GENProtocols

Protocol

Dharmacon Edit-R crRNA Libraries

DRUG DISCOVERY GENOME EDITING

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Abstract

Libraries of predesigned synthetic crRNAs provide an opportunity to apply the CRISPR-Cas9 system for functional gene knockout analysis, or dCas9-VPR system for transcriptional gene activation, in an arrayed format. Dharmacon crRNA libraries for functional gene knockout consist of Edit-R synthetic crRNAs which are predesigned using the proprietary Edit-R CRISPR RNA algorithm.

Introduction

The Edit-R CRISPR RNA algorithm has been trained on functional knockout data and has demonstrated the ability to select guide RNA target regions more likely to give functional knockout of the protein, not just create a cut. The algorithm additionally includes specificity scoring using an internal alignment tool for complete offtarget identification. All Edit-R CRISPRa crRNAs for transcriptional activation are predesigned with the algorithm described by Horlbeck et. al (1), who performed a systematic study of guide RNAs next to transcriptional start sites (TSS) to identify features that contribute to CRISPRa activity.

Reagents and Equipment

Edit-R crRNA Libraries are available as the following:

- Catalog libraries of predefined gene family collections for human and mouse
  - » Four crRNA per gene as an individual crRNA or a pool of four crRNA per well, at 0.1 nmol, 0.2 nmol or 0.5 nmol/well.
  - » Provided in NUNC Polystyrene 96-well V-bottom plates (Cat #249952) or 384-well plates (Thermo Scientific Abgene Cat #AB-0781) with outer columns left open for inclusion of untreated cells and screenspecific positive and non-targeting controls.
- Cherry-pick crRNA libraries based on a customer's gene list. Learn more or get started.
  - » Multiple individual crRNAs or a crRNA pool, minimum 20 wells » Customizable plate layout; Edit-R catalog control crRNAs may be added to any wells within the plate(s).
  - » Provided in NUNC Polystyrene 96-well V-bottom plates (Cat #249952) or 384 well plates (Thermo Scientific Abgene Cat #AB-0781)

Materials

- Plates of synthetic crRNAs, up to 2 nmol per well in 96-well plates or up to 0.5 nmol per well in 384-well plates.

Additional required materials are listed below and are not provided with crRNA library purchase.

- Edit-R tracrRNA, (Dharmacon, Cat #U-002005-XX). tracrRNA is required for use with all synthetic crRNA reagents. If

working with the CRISPRa dCas9-SAM system, Edit-R SAM tracrRNA (Dharmacon Cat #U-102005-XX) should be used in place of the standard tracrRNA.

- DharmaFECT Transfection Reagent (formulation is dependent on specific cell line of interest)
  - » DharmaFECT 1 Cat #T-2001-XX
  - » DharmaFECT 2 Cat #T-2002-XX
  - » DharmaFECT 3 Cat #T-2003-XX
  - » DharmaFECT 4 Cat #T-2004-XX
- 10 mM Tris pH 7.4 nuclease-free buffer solution (Dharmacon, Cat #B006000-100)
- 96-well tissue culture plates
- 96-well V-bottom polystyrene standard storage plates or deep well plates (for example, NUNC Cat #249952 or Cat #12-565-553)
- Assay for assessing cell viability such as CellTiter-Blue® Cell Viability Assay (Promega Corp., Cat #G8081)
- Positive control crRNA and detection primers for assessment of gene editing
- Non-targeting control crRNA
- Assay-specific positive control crRNA (defined by researcher)
- Positive control crRNAs for CRISPRa
- Non-targeting control crRNAs for CRISPRa
- Growth medium: antibiotic-free cell culture medium (with serum and/or supplements) recommended for maintenance of the cells of interest
- Serum-free and antibiotic-free cell culture medium for preparation of transfection mix (for example, MEM-RS, HyClone Cat # SH30564)

For phenotypic analysis with the arrayed synthetic crRNA libraries for gene knockout we strongly recommend using cell lines that constitutively express Cas9 nuclease. Transfection of crRNA:tracrRNA into a cell line that is constitutively expressing Cas9 nuclease results in a higher percentage of edited cells thus allowing for easier downstream high throughput phenotypic analysis of the edited cell population. For generation of the Cas9 stable expressing cells please follow recommendations in the Gene Engineering with Lentiviral Cas9 Particles and Synthetic CRISPR RNAs manual.

