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Version 1

CRISPR Editing of Immortalized Cells with RNPs using Lipofection V.1

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Synthego¹, Synthego¹

¹Synthego

Synthego



Brittany Enzmann

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Synthego uses this protocol and it is 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 standard immortalized cell lines (adherent or suspension). Although optimized for HEK293 (human embryonic kidney 293 cells), this protocol is applicable to many other cell lines (A549, U2OS, HeLa, CHO, MCF-7). RNP delivery is accomplished using LipofectamineTM CRISPRMAXTM Transfection Reagent. 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 quality and consistently drive high editing efficiencies. Data that demonstrate editing efficiency in HEK293 cells are included.

Attachments



Immortalized Cell Li...

394KB



Guidelines

This protocol is meant to serve as a starting point for lipofection of immortalized cells in a 24-well plate format. It may be necessary to experimentally optimize volumes and ratios for RNP formation for each cell type and for other culture plate formats. It is critical to add reagents in the order recommended in the steps. Prepare the RNP complexes with the LipofectamineTM Cas9 PlusTM Reagent and Opti-MEMTM I Reduced Serum Medium in a separate tube (Tube 1) before adding diluted CRISPRMAXTM Reagent (Tube 2).

Reaction volumes are for EACH WELL and should be scaled up proportionally for the number of wells to be used.

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

All Synthego and CRISPRMAXTM reagents should be stored according to the manufacturer's recommendations.

This protocol was optimized in HEK293 cells and can be used for other common cell lines such as A549, U2OS, HeLa, CHO, MCF-7.

Successful transfection is critically dependent on cell density. It may be necessary to optimize cell seeding densities in order to determine the most appropriate level of confluence for transfection.

For fast growing cells, seed fewer cells. Cell seeding is based on the rate of cell growth. Suggested starting cell numbers are listed in the protocol below.

In order to maximize CRISPR editing, be sure to include trypsinization (**Step 9**) in order to perform a reverse transfection of RNPs.

Use cells at lowest passage number possible.



Cas9 nuclease can be diluted in Opti-MEMTM I Reduced Serum Medium in order to achieve a working concentration according to the plate volume.

Synthego recommends sgRNA:Cas9 ratio of 1.3:1 for RNP formation. It may be necessary to optimize ratios for different cell lines/conditions.

RNP complexes are formed in Opti-MEMTM I Reduced Serum Medium and can be added directly to cells in culture medium irrespective of antibiotics. Following transfection, it is not necessary to remove RNP complexes or to add or change medium.

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.

Suggested Controls



Control	Description	Purpose
Mock	Cells transfected without Cas9 and sgRNA	Wild type sequence for control, other negative controls. Control toxicity from RNP, possible viability issues associated with gene of interest.
Negative	Cas9 complexed with a non-targeting sgRNA or no sgRNA	Ensure that there are no false effects (no effect expected=wild type sequence)
Positive	sgRNA with high editing efficiency (e.g., CDC42BPB, RELA)	Ensure all reagents, protocols work (effect expected).
Transfection	pMAX GFP (Lonza), GFP mRNA (SBI)	Assess transfection efficiency

Timeline

Pre-Lipofection	Setup & Lipofection	Post-Lipofection	
Day 1 Seed cells Incubate (1 day)	Day 2 Assemble RNP Complexes Prepare Transfection Solution Make RNP-Transfection Solution Prepare Cell Suspension Transfect Cells Incubate (3 days)	Day 3	Day 4

Note: cell seeding may take 1-2 days and incubation after transfection may take 2-3 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

MATERIALS

- ☒ PBS buffer **Thermo Fisher Scientific Catalog #10010023**
- ☒ Opti-MEM™ Reduced Serum Medium **Thermo Fisher Scientific Catalog #31985062**
- ☒ 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)
- ☒ Microcentrifuge tubes **Eppendorf**
- ☒ Tissue culture plates **Thermo Fisher Scientific**
- ☒ Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent **Thermo Fisher Scientific Catalog #CMAX00001**
- ☒ Trypsin **Thermo Fisher Scientific**

Troubleshooting

Safety warnings

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



Pre-Lipofection - Seed cells

- 1 Seed cells and incubate in 37°C/5% CO₂ incubator overnight so that they are 30-70% confluent on the day of transfection (Day 2).

 37 °C 5% CO₂ incubator

Setup & Lipofection - Assemble RNP Complexes (1.3:1 sgRNA to Cas9 ratio)

- 2 Dilute sgRNA and Cas9 to 3 µM stock concentrations (3 pmol/µl).

Note

If you followed the **Synthego QuickStart Guide**, you may have 30 µM stock concentrations (30 pmol/µl), so be sure to dilute further to 3 µM (3 pmol/µl).

- 3 Prepare RNPs in microcentrifuge tube (Tube 1). Use the quantities (per reaction) in the table below.

