PROTOCOL

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INSPIRED CELL SOLUTIONS

Dharmacon™ Edit-R™ sgRNA Libraries

A protocol for transfection of arrayed synthetic sgRNA plates for gene knockout

Guidelines for using arrayed sgRNA plates for gene knockout

Product description

Libraries of predesigned synthetic single guide RNAs (sgRNAs) provide an opportunity to use the CRISPR-Cas9 system for functional gene knockout analysis, in an arrayed format. Dharmacon sgRNA libraries for functional gene knockout consist of Edit-R synthetic sgRNAs which are predesigned using the proprietary Edit-R CRISPR RNA algorithm. This algorithm was 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 off-target identification.

Edit-R sgRNA Libraries are available as the following:

- Catalog libraries of predefined gene family collections for humano
  - » as a pool of three sgRNA per well,
  - » at 0.1 nmol, 0.25 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 screen-specific positive and non-targeting controls.
  - » ECHO qualified plates are available upon request.
- **Cherry-pick sgRNA libraries** based on a customer's gene list.
  - » Multiple individual sgRNAs or a sgRNA pool of three constructs, minimum 20 wells. Learn more or get started
  - » Customizable plate layout; Edit-R catalog control sgRNAs 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). ECHO qualified plates are available upon request

Materials

Plates of synthetic sgRNAs, up to 0.5 nmol per well in 96-well plates or 384-well plates. Additional required materials are listed below and are not provided with sgRNA library purchase.

- 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 #B-006000-100)
- 96-well tissue culture plates
- 96-well V-bottom polystyrene standard storage plates or deep well plates (for example, Thermo Scientific™ 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 sgRNA and detection primers for assessment of gene editing
- Non-targeting control sgRNA
- Assay-specific positive control sgRNA (defined by researcher)
- Growth medium: antibiotic-free cell culture medium (with serum and/or supplements) recommended for maintenance ofthe 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 sgRNA libraries for gene knockout, we strongly recommend using cell lines that constitutively express Cas9 nuclease. Transfection of sgRNA 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.

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

Guidelines for resuspension of the sgRNA

1. The sgRNA 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, sgRNAs can be stored as dry pellets(unopened) at 4 °C for several weeks.
2. Briefly centrifuge sgRNA plates to ensure that the sgRNA is collected at the bottom of the well.
3. Wipe adhesive foil cover with 70% ethanol or RNase-decontamination solution.
4. Carefully peel back the foil seal to gain access to wells. Use caution and avoid shredding the seal.
5. If you are starting with a plate of 0.5 nmol per well, resuspend arrayed sgRNAs to 10 µM solution by adding 50 µL ofnuclease-free 10 mM Tris pH 7.4 buffer to each well (for different quantities of sgRNAs see Appendix, Table 3).
6. Pipette solution up and down 3–5 times while avoiding introduction of bubbles.
7. Seal the plate and place it on an orbital mixer/shaker for 70–90 minutes at room temperature.
8. Briefly centrifuge plates to collect solution at the bottom of the wells.
9. From the master sgRNA plate, generate daughter sgRNA plates of 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.
10. sgRNA plates may now be used immediately, aliquoted into single-use plates (5 µL/well is recommended for directuse in lipid transfection—see next section) or stored at –20 °C in a manual defrost or non-cycling freezer.
11. For storage, seal plates with appropriate adhesive or heat seals.

Transfection protocol of arrayed sgRNA plates

The following is a general protocol for transfection of arrayed sgRNA libraries using stable Cas9-expressing mammalian cells in 96-well plates. Optimal plating density for each transfection method will depend on growth characteristics of specific cell lines andassay requirements. Exact parameters for sgRNA transfection in your cells of interest should be empirically determined throughcareful optimization prior to experimentation (see Appendix for Optimization of transfection conditions with sgRNA). Catalog sgRNAlibrary 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 sgRNA
3. Non-targeting sgRNA (negative control)

