# Electroporation of Cas9/Synthetic RNA Ribonucleoprotein (RNP) Complexes for CRISPR/Cas9 Genome Editing (Thermo Neon™ System)

## BACKGROUND

This protocol describes how to transfect cultured cells with ribonucleoprotein (RNP) complexes that consist of purified Cas9 nuclease duplexed with synthetic guide RNA (gRNA; synthetic sgRNA or annealed crRNA and tracrRNA) using the Thermo Fisher Neon™ Electroporation system. Delivery of RNPs means that CRISPR components exist only transiently inside the cell, limiting Cas9 and guide RNA expression – this allows for the highest levels of editing efficiency and greatly reduces the chances of possible off-target and toxic effects. Furthermore, the use of synthetic guide RNAs eliminates the risk of incorporating foreign DNA into the host genome, which can occur when using plasmid-based guides. Synthego synthetic guide RNAs are of the highest quality and offer a superior alternative to *in vitro* transcribed (IVT) guide RNAs that are of variable quality and produce inconsistent editing results.

## MATERIALS REQUIRED

| Reagent/Material | Vendor |
|---|---|
| Synthego CRISPRevolution synthetic guide RNA (sgRNA or crRNA/tracrRNA) | Synthego |
| Synthego 2NLS-Cas9 nuclease | Synthego |
| Neon™ Electroporation System | Thermo Fisher (#MPK5000 and variants) |
| Neon™ 10µl Electroporation Kit with Resuspension Buffer | Thermo Fisher (#MPK1025 and variants) |
| DMEM, high glucose, with GlutaMAX™ | Thermo Fisher (#10566 and variants) |
| Fetal Bovine Serum (FBS) | Thermo Fisher (#10437010 and variants) |
| TrypLE™ Express Enzyme (Trypsin) | Thermo Fisher (#12605010 and variants) |
| 1X PBS (without $Ca^{2+}$ and $Mg^{2+}$) | Common lab supplier, or make in house |
| Sterile tissue culture plates (24-well) | Common lab supplier |
| Microcentrifuge tubes | Common lab supplier |

## IMPORTANT CONSIDERATIONS

- Wearing gloves and using nuclease-free tubes and reagents is recommended in order to avoid RNase contamination.
- All Synthego and Neon™ 10µl Electroporation Kit reagents should be stored according to the manufacturer's recommendations.
- This protocol contains guidelines for electroporating with adherent HEK293T cells. General recommendations are given for suspension cells below. For specific electroporation settings for your cell type, we suggest consulting the Thermo Fisher Neon™ cell type guide, available online at: www.thermofisher.com/us/en/home/life-science/cell-culture/transfection/transfection---selection-misc/neon-transfection-system/neon-protocols-cell-line-data.html. It may be necessary to experimentally optimize this protocol for a particular cell line.
- Use Resuspension Buffer R for all cell types, except primary suspension blood cells (use Resuspension Buffer T).
- Successful electroporation is critically dependent on cell density. It may be necessary to experimentally optimize cell seeding densities in order to determine the most appropriate level of confluence for electroporation.
- 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.
- Use cells at lowest passage number possible.
- This protocol is optimized for cell culture in 24-well tissue culture plates. For other cell volumes, it may be necessary to optimize reaction volumes and concentrations.
- Synthetic guide RNA (sgRNA or crRNA/tracrRNA) should be dissolved in an appropriate buffer and diluted to a working concentration using nuclease-free water. If using the two-component system of crRNA/tracrRNA, these should be annealed together beforehand. Please consult the Synthego Quick Start Guide for best practices related to dissolving, annealing and storing synthetic guide RNAs.
- Synthego Cas9 nuclease (20pmol/µl) can be diluted in Resuspension Buffer in order to achieve a working concentration for each plate volume.
- RNP complexes are stable at room temperature for up to 2 hours, and at 4°C for several weeks. Note that RNPs stored at 4°C may become susceptible to contamination from microbial growth after long periods of time.
- RNP complexes are formed in Resuspension Buffer R 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 media.

