EditCo

PROTOCOL

CRISPR Editing of Immortalized Cell Lines with RNPs using Nucleofection

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 Lonza 4D Nucleofector™ unit with 16-well Nucleocuvette™ Strips. An option for knock-in 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 efficiencies.

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
Chemically modified sgRNA	EditCo
Cas9 2NLS nuclease *(S. pyogenes)*	EditCo
Positive control (optional)	Recommended: human *RELA* sgRNA, *CDC42BPB* sgRNA (EditCo)
Transfection control (optional)	Recommended: pMAX GFP (Lonza), GFP mRNA (SBI)
TE buffer	Included with EditCo sgRNA
Nuclease-free water	Included with EditCo sgRNA
4D-Nucleofector™ System with X Unit	Lonza
4D-Nucleofector® X Kit S (32 RCT) specific for cell type	Lonza
Cell counter	Multiple vendors (e.g., Thermo Fisher Scientific)
Normal growth medium	Cell-type dependent
TrypLE Express or preferred cell dissociation reagent	Multiple vendors (e.g., Thermo Fisher Scientific)
1X PBS, cell culture grade	Multiple vendors (e.g., Thermo Fisher Scientific, Lonza)
12-well tissue culture plates	Corning
Microcentrifuge tubes	Multiple vendors (e.g., Eppendorf)
ssDNA HDR template (optional)	Multiple vendors

Important Considerations

Working with RNA and RNPs

- 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 and Nucleofector™ 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 for best practices related to dissolving and storing synthetic sgRNAs.
- RNPs can be formed directly in Nucleofector™ solution.
- 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.

Suggested Controls

Control	Description	Purpose
Mock	Cells transfected without Cas9 and sgRNA	Wild type sequence for comparison with experimental and other negative controls. Control 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	Ensure that there are no false positives due to contamination (no effect expected=wild type).
Positive	sgRNA with high editing efficiency (e.g., *CDC42BPB*, *RELA*)	Ensure all reagents, protocol, and equipment are functioning (effect expected).
Transfection	pMAX GFP (Lonza), GFP mRNA (SBI)	Assess transfection efficiency (without the use of RNPs).

Timeline

Pre-Nucleofection		Setup & Nucleofection	Post-Nucleofection		
Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Seed Cells Incubate (2 days)		Prepare Destination Plate Assemble RNP complexes Prepare Cell Suspension Prepare Cell/RNP Solution Transfer Cell/RNP Solution to the Nucleocuvette™ Strip Transfect Cells Add Recovery Medium Plate Cells Incubate (3 days)			Analysis

Protocol

1. Pre-Nucleofection

1.1. Seed Cells

a. Subculture cells 2 days before nucleofection and seed cells in an appropriately sized vessel so that they are 70-80% confluent on the day of transfection. Each nucleofection reaction will require ~1.5 x 10^5 cells.

Note: Culturing cells for additional days may be necessary to reach the desired confluency.

2. Setup & Nucleofection

2.1. Prepare Destination Plate

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

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

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

b. EditCo recommends sgRNA:Cas9 ratios between 3:1 and 9:1 for RNP formation. Below is an example experiment using a sgRNA:Cas9 ratio of 9:1.

RNP Components, Molarity, & Volume

Component	Molarity	Volume
Nucleofector™ Solution	-	18 µl
sgRNA	30 µM (pmol/µl)	6 µl (180 pmol)
Cas9	20 µM (pmol/µl)	1 µl (20 pmol)
Total volume	**-**	**25 µl**

c. Incubate RNPs for 10 minutes at room temperature.

2.3. Prepare Cell Suspension

Note: For suspension cells, spin down cells before aspiration of culture medium and washes (step a below). Skip steps b and c below. Resuspend in growth medium for counting.

a. Aspirate cell culture medium and wash cells 1-2 times with appropriate volume of 1X concentration of PBS.
b. Add appropriate amount of 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 at least 2X volume of growth medium.

d. Count the cells to determine the cell density.

e. Aliquot enough cells to have 1.5 x 10^5 cells/reaction. Centrifuge cells at 90 x g for 8-10 minutes at room temperature. The cell pellets will not be packed tightly, so care is required when removing the supernatant.

2.4. Prepare Cell/RNP Solution

a. Resuspend the cell pellet in 5 µl Nucleofector™ Solution per reaction. Add 5 µl of cell suspension to 25 µl of RNP solution to make 30 µl of cell-RNP solution per reaction.

Note: Work quickly, but carefully, and avoid leaving cells in the Nucleofector™ Solution for longer than 15 minutes. Avoid bubble formation.

2.5. Transfer Cell/RNP Solution to the Nucleocuvette™ Strip

a. For each reaction, transfer all 30 µl of cell-RNP solution to a well of the Nucleocuvette™ strip and click the lid into place.

b. Gently tap the Nucleocuvette™ strip on the benchtop to make sure that each sample covers the bottom of each well and that there are no bubbles in the wells.

2.6. Transfect Cells

a. Pre-program the Nucleofector™ depending on the cell type per reaction.

Note: Make sure that the entire Nucleofector™ Supplement is added to the Nucleofector™ Solution (according to manufacturer's protocol) and that the mixture is not more than 3 months old.

b. Place the Nucleocuvette™ strip with closed lid into the retainer of the 4D-X Core unit. Check for proper orientation of the Nucleocuvette™ strip. Larger cutout is the top (A1 and A2) and smaller cutout is the bottom (H1 and H2).

c. Press “Start” on the display of the core unit. After run completion, the screen should display a green “+” over the wells that were successfully transfected. Remove the cuvette strips from the Core unit.

Note: Some cell types require a 10-minute incubation at room temperature after nucleofection. Please consult the optimized Lonza protocol to see if this is a necessary step for your cell line.

2.7. Add Recovery Medium

a. Carefully resuspend the cells in each well of the Nucleocuvette™ strip with 70 µl of pre-warmed growth medium, and mix gently by pipetting up and down 2-3 times.

2.8. Plate Cells

a. Transfer all 100 µl to the pre-warmed 12-well tissue culture plate (prepared in step 2.1).

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

c. Replace medium after 24 hours.

3. Post-Nucleofection

3.1 Analysis

a. Extract DNA from cells 48 hours after transfection.

b. Conduct analyses to determine editing efficiency: PCR, Sanger sequencing, and ICE analysis. Next-Gen Sequencing, FACS, or functional tests may be conducted as alternatives.

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.