CRISPR Editing of Immortalized Cell Lines with RNPs Using Lipofection for 24-well Plates

Developed by EditCo

Introduction

After you have performed a CRISPR experiment, you can evaluate the genotype of the edited cells with either Next Generation Sequencing (NGS) or Sanger sequencing.

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 may be applicable to many other cell lines (e.g., A549, U2OS, HeLa, CHO, MCF-7). RNPdelivery is accomplished using Lipofectamine™ CRISPRMAX™ Transfection Reagent. Chemically modified sgRNAs are designed to resist degradation by exonucleases and prevent innate intracellular immunecascades that can lead to cell death. This protocol may be used to transfect EditCo's multi-guide Gene Knockout Kit.

Note: EditCo has achieved the best indel frequency and transfection efficiency with sgRNA using nucleofection/ electroporation. For best results with knockouts and for knock-ins, refer to EditCo's Nucleofection or Neon Electroporation protocols found at EditCo.com/resources.

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

Materials Required

Material	Ordering Information
Target-specific chemically modified sgRNA or multi-guide sgRNA	Gene Knockout Kit (EditCo)
Cas9 2NLS nuclease *(S. pyogenes)*	EditCo, available at checkout
Positive control sgRNA or multi-guide sgRNA (optional)	Controls Kit or Transfection Optimization Kit (Multi-guide) (EditCo, available at checkout)
Transfection control (optional)	Recommended: pMAXGFP™ (Lonza)
TE buffer	Included in EditCo kits
Nuclease-free water	Included in EditCo kits
Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent (includes Cas9 Plus Reagent and CRISPRMAX™ Transfection Reagent)	Thermo Fisher Scientific, Catalog #CMAX00001
Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific, Catalog #31985062
Normal growth medium	Cell-type dependent
24-well cell culture plates	Corning, Catalog #3526
TrypLE Express or preferred cell dissociation reagent	Multiple vendors (e.g, Thermo Fisher Scientific)
1X PBS, cell culture grade	Multiple vendors (e.g, Thermo Fisher Scientific)
Cell counter	Multiple vendors (e.g., Thermo Fisher Scientific)
Microcentrifuge tubes	Multiple vendors (e.g., Eppendorf)

Note: All protocols outlined have been validated using materials mentioned in this manual. Materials other than the ones outlined in our manual may require additional optimization by the user.

General Guidelines

1. All EditCo and CRISPRMAX™ reagents should be stored according to the manufacturer's recommendations.
2. This protocol was optimized in HEK293 cells and can be used for other common cell lines such as A549, U2OS, HeLa, CHO, and MCF-7.
3. 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.
4. Cell seeding is based on the rate of cell growth. For fast growing cells, seed fewer cells. Suggested starting cell numbers are listed in the protocol below.
5. In order to maximize CRISPR editing of adherent cells, be sure to trypsinize the cells prior to lipofection (Step 2.5b).
6. Use cells at lowest passage number possible.
7. Cas9 nuclease can be diluted in Opti-MEM™ I Reduced Serum Medium in order to achieve a working concentration according to the plate volume.

8. 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 add/change medium.
9. 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 Lipofectamine™ CRISPRMAX™ Reagent (Tube 2).
10. It is critical to add reagents in the order presented in the protocol.

Visit EditCo.com/resources for analysis and clonal expansion protocols.

Optimization Guidelines

This protocol is meant to serve as a starting point for lipofection of immortalized cells.

EditCo recommends sgRNA:Cas9 ratio of 1.3:1 for RNP formation for 24-well plates, but it may be necessary to optimize ratios for different cell lines/conditions/ culture plate formats. You may also vary the volume of Lipofectamine™ reagent or cell number for your cell type. It is important to note that while increasing the amount of Lipofectamine™ may improve overall transfection and editing efficiency, it may also decrease the viability of the cells. If the intention of the editing experiment is to expand the cells after CRISPR editing, the viability of the cells will be a critical consideration in this optimization process.

