Cas9 RNP nucleofection for cell lines using Lonza 4D Nucleofector Version 3

Mark Dewitt & Julia Wong

Abstract

This protocol, based on published work, demonstrates how to delivery Cas9 RNP-based gene editing reagents to cultured mamallian cells by electroporation with a Lonza 4d Nucleofector. Consider consulting some of the following papers:

1. RNP delivery paper upon which this work is based (Open Access):

https://elifesciences.org/content/3/e04766

2. Paper by an IGI post-doc that details the rationale behind HDR donor design:

https://www.ncbi.nlm.nih.gov/pubmed/26789497

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Before start

You will need the following materials:

1. Purified Cas9 NLS protein, 40 µM

2. Purified sgRNA from in vitro transcription, >25 µM

3. single-stranded DNA HDR donor, 100 µM (as an IDT Ultramer)

4. Lonza 4d Nucleofector with X Unit

5. Lonza 'S' kit: electroporation solution and 16 reaction small-sized cuvettes

Protocol

Prepare RNP mix

Step 1.
Bring 100 pmol of Cas9 to a final volume of 5 µL using Cas9 buffer (20 mM HEPES-KOH pH 7.5, 150 mM KCl, 10% glycerol, 1 mM TCEP). For 40 µM stock: 2.5 µL.

NOTES
Jacob Corn 21 Mar 2017

Preparing RNP mix notes: Cas9-NLS is stored in -80°C, sgRNAs are prepped by runoff transcription, Cas9 buffer is kept in the TC hood and must be kept sterile. TCEP is optional for same-day application.

Prepare RNP mix

Step 2.
Bring 120 pmol sgRNA to a final volume of 5 µL using Cas9 buffer. This means you will need a minimum sgRNA concentration of 24uM.

PROTOCOL

. In vitro transcription of guide RNAs

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NOTES
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See T7 transcription protocol posted separately for detailed methods to synthesize sgRNA by runoff transcription.

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Select the nucleofection kit that matches your cell type, using the Lonza cell line and nucleofection database, or by browing the nucleofector interface itself.

Design sgRNA and order PCR oligos.

Step 2.1.

Add the desired protospacer sequence to the T7FwdVar oligo and order the oligo from your favorite oligonucleotide supplier. There are many programs available for protospacer design that attempt to optimize on- and/or off-target activity. Which program is most useful depends upon many factors including type of editing, organism being edited, etc. Choice of protospacer design program is beyond the scope of this protocol.

The transcript will start with the bolded G just 5' of the dashes in the T7FwdVar oligo. T7 RNA polymerase requires a 5' G for proper transcript initiation. If your protospacer has a G at the 5' end, you can omit it from the T7FwdVar design to avoid duplication of the G. If your protospacer has a C, T, or A at the 5' end, add the whole protospacer sequence to T7FwdVar. In this case, there will be an extra G added to the 5' end of the protospacer, but literature indicates this will have minimal effect unless your guide is very short.

Primers:

T7FwdVar oligo (5’-GGATCCTAATACGACTCACTATA**G**--protospacer sequence—GTTTTAGAGCTAGAA-3’)
T7RevLong oligo (5’-AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3’)
T7FwdAmp primer (5’-GGATCCTAATACGACTCACTATAG-3’)
T7RevAmp primer (5’-AAAAAAGCACCGACTCGG-3’)

Making in vitro transcription DNA template

Step 2.2.

For each T7FwdVar oligo you designed, set up the following PCR (total volume should be 20.0 µL). Make sure everything is RNase free and filter tips are used. Furthermore, whipe down everything (in every step of protocol) with RNase Away to ensure no contamination.

13.4 µl DEPC-treated H_2O
4.0 uL 5x Phusion HF Buffer
0.8 µl 10 mM dNTPs
0.4 µl T7FwdVar (1 µM)
0.4 µl T7RevLong (1 µM)
0.4 µl T7FwdAmp (100 µM)
0.4 µl T7RevAmp (100 µM)
0.2 µl Phusion HF DNA polymerase (2u/µl)

If making multiple sgRNA templates, prepare a master mix with all components except T7FwdVar. Include a no template control (omit T7FwdVar).

Primers:

T7FwdVar oligo (5’-GGATCCTAATACGACTCACTATA**G**--protospacer sequence—GTTTTAGAGCTAGAA-3’)
T7RevLong oligo (5’-AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3’)
T7FwdAmp primer (5’-GGATCCTAATACGACTCACTATAG-3’)
T7RevAmp primer (5’-AAAAAAGCACCGACTCGG-3’)

Making in vitro transcription DNA template

Step 2.3.

Run PCR:
95˚ 30 sec

95˚ 10 sec
57˚ 10 sec
72˚ 10 sec
34x steps 2-4
72˚ 2 min
4˚ hold

No PCR cleanup necessary at this point

In vitro T7 transcription

Step 2.4.

