PROTOCOL

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INSPIRED CELL SOLUTIONS

Enrichment of transfected cells with Dharmacon™ Edit-R™ Fluorescent Cas9 Nuclease mRNA

General considerations

Edit-R Fluorescent Cas9 Nuclease mRNA enables both transfection optimization and enrichment for gene editing experiments. Using the fluorescent mRNA for expression of both Cas9 and a fluorescent protein, either mKate2 (Evrogen, Moscow, Russia) or EGFP (University of California, San Diego), two separate proteins are translated. For enrichment experiments, we recommend performing the transfection experiment in a 6-well tissue culture dish so that a sufficient number of cells can be harvested for FACS analysis according to commonly used instrument protocols. For optimal enrichment of edited cells, we recommend sorting the cells for high mKate2 or EGFP expression levels (selecting the top 10% fluorescence) in addition to negative and dim fluorescence for comparison. Ensure that each fraction has enough of the total cell population such that desired fractions can be easily expanded for downstream applications and testing.

Materials required

Edit-R CRISPR-Cas9 materials for gene engineering can be ordered at dharmacon.horizondiscovery.com.

- Edit-R Fluorescent Cas9 Nuclease mRNA (choose one)
  1. Edit-R mKate2 Cas9 Nuclease mRNA, 20 µg (1 µg/µL; Cat #CAS11859)
     Or
  2. Edit-R EGFP Cas9 Nuclease mRNA, 20 µg (1 µg/µL; Cat #CAS11860)
- Synthetic targeting guide RNA (choose one):
  1. Edit-R synthetic crRNA and tracrRNA oligos:
     a. crRNA, predesigned for your gene of interest in a variety of sizes, or designed and ordered using the Dharmacon CRISPR Design Tool
     b. tracrRNA, 5, 20 or 50 nmol (Cat #U-002005-XX)
     Or
  2. Edit-R synthetic sgRNA, custom ordering using the Dharmacon CRISPR Design Tool

We recommend testing at least three to five guide RNA designs per gene of interest to identify the one with highest editing efficiency that also results in complete knockout of functional protein.

- Non-targeting control guide RNA (choose one):
  1. Edit-R crRNA Non-targeting Control (Cat #U-007501-XX, U-007502-XX, U-007503-XX, U-007504-XX, U-007505-XX)
     Or
  2. Custom synthetic sgRNA non-targeting control ordered using the Dharmacon CRISPR Design Tool
- DharmaFECT™ Duo transfection reagent (1 mg/mL; Cat #T-2010-xx)

Reagents to be supplied by user

Lipid-mediated transfection and electroporation experiments require standard cell culture reagents and instruments appropriate for maintenance of cells. The following additional materials are required but not supplied.

- Electroporation instrument
- Electroporation reagents (buffer, cuvettes, transfer pipettes)
- Flow cytometer
- Multi-well tissue culture plates or tissue culture dishes
- Antibiotic-free complete medium: Cell culture medium (including serum and/or supplements) recommended for maintenance and passaging of the cells of interest without antibiotic
- Serum-free medium: Cell culture medium without serum or antibiotic but containing supplements recommended for maintenance of the cells such as HyClone™ HyQ-RS medium (HyClone, Cat #SH30564.01)
- Assay(s) for detecting gene editing events in a cell population
- 10 mM Tris-HCl Buffer pH 7.4 (Tris buffer), nuclease-free (Cat #B-006000-100)
- Phosphate-buffered saline (PBS)

General protocol for lipid transfection of Edit-R Fluorescent Cas9 Nuclease mRNA and synthetic guide RNAs and enrichment of transfected cells

The following is a general protocol using Edit-R Fluorescent Cas9 Nuclease mRNA to enrich for transfected cells using fluorescence activated cell sorting (FACS). Exact reagent amounts and parameters for both lipid-mediated transfection and electroporation should be empirically determined through careful optimization in cells of interest prior to experimentation. The protocol below describes delivery conditions in U2OS cells using the DharmaFECT Duo transfection reagent and is given for illustrative purposes only. For further details regarding transfection recommendations please see the full Technical Manual.

All steps of the protocol should be performed in a laminar flow cell culture hood using sterile technique.