For phenotypic analysis with the arrayed CRISPRa crRNAs libraries for gene activation, we recommend using cell lines that constitutively express dCas9- VPR transcriptional activator. For generation of the dCas9-VPR stable cells follow recommendations in the Dharmacon Edit-R CRISPRa transcriptional activation system with synthetic guide RNA Technical Manual. The arrayed CRISPRa crRNA libraries may also be used for gene activation in cells expressing the dCas9-SAM transcriptional activation system, in which case Edit-R SAM tracrRNA should be used in place of Edit-R tracrRNA.

The phenotypic analysis, including assay optimization and analysis, is cell line and assay-specific and requires optimization by the researcher.

Procedure

Guidelines for resuspension of the crRNA and generating crRNA:tracrRNA plates

1. The crRNA libraries are shipped at ambient temperature as dry pellets of RNA in each well and should be stored at –20 °C upon arrival in a manual defrost or non-cycling freezer. If necessary, crRNAs can be stored as dry pellets (unopened) at 4 °C for several weeks.
2. crRNA and tracrRNA are used in equimolar amounts, so be sure to purchase enough tracrRNA for the number and quantity of wells in your library. Calculate the nmol amount of tracrRNA = # wells × nmol quantity per well. For example for a 0.5 nmol library of 100 wells you will need 50 nmols of tracrRNA (0.5 nmol × 100 wells = 50 nmols of tracrRNA). Make 10 µM working concentration of tracrRNA in Tris pH7.4 buffer as noted in Table 3 of Appendix.
3. Briefly centrifuge crRNA plates to ensure that the crRNA is collected at the bottom of the well.
4. Wipe adhesive foil cover with 70% ethanol or other RNasedecontamination solution.
5. Pierce or carefully peel back the foil seal to gain access to wells. Use caution and avoid shredding the seal.
6. If you are starting with a plate of 0.5 nmol per well, resuspend arrayed crRNAs to 10 µM solution by adding 50 µL of 10

µM tracrRNA solution in nuclease-free 10 mM Tris pH 7.4 buffer to 0.5 nmol of crRNA (for different quantities of crRNAs see Appendix, Table 3). This will generate 10 µM of crRNA:tracrRNA master plates.
7. Pipette solution up and down 3–5 times while avoiding introduction of bubbles.
8. Seal the plate and place it on an orbital mixer/shaker for 70–90 minutes at room temperature.
9. Briefly centrifuge plates to collect solution to bottom of the wells.
10. From the master crRNA:tracrRNA plate, generate daughter crRNA:tracrRNA plates with 2 µM working concentration using nuclease-free, 10 mM Tris pH 7.4 buffer. This eliminates the subsequent requirement for pipetting of very small volumes.
11. crRNA:tracrRNA plates may now be used immediately, aliquoted into single-use plates (5 µL/well is recommended for direct use in lipid transfection—see next section) or stored at –20 °C in a manual defrost or non-cycling freezer.
12. For storage, seal plates with appropriate adhesive or heat seals.

Alternatively, the crRNAs can be resuspended in 10 mM Tris buffer to generate master plates of 10 µM crRNA, and the tracrRNA can be added during the generation of the daughter plates. Use equimolar amounts of crRNA and tracrRNA to generate 2 µM crRNA:tracrRNA working plates.

