GE Healthcare

Dharmacon™ Edit-R™ crRNA Libraries

A guide for using arrayed crRNA plates

Protocol

Table of Contents

1 Guidelines for using arrayed crRNA plates

Product description

Libraries of predesigned synthetic crRNAs provide an opportunity to apply the CRISPR-Cas9 system for functional gene knockout analysis in an arrayed format. Dharmacon crRNA libraries consist of Edit-R synthetic crRNAs 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 crRNA Libraries are available as the following:

- Catalog libraries of predefined gene family collections for human and mouse
  - » Four crRNA per gene, 80 wells per plate, columns 1 and 12 left open, 0.5 nmol per well in 96-well plates
  - » Provided in NUNC Polystyrene 96-well V-bottom plates (Cat #249952)
- Cherry-pick crRNA libraries based on a customer's gene list. Learn more or get started at http://dharmacon.gelifesciences.com/cherry-pick-libraries/
  - » One to five crRNA per gene (minimum of three is recommended) in 96-well plates, minimum 40 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)

Materials

- Plates of synthetic crRNAs, up to 2 nmol per well in 96-well plates

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

- Edit-R tracrRNA, 5, 20 or 50 nmol (Dharmacon, Cat #U-002005-XX). tracrRNA is required for use with all synthetic crRNA reagents.
- 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, NUNC Cat #249952 or Cat #12-565-553)
- Assay for assessing cell viability such as CellTiter-Blue® Cell Viability Assay (Promega Corp., Cat #G8081)
- Assay(s) for detecting gene editing events in a cell population (http://dharmacon.gelifesciences.com/uploadedFiles/Resources/crrna-positive-controls-protocol.pdf)
- Positive control crRNA and detection primers for assessment of gene editing (http://dharmacon.gelifesciences.com/gene-editing/crispr-cas9/edit-r-synthetic-positive-crrna-controls-and-detection-primers/)
- Non-targeting control crRNA (http://dharmacon.gelifesciences.com/gene-editing/crispr-cas9/edit-r-synthetic-crrna-non-targeting-controls/)
  Assay-specific positive control crRNA (defined by researcher)
- 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 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. For generation of the Cas9 stable expressing cells please follow recommendations in the **Gene Engineering with Lentiviral Cas9 Particles and Synthetic CRISPR RNAs** manual (http://dharmacon.gelifesciences.com/uploadedFiles/Resources/edit-r-lenti-cas9-particles-and-synthetic-crispr-rnas-manual.pdf).

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

Guidelines for resuspension of the crRNA

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. Briefly centrifuge plates to ensure that the crRNA is collected at the bottom of the well.
3. Wipe adhesive foil cover with 70% ethanol or other RNase-decontamination solution.
4. Pierce or 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 crRNAs to 10 µM solution by adding 50 µL nuclease-free 10 mM Tris pH 7.4 buffer to 0.5 nmol of crRNA (for different quantities of crRNAs 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 to bottom of the wells.
9. From the master crRNA plate, generate plates with 1 µM working concentration of crRNAs using nuclease-free, 10 mM Tris pH 7.4 buffer. This eliminates the subsequent requirement for pipetting of very small volumes.
10. crRNA plates may now be used immediately, aliquoted into single-use plates 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 of arrayed crRNA

The following is a general protocol for transfection of arrayed crRNA libraries using stable Cas9-expressing mammalian cells in 96-well plates in triplicate at 25 nM final concentration of the crRNA:tracrRNA complex. Optimal plating density 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. Negative control: non-targeting crRNA

Day 1

1. Plate cells at cell density appropriate for the screen assay in 96-well plates using growth medium. To screen in triplicate, create three cell plates for each arrayed crRNA plate.
2. Incubate cells at 37 °C in a humidified CO_2 incubator overnight.

Day 2

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 for the ease of pipetting.

3. Transfer 10 µL of 1 µM working crRNA solution to each well of a 96-well V-bottom transfection mix plate (NUNC polystyrene 96 well V- bottom plates Cat #249952 or other appropriate plates may be used).

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).

