CRISPR Editing of Human iPS cells with RNPs using Nucleofection

Developed by researchers at Ludwig Maximilian University of Munich in conjunction with 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 induced pluripotent stem (iPS) cells derived from human fibroblasts. RNP delivery is accomplished using the Lonza 4D Nucleofector™ unit with 20 µl Nucleocuvette™ strips. Chemically modified sgRNAs are designed to resist exonucleases and innate intracellular immune cascades that can lead to cell death. Although primary cells can be challenging to transfect and manipulate, 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	Synthetic sgRNA Kit (EditCo)
Cas9 2NLS nuclease (S. pyogenes)	EditCo, available at checkout
Positive control sgRNA (optional)	Controls Kit (EditCo, available at checkout)
Transfection control (optional)	Recommended: pMAXGFP™ (Lonza)
TE buffer	Included in EditCo kits
Nuclease-free water	Included in EditCo kits
6-well plate	Corning
Matrigel® Matrix	Corning
mTeSR™ 1 cGMP feeder-free maintenance medium, 500 ml Kit	STEMCELL Technologies, Catalog #85850
ROCK inhibitor	Multiple vendors (e.g., STEMCELL Technologies)
Accutase®, 100 ml	Innovative Cell Technologies, Inc., Catalog #AT104
Corning® 15 ml Centrifuge Tubes	Corning
Cell counter	Multiple vendors (e.g., Thermo Fisher Scientific)
PBS buffer	Multiple vendors (e.g., Thermo Fisher Scientific)
4D-Nucleofector ™ System with X Unit	Lonza
P3 Primary Cell 4D-Nucleofector® Kit S (32 RCT)	Lonza, Catalog #V4XP-3032

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. Refer to EditCo.com/resources to find 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	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 control	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 control	sgRNA that has validated high editing efficiency.	Ensures that all reagents, protocol, and equipment are functioning (effect expected).
Transfection	pMAXGFP™ vector (Lonza), GFP mRNA (SBI)	Assess transfection efficiency (without the use of RNPs).

Timeline

Pre-Nucleofection	Setup & Nucleofection	Post-Nucleofection
Day 1 Seed Cells Incubate (2 days)	**Day 3** 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 (4 days)	**Day 7** Analysis

Protocol

1. Pre-Nucleofection

1.1. Seed Cells

1. Culture iPS cells on Matrigel® Matrix-coated plates until they are semiconfluent.

2. Setup & Nucleofection

2.1. Prepare Destination Plate

1. Coat a new 6-well plate with Matrigel® Matrix and incubate according to the manufacturer's instructions.
2. Aspirate Matrigel® Matrix from the plates after coating.
3. Fill each well with 2 ml mTeSR1 medium + 10 µM ROCK inhibitor. Incubate at 37°C until nucleofection.
4. Prepare 70 µl additional pre-warmed mTeSR1 medium + 10 µM ROCK per reaction for cell recovery after nucleofection.
5. 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.

2.2. Assemble RNP complexes (7.5:1 sgRNA to Cas9 ratio)

1. Hydrate sgRNA (3 nmol) in 30 µl 1X nuclease-free TE buffer to make 100 µM (100 pmol/µl) sgRNA. Concentration of Cas9 nuclease: 20 µM (20 pmol/µl).
2. Mix the components following the proportions given in the table for a single reaction (scale up appropriately).

RNP Components, Molarity, & Volume		
Component	**Molarity**	**Volume**
sgRNA	100 µM (pmol/µl)	3 µl (300 pmol)
Cas9	20 µM (pmol/µl)	2 µl (40 pmol)
Total volume	**-**	**5 µl**

3. Incubate the RNPs at room temperature for 10 minutes, then place on ice until use.

2.3. Prepare Cell Suspension

1. Prior to electroporation, detach the iPS cells from the Matrigel® Matrix plates by incubating with pre-warmed Accutase® for 3-5 minutes at 37°C.

2. Dissociate the cells into a single cell suspension by pipetting the suspension carefully up and down 4-6 times.

3. Add the same amount of mTeSR1 medium to stop Accutase®.

4. Harvest the cells to an appropriately sized tube.

5. Count an aliquot of the detached cells and determine cell density.

6. Transfer the required number of cells (5 x 105 cells per reaction) to a Corning® 15 ml Centrifuge Tube.

7. Centrifuge cells at 115 x g for 3 minutes at room temperature.

8. Aspirate the supernatant completely. The cell pellets will not be packed tightly so care is required when removing supernatant.

9. Add 5 ml of PBS buffer and wash cells by gently pipetting up and down.

10. Centrifuge at 115 x g for 3 minutes at room temperature. Aspirate the supernatant.

11. Resuspend cells in Lonza P3 Nucleofector™ solution at 20 µl/reaction. Work quickly, but carefully, and avoid leaving cells in the Nucleofector™ solution for longer than 15 minutes. Avoid bubble formation.

2.4. Prepare Cell/RNP Solution

1. Mix 20 µl of cells with 5 µl RNPs (prepared in Step 2.2) for each reaction in an appropriate tube/plate.

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

1. Transfer each cell-RNP solution (25 µl) to each well of the Nucleocuvette™ strip. Click the lid into place.

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

   Note: The total transfection volume (with cell suspension) to be transferred to the Nucleocuvette™ strips can be adjusted to 20 µl to match Lonza's recommendations. Optimize the total volume of RNP complexes by keeping the pmols of guide RNA and Cas9 proportional to that recommended in the Table on pg 4.

2.6. Transfect Cells

1. 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).

2. Use the electroporation protocol “CA137.” 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 Nucleocuvette™ strip from the core unit.

2.7. Add Recovery Medium

1. Use a multichannel pipette to add 70 µl pre-warmed mTeSR1 medium + 10 µM ROCK into each well of the Nucleocuvette™ strip to recover the cells.

2.8. Plate Cells

1. Transfer cells from each well to the pre-warmed 6-well plate (prepared in Step 2.1) and incubate overnight in a humidified 37°C/5% CO_2 incubator.

2. Change with fresh mTeSR1 (without ROCK inhibitor) 24 hours after nucleofection and daily until time for cell harvest.

3. Incubate the cells for 4 days in a humidified 37°C/5% CO_2 incubator.

3. Post-Nucleofection

3.1. Analysis

1. Extract DNA from cells.

2. Conduct analyses to determine editing efficiency: PCR, Sanger sequencing, and Inference of CRISPR Edits (ICE) analysis. Please visit EditCo.com/resources for a Genotyping protocol (primer design, DNA extraction, and PCR) to prepare DNA for Sanger sequencing, and a protocol on how to run and interpret an ICE analysis. Next-Gen Sequencing, FACS, Western blot, or functional assays may also be conducted.

   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.