The following is a general protocol for transfection of arrayed crRNA:tracrRNA libraries using stable Cas9-expressing or dCas9-VPR-expressing mammalian cells in 96-well plates in triplicate at 25 nM final concentration of the crRNA:tracrRNA complex. Optimal plating density for each transfection method will depend on growth characteristics of specific cell lines and assay requirements and these parameters should be determined experimentally. Exact parameters for crRNA:tracrRNA transfection in your cells of interest should be empirically determined through careful optimization prior to experimentation (see Appendix for Optimization of transfection conditions with crRNA:tracrRNA). Catalog crRNA library plates are supplied with columns 1 and 12 empty to allow addition of researcher-defined controls. We suggest including the following controls:

1. Untreated cells
2. Positive control crRNA
3. Non-targeting crRNA (negative control)

The protocol is provided for transfection of one arrayed crRNA plate in triplicate for a final 25 nM concentration of the crRNA and tracrRNA. Calculations are done for quadruplicates providing excess volume for the ease of pipetting. This protocol is written for a direct use of the daughter plates containing 5 µL of 2 µM crRNA:tracrRNA complex for preparation of the transfection mix. This protocol uses the least amount of pipetting and liquid-handling steps.

Positive and negative crRNA controls can be added to empty wells of the V-bottom transfection mix plate (columns 1 and 12 in catalog libraries).

1. Prepare transfection reagent working solution by diluting the transfection reagent stock solution in serum-free medium. Preparing 9 mL volume will allow for transfection of one crRNA library plate in triplicate and includes excess for ease of pipetting. For example, if the optimal amount of transfection reagent was determined to be 0.1 µL per well of cells, add 48 µL of transfection reagent stock solution to serum-free medium for a total volume of 9 mL See table 1 for additional volumes in plate format, or table 2 for individual well recommendations.
2. Add 75 µL of transfection reagent working solution to each well of 96- well V-bottom daughter plate containing 5 µL of 2 µM crRNA:tracrRNA complex. This brings the total volume to 80 µL and the concentration of the crRNA and tracrRNA to 250 nM.
3. Immediately mix by pipetting gently up and down and incubate for 20 minutes at room temperature.
4. Briefly mix the transfection mix (by pipetting) in the V-bottom plates after 20 min incubation.
5. Add transfection mix to your cells.
    - a. For the Forward transfection method, seed cells a day in advance. Before transfection replace the media with 80 µL of fresh cell media and add 20 µL of transfection mix from the 96-well V-bottom transfection mix prepared in step 3 plate to corresponding wells of the 96-well tissue culture plate. This will bring the volume to 100 µL and the final concentration of the crRNA:tracrRNA complex to 25 nM. Repeat this step for the other two plates to obtain triplicates.

- b. For the Reverse transfection method, add 20 µL of the transfection mixture to each well of three new 96-well tissue culture plates and add 80 µL of the cell suspension prepared in step 4b to each well of the three 96-well tissue culture plates. This will bring the volume to 100 µL and the final concentration of the crRNA:tracrRNA complex to 25 nM.

6. Incubate transfected cells at 37 °C in a humidified CO2 incubator for 48–96 hours before proceeding with the phenotypic assay or gene analysis

Table 1. Preparation of different concentration working solution of transfection reagent for transfection of one arrayed crRNA plate in triplicate. The highlighted row indicates the experimental conditions used in steps 1–3, above.

Transfection reagent volume per well of cells (µL)	Transfection reagent volume (µL)	Serum-free medium volume (mL)
0.01	6	9
0.025	12	9
0.5	24	9
0.1	**48**	**9**
0.2	96	8.9
0.3	144	8.9
0.4	192	8.8
0.5	240	8.8
0.6	288	8.7

Time Taken

2 hours

Notes and Comments

Optimization of transfection conditions for delivery of crRNA:tracrRNA

To obtain the highest transfection efficiency of the Edit-R crRNA:tracrRNA components with minimal effects on cell viability, we recommend carefully optimizing transfection conditions for each cell line using a positive control crRNA. The transfection optimization can be easily performed in a 96-well format allowing for testing of multiple transfection conditions. Transfection conditions that have previously been optimized for siRNA delivery are a reasonable starting point for crRNA:tracrRNA transfection optimization. Cell-type specific guidelines for the four DharmaFECT formulations can be found in the DharmaFECT

Cell Type Guide.