4. Resuspend Edit-R tracrRNA to 10 µM stock solution in nuclease-free, 10 mM Tris pH 7.4 buffer (for example, add 500 µL to 5 nmol of tracrRNA).
5. Prepare 333 nM tracrRNA working solution by adding 120 µL of 10 µM tracrRNA stock solution to serum-free medium for a total volume of 3.6 mL. This will allow preparation of one arrayed crRNA library plate in triplicate and includes excess for ease of pipetting.
6. Add 30 µL of 333 nM working tracrRNA solution to each well of 96-well V-bottom transfection mix plate containing 10 µL of crRNA, prepared in step 3. This will bring the concentration of the crRNA and tracrRNA to 250 nM.
7. Prepare transfection reagent working solution by diluting the transfection reagent stock solution in serum-free medium for a total volume of 5 mL. This volume will allow preparation 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 50 µL of transfection reagent stock solution to serum-free medium for a total volume of 5 mL. For preparations of other working concentrations of transfection reagent see Table 1.
8. Add 40 µL of transfection reagent working solution to each well of 96-well V-bottom transfection mix plate containing the crRNA:tracrRNA complex. This brings the total volume to 80 µL.
9. Immediately mix by pipetting gently up and down and incubate for 20 minutes at room temperature.
10. Replace the medium in each well of three 96-well tissue culture plates with cells with 80 µL of the appropriate growth medium.
11. Briefly mix the transfection mix (by pipetting) in the V-bottom plates after 20 min incubation.

12. Add 20 µL of transfection mix from the 96-well V-bottom transfection mix plate to corresponding wells of the 96-well tissue culture plate. This will bringthe volume to 100 µL and the final concentration of the crRNA:tracrRNA complex to 25 nM. Repeat this step for the other two cell plates to obtain triplicates.

The transfection mix can alternatively be prepared in deep well 96-well plates (steps 1-8). After the 20 minute incubation (step 9) add 320 µL of growth medium per well in the deep well plates and mix well. Replace the medium in each well of three 96-well tissue culture cell plates with 100 µL of the complete transfection mix.

13. Incubate transfected cells at 37 °C in a humidified CO_2 incubator for 48-72 hours before proceeding with the phenotypic assay or gene editing analysis.

Table 1. Preparation of different concentration working solution of transfection reagent for transfection of one arrayed crRNA plate in triplicate.

Transfection reagent volume per well of cells (µL)	Transfection reagent volume (µL)	Serum-free medium volume (mL)
0.05	25	4.98
0.1	50	4.95
0.2	100	4.90
0.3	150	4.85
0.4	200	4.80
0.6	300	4.70

2 Appendix

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.

Table 2. Preparing samples for 96-well transfection optimization. Mix the diluted crRNA:tracrRNA and diluted transfection regent content and incubate the transfection mix for 20 minutes. Add growth medium and use the total volume to replace the medium in the cell culture plate well. Volumes (µL) are shown per ONE well of a 96-well plate; for triplicate wells multiply all values by 3.5 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 per one well.

	Tube 1: Diluted crRNA:tracrRNA			Tube 2: Diluted DharmaFECT Transfection reagent				
Transfection condition DharmaFECT volume (µL) /well	Serum-free medium (µL)	Volume of 1 µM crRNA (µL)	Volume of 1 µM tracrRNA (µL)	Serum-free medium (µL)	Volume of DharmaFECT (µL)	Transfection mix volume (µL)	Growth medium (µL)	Total volume per 96-well (µL)
0.05 µL/well	5	2.5	2.5	9.95	0.05	20	80	100
0.1 µL/well	5	2.5	2.5	9.9	0.1	20	80	100
0.2 µL/well	5	2.5	2.5	9.8	0.2	20	80	100
0.3 µL/well	5	2.5	2.5	9.7	0.3	20	80	100
0.4 µL/well	5	2.5	2.5	9.6	0.4	20	80	100
0.6 µL/well	5	2.5	2.5	9.4	0.6	20	80	100
0.8 µL/well	5	2.5	2.5	9.2	0.8	20	80	100
Untreated (0 µL/well)	10	0	0	10	0	20	80	100

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 a gene editing assay (see below) to determine the condition that produces the best editing efficiency.

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

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. Detail protocol is provided in: http://dharmacon.gelifesciences.com/uploadedFiles/Resources/crrna-positive-controls-protocol.pdf.

3 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 2) 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
0.5	-	50
2	20	200
5	50	500
20	200	2000
50	500	Exceeds tube volume*

*When tube volume is exceeded, make a 100 µM stock and dilute it 1:10 to obtain the 10 µM stock.

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 plasmids?

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:tracrRNAtransfection 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.

Orders can be placed at:
gelifesciences.com/dharmacon

Customer Support: cs.dharmacon@ge.com
Technical Support: ts.dharmacon@ge.com or
+1 800 235 9880; +1 303 604 9499 if you have any questions.

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