## TIME CONSIDERATIONS

- Growth of cells for electroporation (before electroporation): ~ 2-3 days
- Preparation of cells for electroporation: ~ 30 minutes
- Preparation of RNPs and reagents: ~ 15 minutes
- Electroporation into cells: ~ 10 minutes
- Final incubation (after electroporation): ~ 2-3 days

## EXPERIMENTAL OUTLINE

1. Seed cells (24-well) so that they are 30-70% confluent for electroporation.
2. Prepare RNP complexes (synthetic guide RNA + Cas9 nuclease) in Resuspension R buffer.
3. Prepare cells for electroporation and aliquot.
4. Add the RNP complexes to cells.
5. Electroporate cells.
6. Incubate for 2-3 days.
7. Perform an appropriate genomic editing assay (e.g., PCR followed by genomic cleavage detection assay and/or TIDE analysis; Next-Gen Sequencing)

## PROTOCOL

**Please Note**

It is critical to add reagents in the order recommended below. For amounts of Cas9 nuclease and synthetic guide RNA, a recommended range are both given; it may be necessary to experimentally optimize these volumes and ratios for RNP formation for each cell type.

**Reaction volumes (24-well) are for EACH WELL and should be scaled up proportionally for the number of wells to be used.**

Day 0

**Seed cells so that they are 30-70% confluent for transfection**

For 24-well culture, expect to start with 0.5-1 x $10^5$ cells/well (1-2.5 x $10^5$ cells/well for suspension cells) and a volume of 500µl growth medium/well.

Day 1

1. **Prepare growth media:**

   Prepare growth media (DMEM with GlutaMAX™, supplemented with 10% FBS). Add 500µl of growth medium to each required well of a 24-well plate.

## PROTOCOL (continued)

2. **Prepare RNPs in a microfuge tube:**

   For 24-well culture, mix:
   1pmol of Cas9 nuclease (range: 0.5 – 5pmol)[1]
   1pmol of guide RNA (sgRNA or annealed crRNA:tracrRNA; range: 0.5 – 5pmol)[1]
   5µl Resuspension Buffer R[2]
   ~ 7µl total volume[3]

   [1] You may need to experimentally determine the optimum amounts of Cas9 nuclease and guide RNA. In general, a 1:1 ratio of Cas9 nuclease:guide RNA is suggested for RNP formation.
   [2] Use Resuspension Buffer T for primary suspension blood cells only.
   [3] Estimated total volume. Make up any remaining volume with Resuspension Buffer.

3. **Incubate RNPs for 5-10 minutes at room temperature.**

   Keep RNPs at room temperature until ready to use.

4. **Prepare cells for electroporation.**

   Trypsinize cells using TrypLE™ Express Enzyme and wash once to remove any residual trypsin. Resuspend cells in 10ml of growth media, and count.

   Remove 1-2 x $10^6$ cells to a sterile microfuge tube. Each tube will suffice for ~ 10 electroporations. Pellet cells at 500xg for 5 minutes.

   Wash cells with 1X PBS (without $Ca^{2+}$ and $Mg^{2+}$), and pellet at 500xg for 5 minutes.

   Resuspend cells in 50µl of Resuspension Buffer R (use Resuspension Buffer T for primary suspension blood cells).

5. **Add 5µl of cells to each ~ 7µl RNP reaction tube.**

6. **Electroporate cells by transferring 10 µl of the cell-RNP mix to a 10µl Neon™ tip.**

   Electroporate cells at 1700V, 20ms, 1 pulse.

7. **Transfer electroporated cells into growth media.**

   Dispense cells directly into their assigned well of the 500µl growth media in the 24-well plate.

## PROTOCOL (continued)

8. **Repeat electroporation procedure until all cell-RNP mixes are electroporated and dispensed into their assigned wells.**

9. **Incubate cells for 2-3 days at 37°C.**

Days 2-4

**Visualize and analyze transfected cells:**

Rinse cells with 50-500µl PBS. Analyze an aliquot of cells by lysing cells with 20-250µl lysis buffer, perform PCR of region(s) of interest and perform genomic cleavage assay and/or TIDE analysis.