Day 1

1. Trypsinize and count cells.
2. Plate cells in 6-well plates using growth medium at a cell density so that the cells are 70 to 90% confluent the next day. For example, U2OS cells should be diluted to 100,000 cells in 1 mL of medium for plating at 250,000 cells/well in a 6-well plate.
3. Incubate cells at 37 °C with 5% CO_2 overnight.

Table 1. Antibiotic resistances conveyed by Edit-R Lentiviral sgRNA plasmid.

Sample name	Purpose
Edit-R Fluorescent Cas9 Nuclease mRNA with Non-targeting Control synthetic guide RNA	**Negative control:** Cas9 nuclease mRNA without targeting RNAs
Edit-R Fluorescent Cas9 Nuclease mRNA with gene-specific synthetic guide RNA	**Gene engineering sample:** Cas9 nuclease programmed by RNAs for targeted double-stranded break in gene of interest
Untreated	**No treatment control sample:** confirmation of cell viability, negative FACS sample

Day 2

1. Prepare a 100 ng/µL fluorescent Cas9 mRNA working solution by thawing fluorescent Cas9 mRNA on ice and adding 20 µL of 1 µg/µL stock solution of fluorescent Cas9 mRNA to 180 µL of Tris buffer. Verify the Cas9 mRNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 100 ng/µL.
2. Prepare guide RNA reagents for transfection.
   For crRNA and tracrRNA:
   a. Prepare a 10 µM crRNA stock solution by adding the appropriate volume of Tris buffer to crRNA. Verify the RNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 10 µM.
   b. Prepare a 10 µM tracrRNA stock solution by adding the appropriate volume of Tris buffer to tracrRNA. Verify the RNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 10 µM.
   c. Prepare a 2.5 µM crRNA:tracrRNA transfection complex by adding 25 µL of crRNA and 25 µL of tracrRNA to 50 µL of Tris buffer (total volume is 100 µL).

   For synthetic sgRNA:
   a. Prepare a 2.5 µM synthetic sgRNA stock solution by adding the appropriate volume of Tris buffer to the sgRNA. Verify the RNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 2.5 µM.
3. In a 15 mL conical prepare for each sample to be transfected as described in Table 2 (columns 2-4) for a final 25 nM concentration of the guide RNA and 5 µg/well of Cas9 mRNA.
4. In a separate tube, prepare a 30 µg/mL DharmaFECT Duo working solution by diluting 30 µL of 1 mg/mL stock DharmaFECT Duo transfection reagent in 1 mL serum-free medium and mix gently; this volume is sufficient for 4 wells with 7.5 µL/well in 6-well format. Incubate for 5 minutes at room temperature.

Table 2. Preparing transfection samples for gene editing experiments in a 6-well plate format.

Sample name	Serum-free medium	Synthetic guide RNA transfection complex (2.5 µM)	Fluorescent Cas9 mRNA working solution (100 ng/µL)	DharmaFECT Duo working solution (3 µg/mL)	Growth medium	Total volume per well
Fluorescent Cas9 Nuclease mRNA with non-targeting control synthetic guide RNA	175	25	50	250	2,000	2,500
Fluorescent Cas9 nuclease mRNA with gene-specific synthetic guide RNA	175	25	50	250	2,000	2,500
Untreated	500	0	0	0	2,000	2,500

Volumes (µL) are for a single well of a 6-well plate of U2OS cells to be transfected. It is recommended to prepare sufficient sample volumes for the total number of replicates and to account for pipetting.

5. Add 250 µL DharmaFECT Duo working solution to each sample tube as shown in Table 2 (column 5); this will result in 3 µg/well final concentration. DO NOT add DharmaFECT Duo working solution to the untransfected control, which should contain serum-free medium only. This brings the total volume to 500 µL in each tube. Mix by pipetting gently up and down and incubate for 20 minutes at room temperature.
6. Prepare transfection medium by adding 2,000 µL antibiotic-free complete medium to each sample to bring the total volume in each tube to 2,500 µL (columns 6 and 7).
7. Remove medium from the wells of the 6-well plate containing cells and replace with 2,500 µL of the appropriate transfection medium to each well.

Day 3

8. After 24 hours, trypsinize cells. Collect ¾ of the cells leaving ¼ as a presorted population.
9. Centrifuge and wash cell pellet with PBS to remove medium. Centrifuge again and resuspend cells in appropriate cell sorting buffer.

