PROTOCOL

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Enrichment of transfected cells with CRISPRmod EGFP dCas9-VPR or dCas9-SALL1-SDS3 mRNA

General considerations

CRISPRmod EGFP mRNA enables both fluorescent visualization for transfection optimization and enrichment for gene modulation experiments. Using a single mRNA for co-expression of dCas9-VPR or dCas9-SALL1-SDS3 and a fluorescent protein, EGFP (University of California, San Diego), two separate proteins are translated. For enrichment experiments, we recommend performing the transfection in a 6-well tissue culture dish so that a sufficient number of cells can be harvested for FACS according to commonly used instrument protocols. For optimal enrichment, 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 materials for gene modulation can be ordered at www.horizondiscovery.com.

- CRISPRmod EGFP mRNA
  1. CRISPRmod EGFP dCas9-VPR mRNA, 20 µg (1 µg/µL; Cat #CAS12025)
     Or
  2. CRISPRmod EGFP dCas9-SALL1-SDS3 mRNA, 20 ug (1µg/µL); Cat # CAS12225)
- Synthetic targeting guide RNA (choose one):
  1. CRISPRmod synthetic crRNA and tracrRNA oligos:
     a. CRISPRa crRNA, predesigned for your gene of interest in a variety of sizes, or ordered using the Custom CRISPR Ordering Tool
     b. tracrRNA, 5, 20 or 50 nmol (Cat #U-002005-XX)
     Or
  2. CRISPRmod CRISPRi sgRNA, predesigned for your gene of interest in a variety of sizes, or designed and ordered using the Custom CRISPR Ordering Tool.

We recommend testing three guide RNA designs per gene of interest to identify the most active guide RNA, or using predesigned pooled guide RNA.

- 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. CRISPRmod CRISPRi sgRNA Non-targeting Control (Cat#U-009550-XX-02 or U-009550-XX-05)
- 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 may be 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 or repression 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 EGFP mRNA and synthetic guide RNAs and enrichment of fluorescent cells

The following is a general protocol using CRISPRmod EGFP 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 that will allow the cells to achieve 70 to 90% confluency 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. Recommended samples for a gene modulation experiment with CRISPRmod EGFP mRNA .

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

Day 2

1. Prepare a 100 ng/µL CRISPRmod EGFP mRNA working solution by thawing CRISPRmod EGFP mRNA on ice and adding 20 µL of 1 µg/µL stock solution of EGFP dCas9 mRNA to 180 µL of Tris buffer. Verify the EGFP dCas9-VPR or dCas9-SALL1-SDS3 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. Prepare each transfection sample in a 15 mL conical using the volumes in columns 2-4 of Table 2 for a final 25 nM concentration of guide RNA and 5 µg/well of CRISPRmod EGFP 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 modulation experiments in a 6-well plate format.

Sample name	Serum-free medium	Synthetic guide RNA (2.5 µM)	CRISPRmod EGFP mRNA working solution (100 ng/µL)	DharmaFECT Duo working solution (30 µg/mL)	Growth medium	Total volume per well
CRISPRmod EGFP mRNA with non-targeting control synthetic guide RNA	175	25	50	250	2,000	2,500
CRISPRmod EGFP 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/mL 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 or dCas9-SALL1-SDS3 and fluorescent proteins, or 8-24 hours following 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.

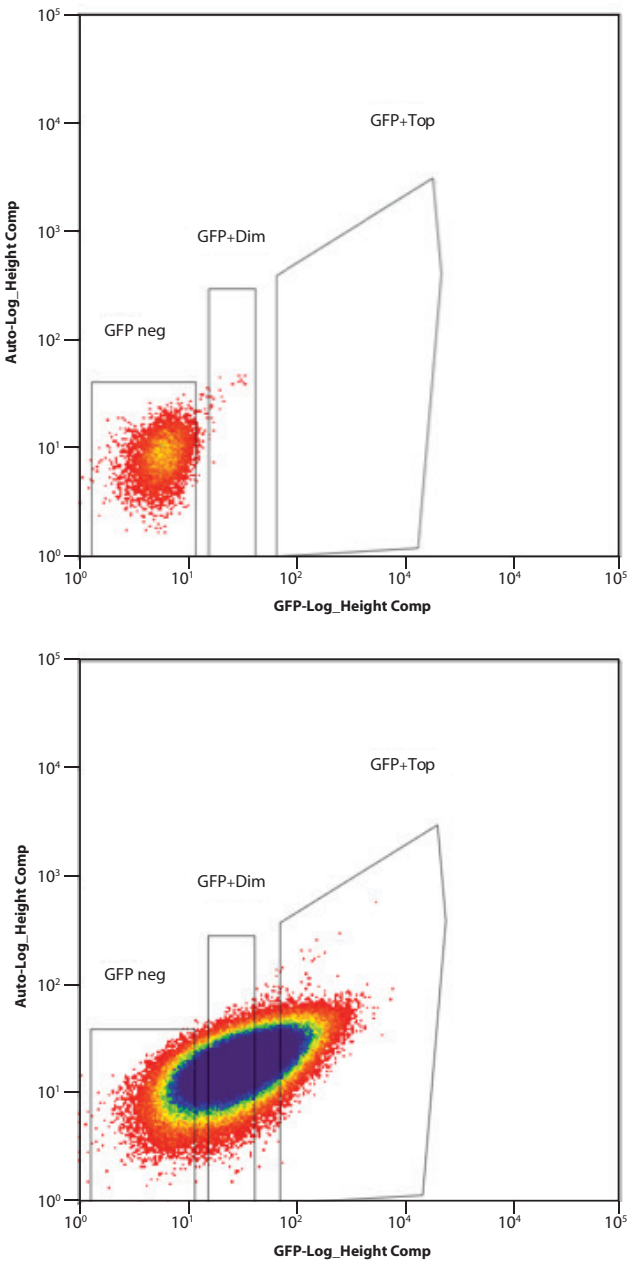

Figure 1. Representative FACS data of the untransfected sample (top) and transfected sample containing CRISPRmod EGFP mRNA (bottom). The untransfected sample should be used to define which cells are negative GFP neg for fluorescence and to be excluded from sorting. Gates are drawn around the dim (GFP+Dim) and top 10% (GFP+Top) 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.

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 24 to 48 hours before proceeding with gene expression analysis.

Gene expression assay recommendations

The most commonly used method for detecting changes in gene expression 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 EGFP?

Fluorescent reporter	Excitation wavelength	Emission wavelength
CRISPRmod 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-protein or the fluorescent protein.

Is CRISPRmod EGFP dCas9-VPR or dCas9-SALL1-SDS3 mRNA a fusion protein?
No, the fluorescent protein and dCas9-VPR or dCas9-SALL1-SDS3 are separate proteins translated from a single mRNA strand.

Can puromycin be used to enrich for cells expressing CRISPRmod mRNA?
Yes. Puromycin selection can be used as an alternative to FACS enrichment. To do so you will need to purchase either Puro dCas9-VPR mRNA (Cat #CAS12026) or Puro dCas9-SALL1-SDS3 mRNA (Cat #CAS12226). Please note that the concentration of puromycin and time needed for selection will need to be optimized for your cell line of interest and method of delivery.

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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