Protocol

Dharmacon™ Edit-R™ sgRNA RNP Electroporation

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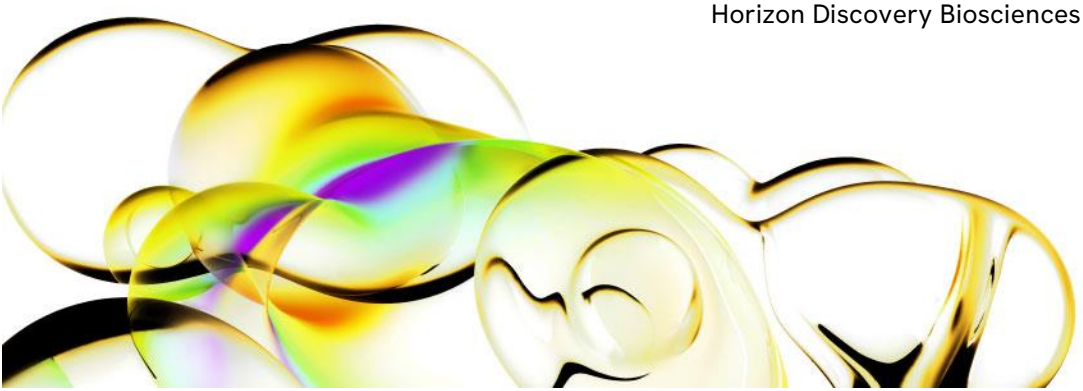
Horizon Discovery Biosciences Ltd. (A Revvity Company)



Protocol for RNP electroporation of arrayed synthetic sgRNAs for CRISPR 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 designed utilizing 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 also includes specificity scoring using an internal alignment tool for complete off-target identification.

Formats

- Catalog libraries of predefined gene family collections.
- As a pool of three sgRNA per well, 1 nmol/well.
- Provided in NUNC Polystyrene 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 desired gene list.
- Multiple individual sgRNAs or a sgRNA pool of three constructs, minimum 20 wells.
- Customizable plate layout; Edit-R catalog control sgRNAs may be added to any wells within the plate(s).

For phenotypic analysis with the arrayed synthetic sgRNA libraries for gene knockout, we strongly recommend using cell lines that constitutively express Cas9 nuclease. When cells constitutively expressing Cas9 nuclease are not feasible, such as for work in primary cells, transfection of sgRNA and Cas9 protein as a ribonucleoprotein (RNP) complex is recommended. Successful electroporation of the Cas9 RNP complex allows for downstream phenotypic analysis of the edited cell population.

Materials

Plates of synthetic sgRNAs, 1 nmol per well in 384-well plates. Additional required materials are listed below and are not provided with sgRNA library purchase:

- Edit-R Cas9 Protein Hybrid NLS (Cat# CAS135XX)* is available as:
  - 50 µg, 100 µg, 500 µg, and 5 x 500 µg sizes

    *Note: This protocol is also recommended for Cas9 nuclease protein NLS (Cat# CAS122XX). Further optimization may be required.
- 10 mM Tris pH 7.4 nuclease-free buffer solution (Dharmacon, Cat #B-006000-100)
- 384-well tissue culture plates
- Assay for assessing cell viability such as CellTiter-Blue® Cell Viability Assay (Promega Corp., Cat #G8081)

- sgRNA controls
  - Positive control sgRNA and detection primers for assessment of gene editing.
    - Edit-R PPIB or DNMT3B positive controls and kits are designed and validated for mismatch detection assays to verify gene editing experiments.
    - Edit-R lethal synthetic sgRNA controls are universal positive controls designed to induce cell death in a dose- and Cas9-dependent manner by targeting multiple repeat regions in the genome.
  - Edit-R non-targeting control sgRNA.
  - Assay-specific positive control sgRNA (defined by the researcher).
- Cell maintenance medium: antibiotic-free cell culture medium (with serum and/or supplements) recommended for maintenance of the cells of interest.
- Electroporation instrument and reagents
  - 384-well Nucleofector™ System
  - P3 Primary Cell 384-well Nucleofector™ Kit, or
  - SF, SE, SG Cell Line 384-well Nucleofector™ Kit (Lonza Cell Line Optimization Kit recommended for prior determination of most suitable buffer for cell line of interest)

CRISPR sgRNA library nucleofection using RNPs

The protocol for RNP electroporation of arrayed sgRNA libraries needs to be carefully optimized. Cell density will depend on the growth characteristics of specific cell lines and assay requirements. Exact parameters for sgRNA electroporation in your cells of interest should be empirically determined through careful optimization before experimentation. Catalog sgRNA library plates are supplied with columns 1, 2, 23 and 24 empty to allow the addition of researcher-defined controls.

