PROTOCOL

Enrichment of transfected cells with Dharmacon™ CRISPRmod CRISPRa Fluorescent dCas9-VPR mRNA

General considerations

CRISPRmod CRISPRa Fluorescent dCas9-VPR mRNA enables both transfection optimization and enrichment for gene modulation experiments. Using the fluorescent mRNA for expression of both dCas9-VPR and a fluorescent protein, 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 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

CRISPRmod CRISPRa dCas9-VPR materials for gene modulation can be ordered at dharmacon.horizondiscovery.com.

- CRISPRmod CRISPRa Fluorescent dCas9-VPR mRNA
  1. CRISPRmod CRISPRa EGFP dCas9-VPR mRNA, 20 µg (1 µg/µL; Cat #CAS12025)
- Synthetic targeting guide RNA (choose one):
  1. CRISPRmod CRISPRa synthetic crRNA and tracrRNA oligos:
     a. CRISPRa 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. CRISPRmod CRISPRa CRISPRa crRNA 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 activation efficiency.

- Non-targeting control guide RNA (choose one):
  1. CRISPRmod CRISPRa crRNA Non-targeting Control (Cat #U-009500-01-XX or #U-009500-10-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 activation 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 CRISPRmod CRISPRa Fluorescent dCas9-VPR mRNA and synthetic guide RNAs for enrichment of transfected cells

The following is a general protocol using CRISPRmod CRISPRa EGFP dCas9-VPR 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.

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 CRISPRmod CRISPRa Lentiviral sgRNA plasmid.

Sample name	Purpose
EGFP dCas9-VPR mRNA with Non-targeting Control synthetic guide RNA	**Negative control:** EGFP dCas9-VPR mRNA without targeting RNAs
EGFP dCas9-VPR mRNA with gene-specific synthetic guide RNA	**Gene engineering sample:** EGFP dCas9-VPR mRNA programmed by RNAs for targeted gene activation in gene of interest
Untreated	**No treatment control sample:** confirmation of cell viability, negative FACS sample

Day 2

1. Prepare a 100 ng/µL EGFP dCas9-VPR mRNA working solution by thawing EGFP dCas9-VPR 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 EGFP dCas9-VPR 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 EGFP dCas9-VPR 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)	EGFP dCas9-VPR mRNA working solution (100 ng/µL)	DharmaFECT Duo working solution (3 µg/mL)	Growth medium	Total volume per well
EGFP dCas9-VPR mRNA with non-targeting control synthetic guide RNA	175	25	50	250	2,000	2,500
EGFP dCas9-VPR 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 dCas9-VPR 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.

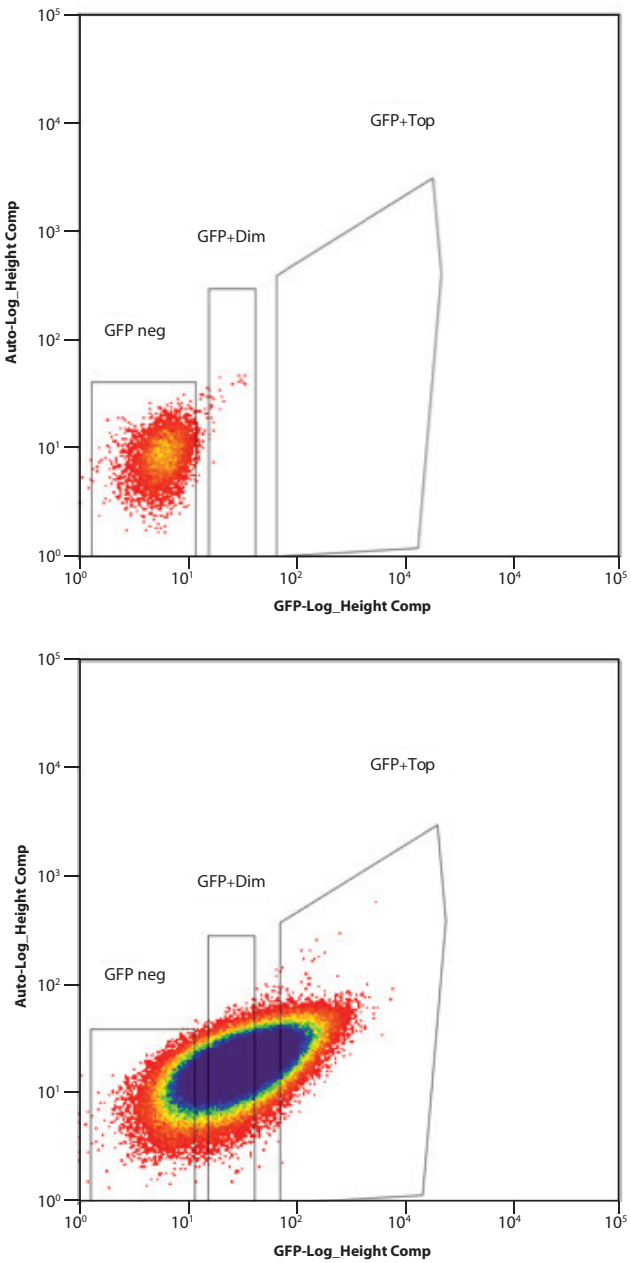

Figure 1. Representative FACS data of the untransfected sample (top) and transfected sample containing CRISPRmod CRISPRa EGFP dCas9-VPR mRNA (bottom). The untransfected sample should be used to define which cells are negative (Neg) for fluorescence and to be excluded from sorting. Gates are drawn around 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 activation assay recommendations

The most commonly used method for detection of editing events in a cell population is RT-qPCR. This assay can be performed using a standard cDNA synthesis kit (ThermoFisher, Cat #K1641) and RT-qPCR protocol (Applied Biosystems TaqMan Assay).

FAQ

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

Fluorescent reporter	Excitation wavelength	Emission wavelength
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 dCas9-VPR protein or the fluorescent protein.

Is CRISPRmod CRISPRa EGFP dCas9-VPR mRNA a fusion protein?
No, the fluorescent protein and dCas9-VPR are separate proteins translated from a single mRNA strand.

For more information

To find the contact information in your country for your technology of interest, please visit us at **horizondiscovery.com/contact-us**

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