Expression of fluorescent protein over time should be examined for your cells to determine the optimal time to sort for enrichment. We suggest a minimum of 24 hours after lipid transfection to allow for translation of the mRNA into Cas9 and fluorescent proteins, and 8-24 hours for electroporation. Cell sorting will need to be performed before turnover of the fluorescent protein, which will be dependent on the half-life in your experimental cells.

10. Use untransfected cells as a negative sorting control to set the sorting parameters (gate) for singlet, non-fluorescent viable cells. Sort cells into populations based on fluorescence intensity relative to the negative control as measured by the cell sorter instrument.

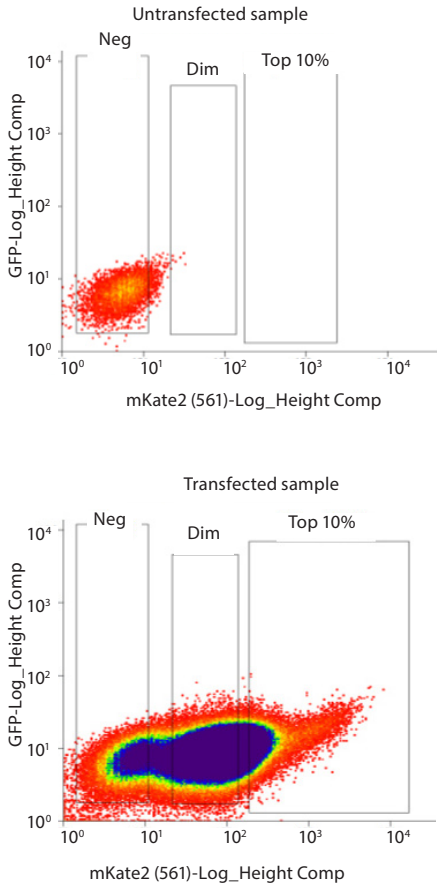

Figure 1. Representative FACS data of the untransfected sample (left) and transfected sample containing Edit-R mKate2 Cas9 Nuclease mRNA (right). The untransfected sample should be used to define which cells are negative (Neg) for fluorescence and to be excluded from sorting. Gates are drawn around the dim and top 10% fluorescent populations to be sorted and collected.

For optimal enrichment, we suggest collecting cells with high fluorescence intensity, selecting the top 10% of fluorescent cells.

Clonal cell lines can be created through sorting single cells into individual wells of a 96-well plate.

11. Expand cell populations in new plates with an appropriate well size corresponding to the number of cells collected.
12. Incubate cells at 37 °C with 5% CO_2 for an additional 48 hours before proceeding with gene editing analysis.

Gene editing assay recommendations

The most commonly used method for detection of editing events in a cell population is a DNA mismatch detection assay (Guschin, 2010; Reyon, 2012; Cong, 2013). This assay can be performed on either purified genomic DNA or whole cell lysate using T7 Endonuclease I. When edited cells are expanded and clonal populations are obtained, the most commonly used method for characterizing gene editing is Sanger sequencing (Reyon, 2012). See this Application Note for an example.

FAQ

What are the maximum excitation and emission wavelengths for mKate2 and EGFP?

Fluorescent reporter	Excitation wavelength	Emission wavelength
mKate2	588 nm	633 nm
EGFP	489 nm	509 nm

What is the best way to determine optimal sorting time?

We suggest observing fluorescence intensity over time by microscopy, but also performing a western blot for Cas9 protein or the fluorescent protein.

Is Edit-R Fluorescent Cas9 mRNA a fusion protein?

No, the fluorescent protein and Cas9 are separate proteins translated from a single mRNA strand.

References

1. L. Cong, F. A. Ran, *et al.* Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science.* **339** (6121), 819-823 (2013).
2. E. Deltcheva, K. Chylinski, *et al.* CRISPR RNA maturation by trans-encoded small RNA and host factor Nuclease III. *Nature.* **471** (7340), 602-607 (2011).
3. D.Y. Guschin, A. J. Waite, *et al.* A rapid and general assay for monitoring endogenous gene modification. *Methods Mol. Biol.* **649**, 247-256 (2010).

If you have any questions, contact

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