Optimization parameters

Editing efficiency will correlate directly with the transfection efficiency, therefore, optimal delivery conditions will increase knockout efficiency. Exact reagents, amounts and parameters for electroporation should be empirically determined through careful optimization in cells of interest, in accordance with the electroporation instrument manufacturer's recommendations.

1. Optimize the number of cells for electroporation specific to your cells of interest and assay requirements. A range between 10^5-10^6 cells can be electroporated per well in a 384-well electroporation plate
2. sgRNA:Cas9 protein amounts and ratio
   - Examine different amounts of sgRNA and Cas9 proteins with different ratios of sgRNA to Cas9 protein. Test 30-150 pmol sgRNA and 15-60 pmol of Cas9 protein per 20 µL of electroporation volume in different ratios of sgRNA to Cas9 protein (4:1, 3:1, and 2:1)
3. Optimization controls
   - Table 1 provides a list suggested control for nucleofection optimization. We recommend assaying each reagent/condition in triplicate.
     i. Positive controls PPIB or DNMT3B can be used to assess editing efficiency by T7E1 and TIDE analysis

ii. Lethal controls provide a quick assessment of electroporation efficiency - when the nucleofection protocol is optimized and induces minimal cell death with the NTC controls, the, electroporation efficiency of the lethal controls correlates with cell death.

iii. Custom control, a known gene that affects the phenotype readout e.g., expression of a marker that can be assessed and detected in the read-out assay.

4. Time course to establish the best readout timing after RNP delivery
   - This can be cell- and assay-specific and we suggest examination between 2 to 7 days post-electroporation.

Table 1. Controls for electroporation experiment

Reagent/condition	Control type
PPIB or DNMT3B positive control sgRNA	Positive control to assess editing efficiency
Non-targeting control sgRNA	Effect of CRISPR reagents without DNA cut
Cas9 only	Non-specific Cas9 effect
Lethal sgRNA control #1 or #2	Electroporation efficiency
Custom control	Assay specific control
Non-electroporated cells	Cell baseline response
Electroporated cells	Effect of electroporation on readout

Guidelines for resuspension of the sgRNA

For automated or high throughput electroporation workflows, higher stock concentrations of sgRNA are required (50–100 μM) to account for the dead volume in liquid handling or acoustic dispensing

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. For a 384-well plate library with 1 nmol of sgRNA per well, resuspend arrayed material to 100 μM solution by adding 10 μL of nuclease-free 10 mM Tris pH 7.4 buffer to each well. Plan for any additional dead volume required for automation or other instrument. If necessary, increase the volume and make lower sgRNA concentration, however, do not go below 50 μM sgRNA solution.
6. Pipette solution up and down 3-5 times while avoiding the 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. sgRNA plates may now be used immediately or stored at –20 °C in a manual defrost or non-cycling freezer.
10. For storage, seal plates with appropriate adhesive or heat seals.

RNP nucleofection protocol

The protocol below is optimized for primary cells and provided as an example. Buffer type, cell density, pulse code, and timelines may vary, and should be optimized for the cells of interest.

1. Prepare electroporation buffer – For primary cells, use 384-well Nuclefector kit (Lonza cat # V5SP-3010)—mix P3 buffer and Supplement 1 according to manufacturer's recommendations
2. Retrieve the sgRNA library and Cas9 protein from the -20° C freezer. Thaw reagents on ice.
3. Prepare the RNP complex—transfer the CRISPR reagents (sgRNA and Cas9 protein) at the required amounts into a 384-well electroporation plate (384-well Nucleocuvette™ plate) using automation. We recommend making replicate plates.

   Note: Do not exceed 20% of electroporation volume (for 384-well Nucleocuvette™ plates, the electroporation volume is 20 µL, so the RNP complex should not exceed 4 µL).

