CRISPR Editing of Immortalized Cell Lines with RNPs using Neon Electroporation

Developed by EditCo

Introduction

This protocol describes how to deliver ribonucleoprotein (RNP) complexes that consist of purified Cas9 nuclease duplexed with chemically modified synthetic single guide RNA (sgRNA) to immortalized adherent or suspension cells. RNP delivery is accomplished using the Thermo Fisher Neon™ Transfection System. A reference for electroporation settings for a wide variety of cell types is included. Chemically modified sgRNAs are designed to resist exonucleases and innate intracellular immune cascades that can lead to cell death. EditCo chemically modified synthetic sgRNAs are of exceptional purity and consistently drive high editing frequencies.

Abbreviations:

CRISPR: clustered regularly interspaced short palindromic repeats
Cas9: CRISPR associated protein 9
sgRNA: single guide RNA
RNP: ribonucleoprotein
PCR: polymerase chain reaction
ICE: inference of CRISPR edits
FACS: fluorescence-activated cell sorting
TE: Tris EDTA
PBS: phosphate-buffered saline
GFP: green fluorescent protein

Materials Required

Material	Ordering Information
Target-specific chemically modified sgRNA	Gene Knockout Kit (EditCo)
Cas9 2NLS nuclease *(S. pyogenes)*	EditCo, available at checkout
Positive control sgRNA or multi-guide sgRNA (optional)	Controls Kit or Transfection Optimization Kit (Multi-guide) (EditCo, available at checkout)
Transfection control (optional)	Recommended: pMAXGFP™ (Lonza)
TE buffer	Included in EditCo kits
Nuclease-free water	Included in EditCo kits
Neon™ Transfection System	Thermo Fisher Scientific, Catalog #MPK5000
Neon™ Transfection System 10 µl Kit Alternative: Neon™ Transfection System Starter Pack	Thermo Fisher Scientific, Catalog #MPK1025 Thermo Fisher Scientific, Catalog #MPK5000S
Cell counter	Multiple vendors (e.g., Thermo Fisher Scientific)
Normal growth medium	Cell-type dependent
1X PBS, cell culture grade	Multiple vendors (e.g, Thermo Fisher Scientific)
Tissue culture plates	Corning, Catalog #3526
Microcentrifuge tubes	Multiple vendors (e.g., Eppendorf)
TrypLE Express or preferred cell dissociation reagent	Multiple vendors (e.g, Thermo Fisher Scientific)

Note: All protocols outlined have been validated using materials mentioned in this manual. Materials other than the ones outlined in our manual may require additional optimization by the user.

General Guidelines

- Wearing gloves and using nuclease-free tubes and reagents is recommended in order to avoid RNase contamination.
- Always maintain sterile technique, and use sterile, filter pipette tips.
- All EditCo reagents should be stored according to the manufacturer's recommendations.
- Synthetic sgRNA should be dissolved in TE buffer and diluted to a working concentration using nuclease-free water. Please consult the EditCo Quick Start Guide to find best practices related to dissolving and storing synthetic sgRNAs.
- RNP complexes are stable at room temperature for up to 1 hour (may be stored at 4°C for up to one week, or at -20°C for up to 1 month). Note that RNPs stored at 4°C may become susceptible to contamination from microbial growth after long periods of time.

Optimization Guidelines

Optimization of editing efficiency for a specific cell type will require empirically determining the number of cells required, amount of Cas9 and ratio of sgRNA:Cas9. This guide is meant to provide a starting point for your CRISPR editing experiments.

EditCo highly recommends optimizing transfection conditions for your cell type using a postive control sgRNA prior to conducting your experiment using your target-specific sgRNA. Our Controls Kit can be used to optimize knockout or knock-in experiments that use one sgRNA per target. The Transfection Optimization can be used for knockout experiments using multi-guide sgRNA (i.e., in conjunction with the Gene Knockout Kit). Both kits are available at EditCo checkout.

Optimization of editing efficiency for a specific cell type may require varying the following:

- The number of cells per reaction
- Amount of Cas9
- Ratio of sgRNA:Cas9
- Electroporation setting*

* For specific electroporation settings for your cell type, we suggest consulting the Thermo Fisher Neon™ Transfection System Protocols and Cell Line Data.

Suggested Controls

Control	Description	Purpose
Mock	No Cas9 or sgRNA	Wild type sequence for comparison with experimental and other negative controls. Controls for toxicity from RNP, cell death from electroporation, or possible viability issues associated with editing the specific gene of interest.
Negative	Cas9 complexed with a non-targeting sgRNA or no sgRNA	Ensures that there are no false positives due to contamination (no effect expected=wild type).
Positive	sgRNA that has validated high editing efficiency	Ensures that all reagents, protocol, and equipment are functioning (effect expected). Used to optimize transfection conditions for a particular cell type.
Transfection	pMAXGFP™ vector (Lonza), GFP mRNA (SBI)	Assess transfection efficiency (without the use of RNPs).