RNP Preparation (Tube 1)		
Component	Molarity	Volume (per reaction)
Opti-MEM™ I Reduced Serum Medium	-	25 µl
sgRNA	3 µM (pmol/µl)	1.3 µl (3.9 pmol)
Cas9	3 µM (pmol/µl)	1 µl (3 pmol)
Lipofectamine™ CRISPRMAX™ Cas9	-	1 µl
Total volume	-	28.3 µl

Note

* You may need to experimentally determine the optimum amounts of sgRNA and Cas9 nuclease. Synthego recommends a ratio of 1.3:1 sgRNA to Cas9 for RNP formation.

**Note**

Note: For knock-ins, see Synthego's [Nucleofection](#) or [Electroporation](#) protocol.

- 4 Incubate RNPs for 5-10 minutes at room temperature.

 00:05:00 Incubation

Setup & Lipofection - Prepare Transfection Solution

- 5 In a separate microcentrifuge tube (Tube 2), dilute CRISPRMAX™ Reagent in Opti-MEM™ I Reduced Serum Medium. Use the quantities (per reaction) in the table below.

Transfection Solution (Tube 2)	
Reagent	
Opti-MEM™ I Reduced Serum Medium	
Lipofectamine™ CRISPRMAX™ Transfection Reagent	
Total volume	

 25 µL Opti-MEM I Reduced Serum Medium

 1.5 µL CRISPRMAX Reagent

- 6 Incubate transfection solution for 5 minutes at room temperature.



00:05:00 Incubation

Setup & Lipofection - Make RNP-Transfection Solution

- 7 Add the transfection solution (Tube 2) directly to RNPs (Tube 1), and mix well by pipetting up and down.
- 8 Incubate for 5-10 minutes at room temperature. Do not exceed 30 minutes.

00:05:00 Incubation

Note

Synthego highly recommends reverse transfection (RNPs are added to wells first and cells are added second), as this method has resulted in high editing efficiencies.

Setup & Lipofection - Prepare Cells

9

Note

For suspension cells, resuspend in growth medium and mix well. Skip steps 9 and 10 below and proceed to step 11.

Wash cells with 1X PBS (enough to cover bottom of each well), then aspirate PBS.

- 10 Add trypsin (enough to cover bottom of each well), incubate for 5 minutes in a humidified 37°C/5% CO₂ incubator. Resuspend cells in an equivalent volume of medium to stop the trypsin reaction.

 00:05:00 Incubation

11 Count cells to determine density.

12 Transfer $0.42 - 1.2 \times 10^5$ cells per reaction to a microcentrifuge tube.

13 Centrifuge cells at 200 x g for 5 minutes.

 00:05:00 Centrifugation

14 Resuspend cells in 500 µL of the growth medium.

 500 µL growth medium

Setup & Lipofection - Transfect Cells

15 Add the RNP-transfection solution mixture to each well of a 24-well tissue culture plate (see table below).
Add cell suspension to each well, and mix by pipetting (see table below).

RNP-Transfection Solution & Cell	
Reagent	
RNP-Transfection Solution	
Cell suspension in growth medium	



🧪 50 µL RNP-Transfection Solution

🧪 500 µL Cell suspension in growth medium

- 16 Incubate cells for 2-3 days in a humidified 37°C/5% CO₂ incubator.

🌡 37 °C Incubation

🕒 48:00:00 Incubation

Post-Lipofection - Analysis

- 17 Extract DNA from cells.

- 18 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).

Expected result

Representative Data

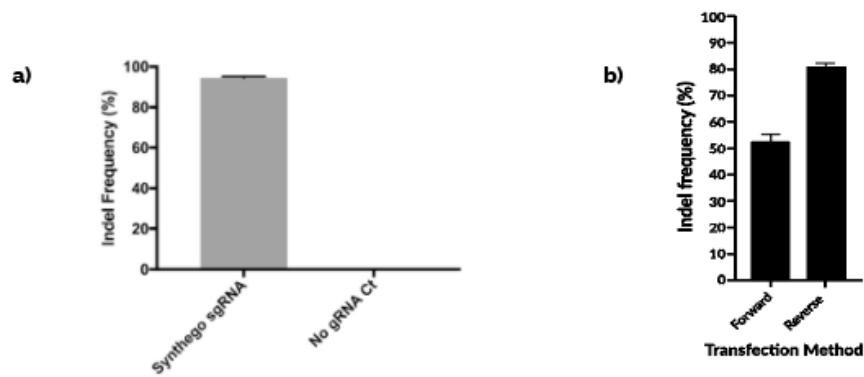


Figure 1. a) The percent indel frequency (mean \pm SD) of Synthego sgRNA targeted to *CDC42BPB*. RNPs were constructed with a sgRNA:Cas9 ratio of 3.91:3 and HEK293 cells (1×10^5 /reaction) were reverse-transfected using Lipofectamine™ CRISPRMAX™ Transfection Reagent. b) Indel frequency (mean \pm SD) in of reverse and forward-transfected HEK293 cells using Lipofectamine™ CRISPRMAX™. The indel frequency was 30% higher in reverse-transfected cells.