   - For example, if it was determined that 120 pmol of sgRNA and 60 pmol of Cas9 protein gave optimal editing in your cells, use 1 µL of Cas9 protein per well (using Cat# CAS135XX at 60.7 µM) with 1.2 uL of 100 µM sgRNA per well for a total of 2.2 µL of RNP.
4. Briefly pulse spin the electroporation plate (up to 300 × g), add P3 electroporation buffer to 10 µL/well and incubate the RNP complex for 10-15 min at room temperature.
5. Prepare cell suspension containing a total number of cells needed for your experiment (number of plates × number of replicates × number of cells/well). Note: Cell numbers should be optimized for reagent amounts, but a range between 10^5-10^6 cells can be electroporated per well in a 384-well electroporation plate.
6. Pellet down the cell suspension by centrifugation (300 × g for 5 min) and wash with PBS (this step helps remove RNAse activity from trypsin or FBS in cell media).
7. Resuspend the cell pellet in complete P3 buffer and aliquot 10 µL of cell suspension per well.
8. Pulse-spin the 384-well electroporation plate up to 300 × g. This step helps removing any air bubbles in the wells prior to electroporation.
9. Electroporate the plate using the 384-well electroporator device from Lonza.

The pulse code for optimizing electroporation efficiency is dependent on cell type. It should be selected following optimization experiments recommended by the manufacturer.

10. Top up the plate with 30 µL/well of pre-warmed cell maintenance media and transfer desired amount of the electroporated cell suspensions into 384-well tissue culture plates with prewarmed cell maintenance media once the electroporation is complete, using automation.
11. Incubate the electroporated cells at 37 °C, 5% CO_2 for 2 to 7 days before performing genomic and/or functional analysis of the gene knockout.

    Note: The editing timeframe will depend on parameters such as the activation state of the cells or the half-life of the protein being targeted. Therefore, multiple timepoints might be required to confirm knockout.

If you have any questions, please visit us at horizondiscovery.com/contact-us
Horizon Discovery, 8100 Cambridge Research Park, Waterbeach, Cambridge, CB25 9TL, United Kingdom

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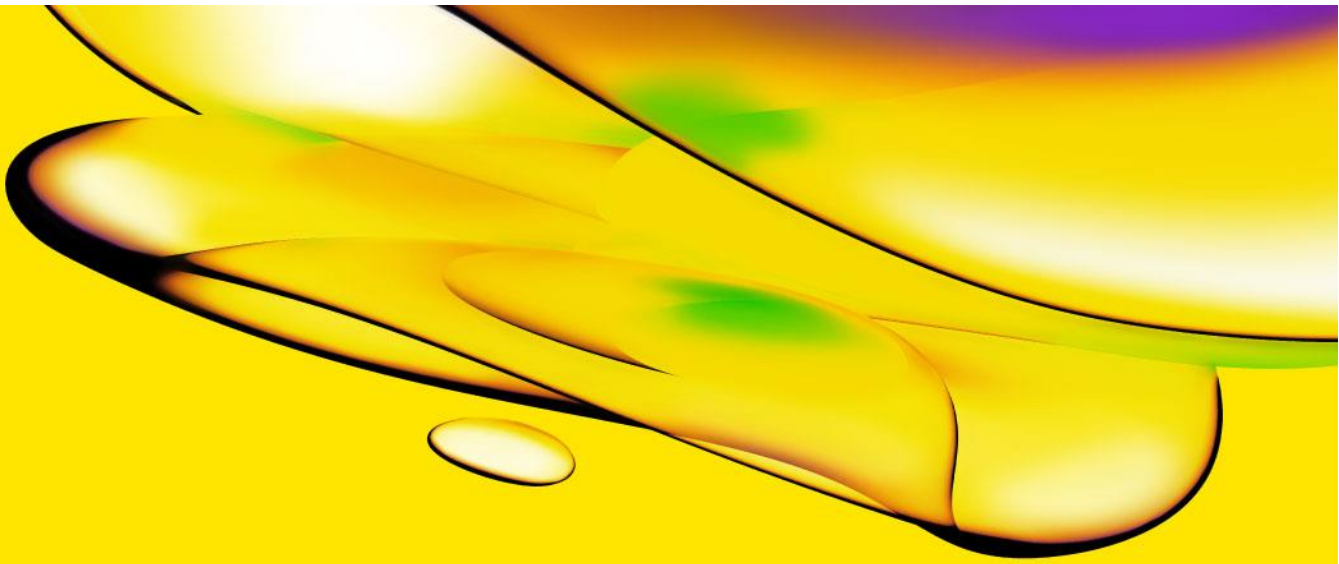

Horizon Discovery Biosciences Ltd. (A Revvity Company)
8100 Cambridge Research Park
Waterbeach, Cambridge, CB25 9TL, United Kingdom

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