for different target cell types. Suggested parameters for different cell types are included in the supplementary material for (Liang et al. 2015).
3. Gently resuspend the cells by tapping, and transfer 7.5 µl of the cell suspension into the PCR tube containing the 7.5 µl of Cas9-sgRNA RNP complex solution.
4. Mix well by gently pipetting up and down.
5. Insert the Neon Pipette into the Neon Tip and confirm that the pipette and tip are tightly connected.
6. Using the Neon Pipette, aspirate the mixture slowly into the Neon Tip.

   NOTE: Avoid any air bubbles in the tip. If you notice air bubbles, place the sample back into the PCR tube and aspirate again into the tip without any air bubbles.
7. Insert the Neon Pipette into the Neon Tube placed in the Neon Pipette Station and run the program.
8. Remove the pipette very carefully and transfer the cells into a cell culture plate with pre-warmed medium.

   NOTE: Use an appropriate well plate for your target-cell type. We had success using 24- and 48-well plates for CD34-positive stem cells and hiPS cells, respectively. hiPS cells typically require greater confluence than regular adherent cells.
9. Shake the plate appropriately to disperse the cells and incubate at 37°C in a humidified incubator with 5% CO_2 until the next necessary procedure.

VI. Electroporation Protocol for 4D-Nucleofector System

Here we provide protocols for performing knockout and knockin experiments in Jurkat and CD34-positive stem cells using the 4D-Nucleofector System with 16-well Nucleocuvette Strips. While these protocols may serve as a helpful starting point for electroporation of other cell types as well, further optimization will be required. Please refer to the 4D-Nucleofector System User Manual and manufacturer's website for more detailed information.

A. Protocol: Preparation of Cells

Cultured target cells are harvested, washed, and resuspended in the appropriate solution.

1. Prepare a sufficient number of fresh cells for your experiment.

   NOTE: Each electroporation requires 2 x 10^5 cells.
2. Take an aliquot of the cell suspension and measure the cell density using your preferred method.
3. Harvest the cells by centrifugation at 400*g* for 5 min in a 15-ml conical tube.

4. Wash once with PBS (without Ca^{2+} and Mg^{2+}), and then resuspend Jurkat cells in SE Nucleofector Solution (with supplement) and CD34-positive stem cells in P3 Nucleofector Solution (with supplement) at a concentration of 1 x 10^7 cells/ml (i.e., 2 x 10^5 cells in 20 µl).

   NOTE: Please refer to the 4D-Nucleofector System User Manual and manufacturer's website for more information about working with other cell types.

   NOTE: 20 µl of cell suspension will be needed per well of the Nucleocuvette Strip.

5. Keep the cell suspension on ice until use.

B. Protocol: Preparation of Cas9-sgRNA RNP Complex

Cas9 and sgRNA components are combined to form RNP complexes for electroporation.

1. Combine the following components in a 200-µl PCR tube to mix the Cas9 protein and sgRNA at a 5:1 mass ratio:

2 µl*	sgRNA (e.g., 1 µg/µl)
1 µl	Guide-it Recombinant Cas9 (10 µg/µl)
3 µl	Total volume

   *The added volume of sgRNA will vary depending on sgRNA concentration. The volume indicated above is based on an sgRNA concentration of 1 µg/µl.

   NOTE: Make a master mix if you are performing multiple electroporations.

   NOTE: The RNP volume required for efficient transfection needs to be optimized for different cell types. Usually ≤10 µl of the RNP solution will be tested for electroporation in each well of the 16-well Nucleocuvette Strip.

2. Mix the reaction well by gently pipetting up and down. Incubate using a thermal cycler preheated to 37°C with the following program:

37°C	5 min
4°C	Hold

C. Protocol: Electroporation

Cas9-sgRNA RNPs are electroporated into target cells.

1. Label wells of Nucleocuvette Strips to be used for electroporation.
2. Combine 20 µl of cell suspension with 5 µl Cas9-sgRNA RNP complex solution in each well of the Nucleocuvette Strip, and mix well by gently pipetting up and down.

   NOTE: If you plan to use donor DNA to induce HDR-mediated knockin, add the donor DNA at this step.

3. Select program CL-120 for Jurkat cells or program D0-100 for CD34-positive stem cells.
4. Insert the Nucleocuvette Strip into the Nucleofector machine and run the program.
5. Add 80 µl of pre-warmed medium to each cuvette and allow electroporated cells to recover for 12 min post-transfection at room temperature.

6. Gently collect the cells along with the media from each well and transfer to individual wells of a 48-well plate containing pre-warmed medium.
7. Shake the plate appropriately to disperse the cells and incubate at 37°C in a humidified incubator with 5% CO_2 until the next necessary procedure.

VII. References

1. Jinek, M., Chylinsky, K., Fonfara, I., Hauer, M., Doudna, J.A., & Charpentier, E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. **337(6096)**, 816–21 (2012).
2. Liang, X. *et al.* Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *J. Biotechnol*. **208,** 44–53 (2015).
3. Sander, J.D. & Joung, J.K. CRISPR-Cas9 systems for genomic editing, regulation and targeting. *Nat. Biotechnol*. **32**, 347-55 (2014).

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