CRISPR Editing of Immortalized Cells with RNPs using Lipofection

Synthego[1], Synthego[1]

[1]Synthego

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Working

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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 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 Lipofectamine™ CRISPRMAX™ 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.

TAGS

CRISPR

lipofection

Show tags

EXTERNAL LINK

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

Immortalized Cell Lipofection.pdf

PROTOCOL STATUS

Working

Synthego uses this protocol and it is working

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 Lipofectamine™ Cas9 Plus™ Reagent and Opti-MEM™ I Reduced Serum Medium in a separate tube (Tube 1) before adding diluted CRISPRMAX™ 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 CRISPRMAX™ 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-MEM™ 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-MEM™ 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 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-Lipofection	Setup & Lipofection	Post-Lipofection		
Day 1	Day 2	Day 3	Day 4	Day 5
Seed cells	Assemble RNP Complexes			Analysis
Incubate (1 day)	Prepare Transfection Solution			
	Make RNP-Transfection Solution			
	Prepare Cell Suspension			
	Transfect Cells			
	Incubate (3 days)			

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

NAME	CATALOG #	VENDOR
PBS buffer	10010023	Thermo Fisher Scientific
Opti-MEM™ Reduced Serum Medium	31985062	Thermo Fisher Scientific
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
Cell counter		Thermo Fisher Scientific
Normal growth medium (Cell-type dependent)		Contributed by users
Microcentrifuge tubes		Eppendorf
Tissue culture plates		Thermo Fisher Scientific
Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent	CMAX00001	Thermo Fisher Scientific
Trypsin		Thermo Fisher Scientific

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% CO_2 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	**Volume (per reaction)**
Opti-MEM™ I Reduced Serum Medium	25 µl
Lipofectamine™ CRISPRMAX™ Transfection Reagent	1.5 µl
Total volume	26.5 µl

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% CO_2 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 x 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 Suspension	
Reagent	**Volume (per reaction)**
RNP-Transfection Solution	50 µl
Cell suspension in growth medium	500 µl

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% CO_2 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

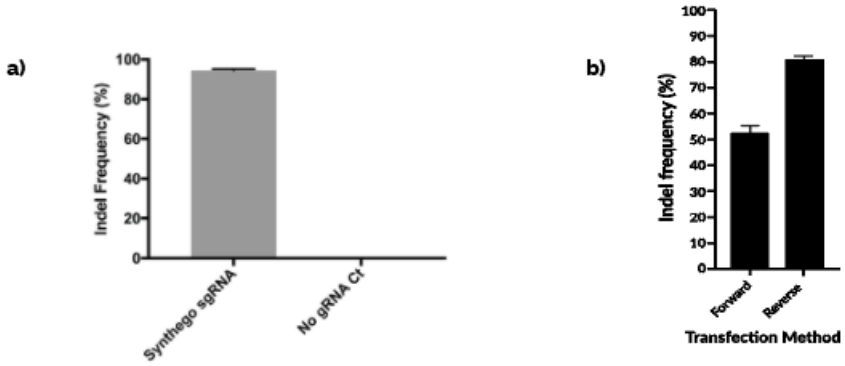

Figure 1. a) The percent indel frequency (mean ±SD) of Synthego sgRNA targeted to *CDC42BPB*. RNPs were constructed with a sgRNA:Cas9 ratio of 3.91:3 and HEK293 cells (1 x 10^5/reaction) were reverse-transfected using Lipofectamine™ CRISPRMAX™ Transfection Reagent. **b)** Indel frequency (mean ±SD) in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