CRISPR Editing of Human iPS cells with RNPs using Nucleofection

Synthego[1]

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Other

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Synthego

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ABSTRACT

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, Synthego chemically modified synthetic sgRNAs are of exceptional purity and consistently drive high editing frequencies.

Developed by researchers at Ludwig Maximilian University of Munich in conjunction with Synthego

TAGS

CRISPR

Cas9

Show tags

EXTERNAL LINK

https://www.synthego.com/resources/ips-cell-nucleofection-protocol

iPS Cell Nucleofection.pdf

PROTOCOL STATUS

Other

This protocol was developed by researchers at Ludwig Maximilian University of Munich in conjunction with Synthego. It has been used by the developers, but has not been tested by Synthego.

GUIDELINES

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

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 Synthego 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 Synthego 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	Day 7
Seed Cells		Prepare Destination Plate				Analysis
Incubate (2 days)		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)				

Additional Information

For an up-to-date list of all Synthego Protocols and other resources, please visit synthego.com/resources

For technical assistance, contact our Scientific Support Team:

Ph: 888.611.6883
Email: support@synthego.com

MATERIALS

NAME	CATALOG #	VENDOR
SF Cell Line 4D-Nucleofector® X Kit S (32 RCT)	V4XC-2032	Lonza
PBS buffer	10010023	Thermo Fisher Scientific
mTeSR™1 500 mL Kit	85850	Stemcell Technologies
Chemically modified sgRNA	Chemically modified sgRNA	Synthego
Cas9 2NLS nuclease (S. pyogenes)	Cas9 2NLS nuclease	Synthego
TE buffer (Included with Synthego sgRNA)		Synthego
Nuclease-free water	R0581	Thermo Fisher Scientific
4D-Nucleofector System with X Unit	AAF-1002X	Lonza
Cell counter		Thermo Fisher Scientific
Positive controls (Recommended: human RELA sgRNA, CDC42BPB sgRNA (Synthego))		Synthego
Transfection controls (Recommended: pMAX GFP (Lonza), GFP mRNA (SBI))		Contributed by users
6-well plate		Corning
Matrigel® Matrix		Corning
ROCK inhibitor		Stemcell Technologies
Accutase®, 100 ml	AT104	Innovative Cell Technologies, Inc
Corning® 15 ml Centrifuge Tubes		Corning

SAFETY WARNINGS

Please refer to the SDS (Safety Data Sheet) for safety warnings and hazard information.

Pre-Nucleofection - Seed Cells

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

Setup & Nucleofection - Prepare Destination Plate

2 Coat a new 6-well plate with Matrigel® Matrix and incubate according to the manufacturer's instructions.

3 Aspirate Matrigel® Matrix from the plates after coating.

4 Fill each well with 2 ml mTeSR1 medium + 10 µM ROCK inhibitor.

2 ml mTeSR1 medium + 10 µM ROCK inhibitor

5 Incubate at 37°C until nucleofection.

37 °C Incubation

6 Prepare 70 µl additional pre-warmed mTeSR1 medium + 10 µM ROCK per reaction for cell recovery after nucleofection.

70 µl mTeSR1 medium + 10 µM ROCK

7 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.

Setup & Nucleofection - Assemble RNP complexes (7.5:1 sgRNA to Cas9 ratio)

8 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).

30 µl 1X nuclease-free TE buffer

Setup & Nucleofection - Assemble RNP complexes

9 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 (100 pmol/µl)	3 µl (300 pmol)
Cas9	20 µM (20 pmol/µl)	2 µl (40 pmols)
Total volume	-	5 µl

3 µl sgRNA

2 µl Cas9

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

00:10:00 Incubation

Setup & Nucleofection - Prepare Cell Suspension

11 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.

37 °C Incubation

00:03:00 Incubation

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

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

14 Harvest the cells to an appropriately sized tube.

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

16 Transfer the required number of cells (5×10^5 cells per reaction) to a Corning® 15 ml Centrifuge Tube.

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

00:03:00 Centrifugation

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

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

5 ml PBS buffer

20 Centrifuge at 115 x g for 3 minutes at room temperature.

00:03:00 Centrifugation

21 Aspirate the supernatant.

22 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.

Setup & Nucleofection - Prepare Cell/RNP Solution

23 Mix 20 µl of cells with 5 µl RNPs (prepared in Step 8) for each reaction in an appropriate tube/plate.

20 µl cells

5 µl RNPs

Setup & Nucleofection - Transfer Cell/RNP Suspension to the Nucleocuvette Strip

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

25 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.

Setup & Nucleofection - Transfect Cells

26 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).

27 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 NucleocuvetteTM strip from the core unit.

Setup & Nucleofection - Add Recovery Medium

28 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.

70 µl pre-warmed mTeSR1 medium + 10 µM ROCK

Setup & Nucleofection - Plate Cells

29 Transfer cells from each well to the pre-warmed 6-well plate (prepared in Step 2) and incubate overnight in a humidified 37°C/5% CO_2 incubator.

37 °C 5% CO2 incubator

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

24:00:00

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

37 °C 5% CO2 incubator

96:00:00 5% CO2 incubator

Post-Nucleofection - Analysis

32 Extract DNA from cells.

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

NOTE

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