Optimization of editing efficiency for a specific cell type may require varying the following:

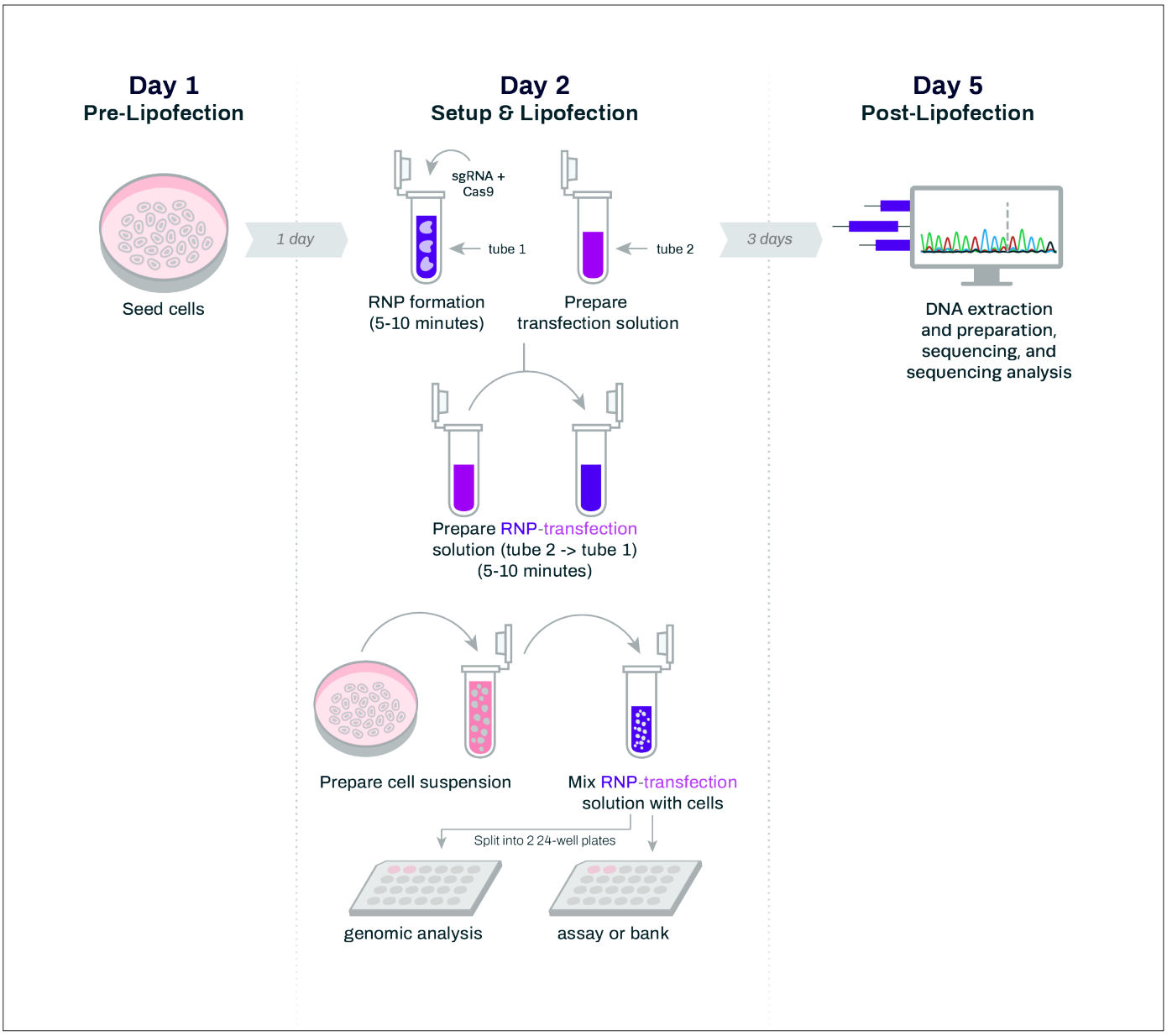
- Volume of Lipofectamine™ reagent
- The number of cells per reaction
- Ratio of sgRNA:Cas9

Suggested Controls

Control	Description	Purpose
Mock	No Cas9 or sgRNA	Wild type sequence for comparison with experimental and other negative controls. Controls for toxicity from RNP, cell death from transfection, or possible viability issues assocaited with editing the specific gene of interest
Negative control	Cas9 complexed with a non-targeting sgRNA or no sgRNA	Ensures that there are no false positives due to contamination (no effect expected=wild type).
Positive control	sgRNA that has validated high editing efficiency.	Ensures that all reagents, protocol, and equipment are functioning (effect expected). Used to optimize transfection conditions for a particular cell type.
Transfection	pMAXGFP™ vector	Assess transfection efficiency (without the use of RNPs).

