Oct 02, 2018

CRISPR Editing of Immortalized Cell Lines with RNPs using Neon Electroporation

DOI
dx.doi.org/10.17504/protocols.io.spredm6

Synthego[1]

[1]Synthego

Synthego

Brittany Enzmann

DOI: https://dx.doi.org/10.17504/protocols.io.spredm6

External link: https://www.synthego.com/resources/immortalized-cell-neon-electroporation-protocol

Protocol Citation: Synthego 2018. CRISPR Editing of Immortalized Cell Lines with RNPs using Neon Electroporation. **protocols.io** https://dx.doi.org/10.17504/protocols.io.spredm6

Protocol status: Working
Synthego uses this protocol and it is working.

Created: August 17, 2018

Last Modified: October 02, 2018

Protocol Integer ID: 14801

Keywords: CRISPR, Cas9, Synthego, crispr editing of immortalized cell line, synthetic sgrna, modified sgrna, modified synthetic single guide rna, synthetic single guide rna, crispr editing, immortalized cell line, rna, neon electroporation this protocol, using neon electroporation, rnp delivery, thermo fisher neontm transfection system, rnp, purified cas9 nuclease, transfection, suspension cell, cell death, ribonucleoprotein, immortalized adherent

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 or suspension cells. RNP delivery is accomplished using the Thermo Fisher Neon^{TM} Transfection System. A reference for electroporation settings for a wide variety of cell types is included. 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 frequencies.

Attachments

PDF

Immortalized Cell Ne...

285KB

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 PBS: phosphate-buffered saline
TE: Tris EDTA
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 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.
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.

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

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-Electroporation		Setup & Electroporation	Post-E	
Day 1 Seed Cells Incubate (2 days)	Day 2	Day 3 Prepare Destination Plate Assemble RNP Complexes Prepare Cells Transfect cells Incubate (3 days)	Day 4	Day

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

MATERIALS

- Chemically modified sgRNA **Synthego** **Catalog #**Chemically modified sgRNA
- Cas9 2NLS nuclease (S. pyogenes) **Synthego** **Catalog #**Cas9 2NLS nuclease
- Positive control (optional); Recommended: human RELA sgRNA, CDC42BPB sgRNA **Synthego**
- Transfection control (optional); Recommended: pMAX GFP (Lonza), GFP mRNA (SBI)
- TE buffer (Included with Synthego sgRNA) **Synthego**
- Nuclease-free water **Thermo Fisher Scientific** **Catalog #**R0581
- Cell counter **Thermo Fisher Scientific**
- Normal growth medium (Cell-type dependent)
- TrypLE Express or preferred cell dissociation reagent **Thermo Fisher Scientific**
- 1X PBS, cell culture grade **Thermo Fisher Scientific**
- Microcentrifuge tubes **Eppendorf**
- Neon™ Transfection System **Thermo Fisher Scientific** **Catalog #**MPK5000
- Neon™ Transfection System 10 µL Kit **Thermo Fisher Scientific** **Catalog #**MPK1025
- Tissue culture plates **Thermo Fisher Scientific**

Troubleshooting

Safety warnings

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

Pre-Electroporation

1 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 1×10^5 - 2×10^5 cells, depending on the cell type.

Note

*For cell type specific information, refer to **Thermo Fisher NeonTM Transfection System Protocols and Cell Line Data**.*

48:00:00 Subculturing cells

Setup & Electroporation - 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 electroporation.

Setup & Electroporation - Assemble RNP Complexes

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

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

Note

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

RNP Components, Molarity, & Volume		
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

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**.

5 Incubate RNPs for 10 minutes at room temperature.

00:10:00 Incubation

Setup & Electroporation - Prepare Cells

6

Note

For suspension cells: spin down cells before each aspiration of culture medium and washes. Skip steps 7 and 8 below.

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

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

00:05:00 Incubation

Note

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

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

9 Count cells to determine the cell density.

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

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

00:05:00 Centrifugation

12 Wash the cells once with 1X PBS.

13 Centrifuge cells for 5 minutes at 500 x g. Aspirate PBS.

00:05:00 Centrifugation

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

50 µL resuspension buffer R

Note

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

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

5 µL cell suspension

7 µL RNP solution

Setup & Electroporation - Transfect Cells

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

10 µL cell-RNP solution

17 Electroporate using cell type optimized conditions.

Note

*Refer to **Thermo Fisher NeonTM Transfection System Protocols and Cell Line Data**.*

18 Immediately transfer cells to a pre-warmed 12-well plate (prepared in step 2).

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

36 °C Incubation

48:00:00 Incubation

Post-Electroporation - Analysis

20 Extract DNA from cells.

21 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).