The optimization experiment should include two to three cell densities and a range of DharmaFECT Transfection Reagent volumes. Our recommendations for the different components in the transfection optimization experiment are as follows:

- 0.05 to 0.8 µL/well of DharmaFECT 1, 2, 3, or 4 in a 96-well plate
- 25 nM positive control (PPIB or DNMT3B) crRNA:tracrRNA per well (recommended range 20 nM to 50 nM).

Use Table 2 for guidance in preparation of samples for 96-well transfection optimization.

At 48–72 hours post-transfection, perform a cell viability assay to determine the highest lipid concentration that has minimal cell toxicity (≥ 80% cell viability is preferred). After assaying for cell viability, we recommend that you carefully wash the cells once with PBS and proceed with either a gene editing assay for the gene knockout libraries (see below) to determine the condition that produces the best editing efficiency or RT-qPCR analysis for the CRISPRa libraries to determine the conditions that produce the best transcriptional activation (see assay recommendation below).Use these optimal determined conditions for subsequent transfection of your selected Cas9 expressing cell lines with the Edit-R crRNA:tracrRNA.

Gene editing assay recommendations for the CRISPR knockout libraries

A commonly used method for detection of insertions and deletions (indels) in a cell population is a mismatch detection assay using T7 Endonuclease I (T7EI). These assays can be performed on either purified genomic DNA or whole cell lysate. A detailed protocol is provided.

Table 2. Preparing samples for 96-well transfection optimization. Volumes (µL) are shown per ONE well of a 96-well plate; for triplicate wells multiply all values by 4 to have sufficient volume for three wells and to account for pipetting error. For the diluted transfection reagent prepare a larger volume to enable accurate pipetting of the small volumes that are required.

	Tube 1: crRNA: tracrRNA (µL/well)	Tube 2: Diluted DharmaFECT Transfection reagent (µL/well)		Final reagent volumes		
Transfection condition DharmaFECT volume (µL/well)	2 µM crRNA: tracrRNA (µL)	Serum-free medium	DharmaFECT Transfection reagent	Transfection Mix volume (µL)	Growth Medium or cell suspension (µL)	Total volume per 96-well (µL)
0.01 µL/well	1.25	18.74	0.01	20	80	100
0.025 µL/well	1.25	18.73	0.025	20	80	100
0.05 µL/well	1.25	18.70	0.05	20	80	100
0.1 µL/well	1.25	18.65	0.1	20	80	100
0.2 µL/well	1.25	18.55	0.2	20	80	100
0.3 µL/well	1.25	18.45	0.3	20	80	100
0.4 µL/well	1.25	18.35	0.4	20	80	100
0.5 µL/well	1.25	18.25	0.5	20	80	100
0.6 µL/well	1.25	18.15	0.6	20	80	100
Untreated	0	20.00	0	20	80	100

Gene expression analysis recommendations for CRISPRa libraries

RNA can be isolated using different methods per manufacturer's instructions. Quantitative RT-qPCR analysis can be performed using gene expression assays according to manufacturer's instructions. Use the expression of a housekeeping gene for normalization of the expression of the gene of interest. Follow best practices for RT-qPCR analysis with appropriate number of technical replicates and proper controls.

Frequently asked questions

How should I store my crRNA and tracrRNAs?

RNA oligonucleotides should be stored at –20 °C or –80 °C in a non-frost free freezer, either as a dried pellet or resuspended in an RNase-free solution buffered to pH 7.4 to help with stability during freeze-thaw cycles. We recommend that RNA

oligonucleotides be resuspended to a convenient stock concentration (Table 3) and stored in small aliquots to avoid multiple freeze-thaw cycles. RNA oligonucleotides should not go through more than four to five freeze thaw cycles. If degradation is a concern, the integrity of the RNA oligonucleotides can be evaluated on an analytical PAGE gel.