Note: Positive and Transfection controls must be ordered separately.

Timeline



Note: Cell seeding may take 1-2 days and incubation after transfection may take 2-3 days. This timeline is for one e xperiment, not including optimization. Optimization may take at least 5 additonal days.

Protocol

Note: This protocol is a reverse transfection (RNP-transfection solution is mixed with cells prior to plating), as this method has resulted in high editing efficiencies (Fig 1 of representative data). For sensitive cell types, a forward transfection (in which cells are seeded in plates a day prior to transfection) may be necessary in order to establish an actively dividing population.

Volumes are for each reaction and should be scaled up proportionally to the number of desired reactions. Individual sgRNA (one per target) or multi-guide sgRNA can be used in this protocol.

1. Pre-Lipofection

1.1 Seed cells

a. Seed cells and incubate in 37°C/5% CO_2 incubator overnight so that they are 30-70% confluent on the day of transfection (may take 1-2 days).

2. Setup & Lipofection

2.1 Prepare Plates

a. Pre-warm 2 x 24-well cell culture plates with 500 µl of normal growth medium in each well.

The same volume of RNP solution + cells (225 µl) will be added to each well of each plate. The cells on the first plate will be lysed and processed to analyze editing efficiency. The cells on the second plate will be cultured for use in assays, banking, and/or single-cell cloning. Consider whether you plan to culture control cells on the second plate.

2.2 Assemble RNP Complexes (1.3:1 sgRNA to Cas9 ratio)

a. Dilute sgRNA and Cas9 to 3 µM working stock concentrations (3 pmol/µl).
b. Prepare RNPs in a microcentrifuge tube (Tube 1). Use the quantities in the table below (28.3 µl reaction volume).

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™ Cas9 Plus Reagent	-	1 µl
Total volume	**-**	**28.3 µl**

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

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

2.3 Prepare Transfection Solution

a. In a separate microcentrifuge tube (Tube 2), dilute Lipofectamine™ CRISPRMAX™ Reagent in Opti-MEM™ I Reduced Serum Medium. Use the quantities in the table below (26.5 µl reaction volume)

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**

b. Incubate transfection solution for 5 minutes at room temperature.

2.4 Prepare RNP-Transfection Solution

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

2.5 Prepare Cells

Note: For suspension cells, re-suspend in growth medium and mix well. Skip steps a and b below and proceed to step c.

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

b. Add TrypLE Express or preferred cell dissociation reagent (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 growth medium to stop the trypsin reaction.

c. Count cells to determine density.

d. Transfer 0.42–1.2 x 10^5 cells per reaction to a microcentrifuge tube.

e. Centrifuge cells at 200 x g for 5 minutes.

f. Resuspend cells in 500 µl of the growth medium.

2.6 Transfect Cells

Note: This protcol is a reverse transfection (RNP-transfection solution is added to wells first and cells are added second).

a. For each reaction, mix the RNP-transfection solution (50 µl) with the cells (500 µl) in a microcentrifuge tube, making a total volume of 550 µl (see the table below). Mix well.

RNP-Transfection Solution & Cell Suspension	
Reagent	**Volume (per reaction)**
RNP-Transfection Solution	50 µl
Cell suspension in growth medium	500 µl
Total volume	**550 µl**

b. Split the 550 µl of RNP transfection solution + cells into the 2 x 24-well plates (step 2.1) by adding 225-250 µl per replicate well. There are now duplicate plates: one for genomic analysis and one for assays/clonal expansion.

b. Incubate the cells in humidified 37°C/5% CO_2 incubator for 2–3 days.
c. Replace medium after 24 hours.

Note: Maintain the second plate by replacing medium and splitting as necessary until clonal expansion, assays, or banking.