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

Note: Positive and negative sgRNA controls can be added to empty wells of the V-bottom transfectionmix 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 96-well sgRNA library plate in triplicate and includes excess forease 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 foradditional volumes for a plate, 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 µLof 2 µM sgRNA. This brings the total volume to 80 µL and the concentration of the sgRNA to 125 nM.
3. Immediately mix by pipetting gently up and down and incubate for 20 minutes at room temperature.
4. Briefly mix the transfection mix in the V-bottom plates after 20 minutes incubation by gently pipetting up and down.
5. Add transfection mix to your cells.
   a. For the Forward transfection method, seed cells a day in advance. Before transfection, replace the medium with80 µL of fresh cell growth medium and add 20 µL of transfection mix from the 96-well V-bottom transfection mixplate prepared in step 4 plate to corresponding wells of the 96-well tissue culture plate. This will bring the volumeto 100 µL and the final concentration of the sgRNA to 25 nM. Repeat this step for the other two plates to obtaintriplicates.
   b. For the Reverse transfection method, add 20 µL of the transfection mixture to each well of three new 96-welltissue culture plates and add 80 µL of the cell suspension 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 sgRNA to 25 nM.
6. Incubate transfected cells at 37 °C in a humidified CO_2 incubator for 48–96 hours before proceeding with the phenotypicassay or gene expression analysis.

Table 1. Preparation of transfection reagent working solutions for transfection of one arrayed sgRNA plate in triplicate. The highlighted row indicates the experimental conditions described 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
0.05	24	9
0.01	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

Appendix

Optimization of transfection conditions for delivery of sgRNA

To obtain the highest transfection efficiency of the Edit-R sgRNA with minimal effects on cell viability, we recommend carefully optimizing transfection conditions for each cell line using a positive control sgRNA. The transfection optimization can be easily performed in a 96-well format allowing for testing of multiple transfection parameters. Transfection conditions that have been previously optimized for siRNA delivery are a reasonable starting point for sgRNA 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 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) sgRNA per well (or 20–50 nM range).

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. Use these determined optimal conditions for subsequent transfection of your selected Cas9 expressing cell lines with the Edit-R sgRNA.

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). This assay 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: sgRNA (µL/well)	Tube 2: Diluted DharmaFECT Transfection reagent (µL/well)			Final reagent volumes		
Transfection condition DharmaFECT volume (µL/well)	2 µM sgRNA (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

Frequently asked questions

How should I store my sgRNA?

RNA oligonucleotides should be stored at –20 °C or –80 °C in a non-frost free freezer, either as dried pellets, or resuspended in an RNase-free solution buffered to pH 7.4 to help maintain 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 sgRNA.

sgRNA amount (nmol)	Volume (µL) of 10 mM Tris pH 7.4 to be added for desired final concentration		
	100 µM stock	10 µM stock	2 µM stock
0.01	*	_	50
0.25	*	25	125
0.5	*	50	*
2	20	200	*
5	50	500	*
20	200	2000	*

*not recommended due to either small volume for proper mixing or large volume that exceeds the tube (well) volume. When tube volume isexceeded, make the larger stock and make the dilution with 10 mM Tris buffer pH7.4 in a separate tube (plate).

What is the stability of the Edit-R sgRNA?

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 sgRNA 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 a 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 sgRNA with the Edit-R Cas9 Nuclease Expression plasmid?

You can perform genome engineering by transient transfection of the synthetic sgRNA 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 format, we have found that sgRNA transfection in a cell line that stably expresses Cas9 nuclease produces higher efficiency gene editing with lower toxicity associated with the transfection. If the generation of Cas9 stable cells is not possible, we recommend co-delivery of the sgRNA with either Cas9 mRNA or Cas9 protein.

What plate layout can I expect for my library?

Catalog 96-well libraries are fulfilled with the following plate layout: 80-wells per plate, columns 1 and 12 left empty. Catalog 384-well libraries are fulfilled as 320-wells per plate, columns 1, 2 and 23, 24 left empty.

NOTE: Please refer to the platemap provided with order for your precise layout, and contact Technical Support with any questions.

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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