CRISPR Editing of Immortalized Cell Lines with RNPs using Nucleofection

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Working

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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 immortalized adherent cells or suspension cells. RNP delivery is accomplished using the Lonza 4D Nucleofector™ unit with 16-well Nucleocuvette™ Strips. A 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. Synthego chemically modified synthetic sgRNAs are of exceptional purity and consistently drive high editing efficiencies.

TAGS

CRISPR/Cas9

sgRNA

Show tags

EXTERNAL LINK

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

Immortalized Cell Nucleofection.pdf

PROTOCOL STATUS

Working

We use this protocol at Synthego and it is working

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 ssODNs: single-stranded donor oligonucleotides HDR: homology-directed repair
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
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 (3 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
Chemically modified sgRNA	Chemically modified sgRNA	Synthego
Cas9 2NLS nuclease (S. pyogenes)	Cas9 2NLS nuclease	Synthego
Positive control (optional); Recommended: human RELA sgRNA, CDC42BPB sgRNA		Synthego
Transfection control (optional); Recommended: pMAX GFP (Lonza), GFP mRNA (SBI)		Contributed by users
TE buffer (Included with Synthego sgRNA)		Synthego
Nuclease-free water	R0581	Thermo Fisher Scientific
4D-Nucleofector System with X Unit	AAF-1002X	Lonza
4D-Nucleofector® X Kit S (32 RCT) specific for cell type	V4XC-1032	Lonza
Cell counter		Thermo Fisher Scientific
Normal growth medium (Cell-type dependent)		Contributed by users
TrypLE Express or preferred cell dissociation reagent		Thermo Fisher Scientific
1X PBS, cell culture grade		Thermo Fisher Scientific
12-well tissue culture plates		Corning
Microcentrifuge tubes		Eppendorf
ssDNA HDR template (optional)	View	Contributed by users

SAFETY WARNINGS

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

Pre-Nucleofection - Seed Cells

1 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.

48:00:00 **Subculturing cells**

Setup & Nucleofection - Prepare Destination Plate

2 Pre-warm 1 ml of normal growth medium in each well of a 12-well cell culture plate per reaction.

1 ml normal growth medium

NOTE

This will serve as the destination plate after nucleofection.

Setup & Nucleofection - Assemble RNP Complexes (9:1 sgRNA to Cas9 ratio)

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

NOTE

Knock-in Option: to knock in small inserts (<50 bp), an ssDNA HDR Template can be added. The recommended length of each homology arm is at least 50 bp. Add 1 µl 60 µM ssDNA HDR Template per reaction to each well. Optimization may be required. To knock in larger inserts and for more information on designing knock-in experiments, see Tips and Tricks: Design and Optimization of CRISPR Knock-in Experiments.

4 Incubate RNPs for 10 minutes at room temperature.

Setup & Nucleofection - Prepare Cell Suspension

5

NOTE

For suspension cells: spin down cells before each aspiration of culture medium and washes (step 5). Skip steps 6 and 7 below.

Aspirate cell culture medium and wash cells 1-2 times with appropriate volume of 1X concentration of PBS.

NOTE

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

Setup & Nucleofection - Prepare Cells

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

00:05:00 **Incubation**

Setup & Nucleofection - Prepare Cell Suspension

7 Neutralize the dissociation reaction with at least 2X volume of normal growth medium.

8 Count the cells to determine the cell density.

9 Aliquot enough cells to have 1.5 x 10^5 cells/reaction.

10 Centrifuge cells at 90 x g for 8-10 minutes at room temperature.

NOTE

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

00:08:00 **Centrifugation**

Setup & Nucleofection - Prepare Cell/RNP Solution

11 Resuspend the cell pellet in 5 µl Nucleofector™ Solution per reaction.

NOTE

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

5 µl **Nucleofector Solution**

12 Add 5 µl of cell suspension to 25 µl of RNP solution to make 30 µl of cell-RNP solution per reaction.

5 µl **cell suspension**

25 µl **RNP solution**

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

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

14 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.

Setup & Nucleofection - Transfect Cells

15 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.

16 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).

17 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.

Setup & Nucleofection - Add Recovery Medium

18 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.

70 µl pre-warmed growth medium

Setup & Nucleofection - Plate Cells

19 Transfer all 100 µl to the pre-warmed 12-well tissue culture plate (prepared in step 2)

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

37 °C Incubation

48:00:00 **Incubation**

Post-Nucleofection - Analysis

21 Extract DNA from cells 48 hours after transfection.

22 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).