Note: Positive and Transfection controls must be ordered separately.

Timeline

Pre-Electroporation		Setup & Electroporation	Post-Electroporation		
Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
Seed cells Incubate (2 days)		Prepare destination plates Prepare sgRNA and RNP Prepare cell suspension (electroporation solution)* Add RNP to cell suspension Transfect cells with electroporator** Replate cells Incubate (3 days) *resuspension buffer R (provided with Neon® Transfection System) **using Neon® 10µL tip			DNA extraction PCR amplification Sanger sequencing ICE analysis

Note: This timeline does not include optimization (which may take about 6 additional days).

Protocol

1. Pre-Electroporation

1.1. Seed Cells

a. Subculture cells 2 days before electroporation and seed cells in an appropriately sized vessel so that they are 70-80% confluent on the day of transfection. Each electroporation reaction will require approximately 1 x 10^5 - 2 x 10^5 cells, depending on the cell type.

Note: For cell type specific information, refer to Thermo Fisher Neon® Transfection System Protocols and Cell Line Data.

2. Setup & Electroporation

2.1. Prepare Destination Plate

a. Pre-warm 1 ml of normal growth medium in each well of a 12-well cell culture plate per reaction. This will serve as the destination plate after electroporation.

2.2. Assemble RNP Complexes (9:1 sgRNA to Cas9 ratio)

EditCo recommends sgRNA:Cas9 ratios between 3:1 and 9:1 for RNP formation. Below is an example using an sgRNA to Cas9 ratio of 9:1 for a single reaction (scale up appropriately).

a. Prepare sgRNA stock at 30 µM and Cas9 nuclease stock at 20 µM, and store at -80°C until use.

b. In appropriate plates/ tubes, assemble RNP complexes in the order shown below.

c. Incubate RNPs for 10 minutes at room temperature.

Note: The sgRNA:Cas9 ratios may need to be determined empirically to achieve optimal editing efficiency.

Component	Molarity	Volume
sgRNA	30 µM (pmol/ µl)	3 µl (90 pmol)
Cas9	20 µM (pmol/ µl)	0.5 µl (10 pmol)
Resuspension buffer	-	3.5 µl
Total volume	**-**	**7 µl**

2.3. Prepare Cells

Note: For suspension cells: spin down cells before each aspiration of culture medium and washes (step a below). Skip steps b and c below.

a. Aspirate cell culture medium and wash cells 1-2 times with 1X PBS.

b. Add TrypLE Express and incubate the cells for ~5 minutes, or until they detach from the plate completely.

Note: Do not shake or hit the flask to dislodge cells, as this may lead to clumping and inaccuracies in cell counting.

c. Neutralize the dissociation reaction with 2X volume of normal growth medium.

d. Count cells to determine the cell density (using cell counter).

e. Transfer 1-2 x 10^6 cells to a sterile microfuge tube. One tube will contain enough cells for ~10 transfections.

f. Centrifuge cells for 5 minutes at 500 x g. Aspirate medium.

g. Wash the cells once with 1X PBS and repeat the centrifugation step. Aspirate PBS.

h. Resuspend the cell pellet in 50 µl of resuspension buffer R (provided with Neon™ Transfection System 10 µl Kit).

Note: Avoid storing the cell suspension for more than 15 minutes at room temperature, as this reduces cell viability and transfection efficiency.

i. Add 5 µl of cell suspension to each RNP solution (7 µl) to make 12 µl of cell-RNP solution per reaction.

2.4. Transfect Cells

a. Aspirate 10 µl of cell-RNP solution to a 10 µl Neon tip.

b. Electroporate using cell type optimized conditions.

Note: Refer to Thermo Fisher Neon® Transfection System Protocols and Cell Line Data

c. Immediately transfer cells to a pre-warmed 12-well plate (prepared in step 2.1)

d. Incubate the cells for 2-3 days in a humidified 37°C/5% CO_2 incubator.

3. Post-Electroporation

3.1 Analysis

a. 72 hours post electroporation, isolate DNA, PCR target region, and Sanger- sequence amplicons. Conduct Inference of CRISPR Edits (ICE) analysis on Sanger sequences to determine editing efficiency. Please visit editco.bio/technical-resources for protocols on genotyping, ICE analysis, and clonal expansion. Next-Gen Sequencing, FACS, Western blot, or functional assays may also be conduced.

Option: If storing cells for future use is desired, split cells into two groups (one for analysis and one for cell culture).

Additional Information

For an up-to-date list of all protocols and other resources, please visit this link.

For technical assistance, contact our Scientific Support Team at technicalsupport@editco.bio.

For common FAQs, please visit this link.