# Nucleofection (electroporation) of Cas9/ synthetic RNA ribonucleoprotein (RNP) complexes for CRISPR/Cas9 genome editing (Lonza Nucleofection™ 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 Lonza 4D Nucleofector™ unit with 16-well Nucleocuvette™ Strips. 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 |
| Lonza Nucleofector 4D Electroporation System | Lonza (#AAF-1002B and variants) |
| Lonza SF Cell Line 4D - Nucleofector™ X Kit S with 16-well Nucleocuvette™ Strip | Lonza (#V4XC-2032) |
| DMEM, high glucose, with GlutaMAX™ | Thermo Fisher (#10566 and variants) |
| Fetal Bovine Serum (FBS) | Thermo Fisher (#10437010 and variants) |
| TrypLE™ Express Enzyme | 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 Nucleofector™ reagents should be stored according to the manufacturer's recommendations.
- This protocol contains guidelines for electroporating with adherent HEK293T cells using the Lonza SF Cell Line 4D - Nucleofector™ X Kit S with 16-well Nucleocuvette™ Strips. For specific electroporation settings for your cell type, we suggest consulting the Lonza Nucleofector™ cell and transfection database, available online at bio.lonza.com/6.html. It may be necessary to experimentally optimize this protocol for a particular cell line.
- 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 nucleofection.
- 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 12-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 Nucleofector™ Solution in order to achieve a working concentration.
- 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 Nucleofector™ Solution and can be added directly to cells in culture medium irrespective of antibiotics. Following nucleofection/electroporation, 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 RNPs and reagents: ~15 minutes
- Preparation of cells for electroporation: ~15 minutes
- Nucleofection (electroporation): ~10 minutes
- Final incubation (after electroporation): ~2-3 days

## EXPERIMENTAL OUTLINE

1. Seed cells (12-well) so that they are 80-90% confluent for electroporation
2. Program the Nucleofector™ and prepare Nucleofector™ Solution
3. Prepare RNP complexes (synthetic guide RNA + Cas9 nuclease) in Nucleofector™ Solution
4. Prepare cells for nucleofection and combine with RNP complex
5. Transfer the cell/RNP complexes to Nucleocuvette™ Strips
6. Nucleofect cells
7. Incubate for 2-3 days
8. 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 12-well culture, expect to start with 1 x $10^5$ cells/well and a volume of 1ml growth medium/well.

Day 1

1. **Prepare growth media and Nucleofector™ Solutions; program Nucleofector™**

   Prepare growth media (DMEM with GlutaMAX™, supplemented with 10% FBS). Add 1ml of growth medium to each required well of a 12-well plate.

   Make sure that the entire Nucleofector™ Supplement is added to the Nucleofector™ Solution (Solution to Supplement ratio is 4.5:1) and that the mixture is not more than 3 months old.

## PROTOCOL (continued)

Enter the CM130 program into the Lonza Nucleofector™ instrument* **before** trypsinizing the cells.

***Program used is dependent on cell type. Please consult the Lonza Nucleofector™ cell and transfection database, available online at http://bio.lonza.com/6.html for more information.**

2. **Prepare each RNP in a separate microfuge tube:**

   For 12-well culture, mix:
   20pmol of Cas9 nuclease[1]
   36pmol of guide RNA (sgRNA or annealed crRNA:tracrRNA)[1]
   23µl Nucleofector™ SF Solution
   ~ 30µl total volume[2]

   [1] You may need to experimentally determine the optimum amounts of Cas9 nuclease and guide RNA.
   [2] Estimated total volume. Make up any remaining volume with Nucleofector™ SF Solution.

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

   Keep RNPs at room temperature until ready to use.

4. **Prepare cells for nucleofection (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 each cell pellet with a single 30µl RNP-Nucleofection Solution tube.

## PROTOCOL (continued)

5. **Nucleofect/electroporate cells**

Transfer cell-RNP solution to Nucleocuvette™ Vessels and click the lid into place. Avoid bubble formation.

Gently tap the Nucleocuvette™ Vessels on the benchtop to make sure the sample covers the bottom of the cuvette.

Place the Nucleocuvette™ Vessel with closed lid into the retainer of the 4D-X Core unit. Check for proper orientation of the Nucleocuvette™ Vessel. Larger cutout is the top (A1 and A2) and the smaller cutout is the bottom (H1 and H2).

Press "Start" on the display of the core unit (recall that protocol was programmed in Step 1, above)

After the program has finished, there should be a green "+" sign over the wells that were successfully transfected. Remove the cuvette strips.

Resuspend the cells in each well of the Nucleocuvette™ with 70µl of pre-warmed growth media, and mix gently by pipetting up and down 2-3 times.

Transfer the cells to the corresponding well of the 12-well plate.

6. **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.

**Find out more at synthego.com**

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