3. Post-Lipofection

a. 72 hours post lipofection, isolate DNA, PCR target region, and Sanger—sequence amplicons.

b. Conduct Inference of CRISPR Edits (ICE) analysis on the Sanger sequences to determine editing efficiency.

Please visit EditCo.com/resources for protocols on genotyping, ICE analysis, and clonal expansion.
Next-Gen Sequencing, FACS, Western blot, or functional assays may also be conduced.

Troubleshooting Guide

Problem	Possible Cause(s)	Recommended Solutions
No cells on plate	Loss of cells during pelleting/removing supernatant before Lipofection	Use caution when aspirating supernatant.
Low viability	Cell culture conditions were suboptimal	Cells should be viable and in culture for several passages. Avoid excessive cell densities or high cell confluencies as this may decrease cell viability post lipofection.
	Too much Lipofectamine™	While increasing the amount of Lipofectamine™ reagent may improve overall transfection and editing efficiency, it may also decrease the viability of the cells.
Low transfection efficiency	Cell number	The optimal number of cells per transfection reaction varies between cell types. Optimize cell number using the GFP plasmid.
Low editing efficiency	Volume of Lipofectamine™	Test different volumes of Lipofectamine™ using a positive control sgRNA in your cell type.
	Concentration of multi-guide sgRNA	Test different concentrations using a positive control sgRNA in your cell type.
	Forward transfection	We recommend reverse transfection for maximum efficiency.
	sgRNA:Cas9 ratio	Optimize ratio of sgRNA:Cas9 using a positive control sgRNA in your cell type.

Representative Data

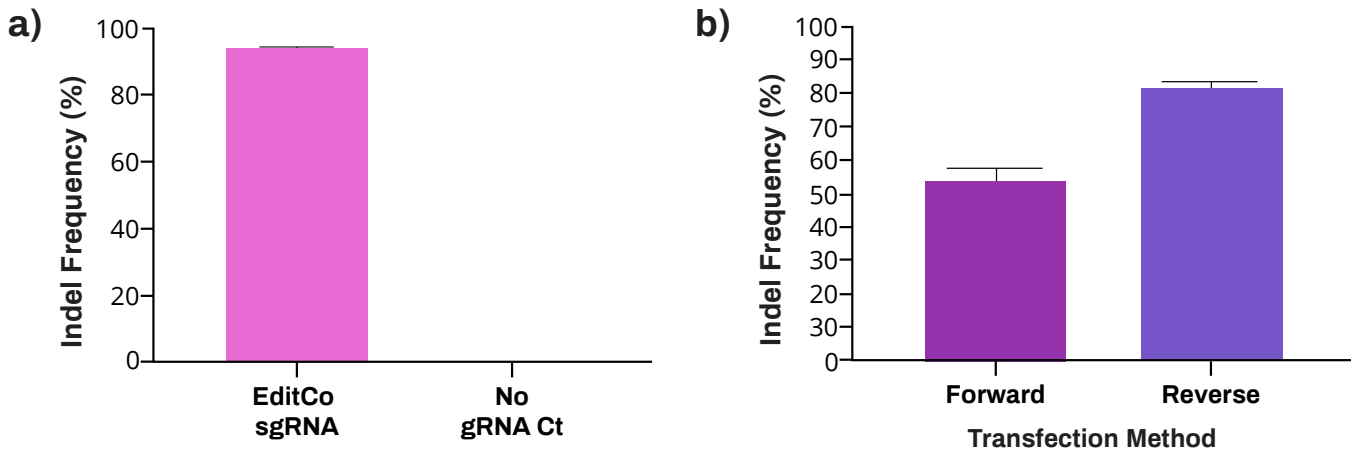


Figure 1: a) The percent indel frequency (mean ±SD) of EditCo sgRNA targeted to CDC42BPB. RNPs were constructed with a sgRNA:Cas9 ratio of 3.91:3 and HEK293 cells (1 x 10^5/reaction) were reverse-transfected using Lipofectamine™ CRISPRMAX™ Transfection Reagent. **b)** Indel frequency (mean ±SD) in of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

Additional Information

For an up-to-date list of all protocols and other resources, please visit this link.

For technical assistance, contact our Scientific Support Team at technicalsupport@editco.bio.

For common FAQs, please visit this link.