Mix the following to make **20 µl** total T7 transcription mix

volume	reagent
2 µl	10x Buffer 1x
2 µl	ATP (100 mM), 10 mM
2 µl	GTP (100 mM), 10 mM
2 µl	CTP (100 mM), 10 mM
2 µl	UTP (100 mM), 10 mM
8 µl	DNA template
2 µl	T7 RNA polymerase mix

In vitro T7 transcription

Step 2.5.

Incubate transcription mix for 18 hours at 37˚ in a thermalcycler

DURATION
18:00:00

Step 2.6.

Add 1 µl of RNase-free DNase; incubated 20 min, room Temp

DURATION
00:20:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.7.

Bring volume to 150 uL with 100% EtOH (this helps binding of small fragments)

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.8.

Add 5X SPRI (we use homemade SeraPure beads for RNA binding)
5*10 (IVT sgRNA)= 50 uL of SPRI Beads
5*20 (IVT sgRNA)= 100 uL SPRI Beads

REAGENTS
Agencourt AMPure XP A63880 by Beckman Coulter

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.9.

Pipette to mix 10 times

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.10.

Incubate 5 minutes at room temperature

DURATION

00:05:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.11.

Place on magnetic stand, 5 min

DURATION

00:05:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.12.

Discard supernatant

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.13.

Wash#1 Add 200 uL, 80% EtOH. Wait 2 min. Remove EtOH.

DURATION

00:02:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.14.

Wash #2: Add 200 uL, 80% EtOH. Wait 2 min. Remove EtOH.

DURATION

00:02:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.15.

Air dry 5-10 min (pellet will change from a glossy/wet to matte/dry.)

DURATION

00:10:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.16.

Elute 20 uL of water or TE. Pipette mix 10 times.

Step 2.17.

Incubate 2 minutes at room temperature

DURATION

00:02:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.18.

Place on magnetic stand, 5 min

DURATION

00:05:00

SPRI Beads clean-up of sgRNAS (96-well plate):

Step 2.19.

Keep Supernatant. Transfer to a new plate / tubes.

Prepare RNP mix

Step 3.

Add Cas9 to sgRNA slowly while swirling pipette tip, should take 30s to 1 minute.

Prepare RNP mix

Step 4.

Allow RNP to form for 10-20 minutes.

DURATION

00:20:00

Prepare Cells

Step 5.

Count cells. (Trypsinize as needed.)

Prepare Cells

Step 6.

For each nucleofection, pipette 200k cells into a 15 mL conical.

Prepare Cells

Step 7.

Spin 100 x g for 10 minutes to pellet cells softly. While the cells are spinning, prepare plate and cuvette.

Prepare Cells

Step 8.

Prepare a 12-well-plate with 1mL media per well, and pre-warm in the incubator.

Nucleofection

Step 9.

Prepare and label wells on 20uL nucleofection strips. Configure Lonza 4d using recommended cell-type program.

REAGENTS

Lonza Nucleofector 4d AAF-1002X by Lonza

SF Cell Line 4D-Nucleofector® X Kit S (32 RCT) V4XC-2032 by Lonza

Step 10.

Pipette off media from cells, gently but completely, using a P200. The pellet is very soft so be careful.

Nucleofection

Step 11.

Resuspend cells in 20 µL of nucleofector solution using a P200.

Nucleofection

Step 12.

Add the entire 10 µL RNP mix to the 20 µL resuspension and mix.

Nucleofection

Step 13.

Add 1uL of 100uM single-stranded donor DNA (100 pmoles) and mix well.

NOTES

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Design the donor to match the guide, according to our NBT paper:

https://www.ncbi.nlm.nih.gov/pubmed/26789497

We order single-stranded donors from IDT, as "Ultramers" and resuspend them to 100 µM final concentration.

Nucleofection

Step 14.
Add nucleofection mixes to the multiwell cuvette, and cap. Pay attention to the orientation of the cap and cuvette in the nucleofector, which is noted in the manufacturer's instructions.

Nucleofection

Step 15.
Insert cuvette into nucleofector and zap.

Nucleofection

Step 16.
Allow cells to sit in nucleofection strips for 10 minutes post-nucleofection. This is supposed to increase efficiency.

DURATION
00:10:00

Nucleofection

Step 17.
Add 80uL of pre-warmed media to each well.

Nucleofection

Step 18.
Pipette mixture out with a P200 into your pre-warmed 12-well plate. This should get the vast majority of cells, but if you wish, you may wash out the rest with media from the same well, chemistry-style.

Nucleofection

Step 19.
Allow cells 24 hours to settle and recover before attempted downstream analysis. Consider including un-zapped controls to test viability.

DURATION
24:00:00