Table 3. Making stock solutions of crRNA and tracrRNA.

crRNA or tracrRNA amount (nmol)	Volume (µL) of 10 mM Tris pH 7.4 to be added for desired final concentration		
	100 µM stock	10 µM stock	4 µM stock
0.1	*	_	25
0.25	*	25	62.5
0.5	*	50	*
2	20	200	*
5	50	500	*
20	200	2000	*
50	500	*	*
200	2000	*	*

* not recommended due to either small volume for proper mixing or large volume that exceeds the tube (well) volume when tube volume is exceeded, make the larger stock and make the dilution with 10 mM Tris buffer pH7.4 in a separate tube (plate) 4 µM stock is provided so that mixing of equal volumes of crRNA and tracrRNA can generate the working dilution of 2 µM crRNA:tracrRNA for lipid mediated transfection.

What is the stability of the Edit-R crRNA and tracrRNAs? Dried RNA oligonucleotide pellets are stable at room temperature for two to four weeks, but should be placed at –20 °C or –80 °C for long-term storage. Under these conditions, the dried tracrRNA and crRNAs will be stable for at least one year. Maintaining sterile, RNase- and DNase-free conditions is always recommended as a critical precaution.

Can I use my siRNA transfection protocols to transfect Edit-R synthetic crRNA and tracrRNAs?
Previously optimized protocols to transfect synthetic siRNA into your cells of interest can be a good starting point for transfection of synthetic crRNA:tracrRNA.

Can I use a different transfection reagent other than DharmaFECT Transfection Reagents to deliver the Edit-R components into my cells?
We cannot predict the performance of other transfection reagents, nor can we troubleshoot experiments performed with any

reagent other than DharmaFECT Transfection reagents. However, other suitable transfection reagents designed for RNA transfection could be utilized provided transfection conditions are carefully optimized for each cell line of interest.

Can I co-transfect arrayed synthetic crRNA:tracrRNA with the Edit-R Cas9 Nuclease Expression plasmid?
You can perform genome engineering by transient transfection of the synthetic crRNA:tracrRNA with the Edit-R Cas9 Nuclease Expression plasmids using DharmaFECT Duo Transfection Reagent. However, for performing phenotypic analysis in the cell population in a high-throughput manner, we have found that crRNA:tracrRNA transfection in a cell line that stably expresses Cas9 nuclease produces higher efficiency gene editing in the cell population with lower toxicity associated with the transfection. If the generation of Cas9 stable cells is not possible we recommend codelivery of the crRNA:tracrRNA with either Cas9 mRNA or Cas9 protein.

Can I co-transfect the Edit-R CRISPRa crRNA:tracrRNA components with the Edit-R CRISPRa Lentiviral dCas9-VPR expression plasmid?
We do not recommend doing a co-transfection of Edit-R Lentiviral dCas9-VPR Expression plasmids with synthetic guide RNAs due to the transient nature of transcriptional activation with the non-expressed guide RNAs and the longer time necessary to perform blasticidin selection (typically > 5 days) to remove the non-transfected cells. For transient expression experiments, or to avoid the use of lentiviral particles, we recommend doing a co-transfection of the Edit-R sgRNA plasmid with the Lentiviral dCas9-VPR expression plasmid which then allows for selection of both components.

Can I use Edit-R CRISPRa crRNA in the dCas9-SAM system?
Yes. Synthetic Edit-R CRISPRa crRNA may be used with synthetic Edit-R SAM tracrRNA for activation in cells expressing the dCas9-SAM system.

Can I use the Edit-R SAM tracrRNA with the VPR system?
No. Because of the added MS2 aptamer sequence, Edit-R SAM tracrRNA is specifically designed for use in activation experiments with cells expressing the SAM components dCas9-VP64 and MS2-p65-HSF1. If working in the dCas9-VPR system, the standard Edit-R tracrRNA (Cat# U-002005-XX) should be used for synthetic crRNA: tracrRNA experiments.

Can I co-transfect arrayed synthetic crRNA:tracrRNA with the Edit-R dCas9-VPR mRNA?
Yes, dCas9-VPR mRNA can be used with arrayed synthetic crRNA:tracrRNA for activation in cells not stably expressing the dCas9-VPR system.

References

1. M. A. Horlbeck et al., Compact and highly active next-generation libraries for CRISPR-mediated gene repression and activation. eLife. 5, e19760 (2016).