Cas9/sgRNA ribonucleoprotein nucleofection using Lonza 4D nucleofector Version 3

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Abstract

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Before start

Mg2+ is required for cleavage by Cas9. Avoid buffer containing high concentration of EDTA as it can chelate Mg2+.

Don't leave cells in nucleofection solution for a long time as it might be toxic to the cells.

Protocol

Prepare cells (part 1)
Step 1.
Trypsinize cells and spin down at 100 x g for 5 minutes.

Prepare cells (part 1)
Step 2.
Remove trypsin and resuspend cells in an appropriate amount of fresh media.

Prepare cells (part 1)
Step 3.

Count cells. Record the cell concentration (cells/uL). In the meantime, put media containing cells in a 37C water bath.

Form the crRNA : tracrRNA duplex (if needed)
Step 4.

Resuspend RNA oligos (cr and tracr) in IDT duplex buffer to final concentrations of 200 uM.

Form the crRNA : tracrRNA duplex (if needed)
Step 5.

Mix the two oligos in equimolar concentrations to a final duplex concentraition of 100 uM. For

example, mixing 1.25 uL of 200 uM crRNA and 1.25 uL of 200 uM tracrRNA yields 2.5 uL of 100 uM guide duplex.

Form the crRNA : tracrRNA duplex (if needed)

Step 6.

Heat at 95C for 5 minutes. Let tubes cool down to RT before proceeding.

Prepare ribonucleoproteins RNPs mix (part 1)

Step 7.

Add 2.5 uL of 40 uM Cas9 (100 pmol) to 2.5 uL of Cas9 buffer (20 mM HEPES-KOH pH 7.5, 150 mM KCl, 10% glycerol, 1 mM TCEP-can make this ahead of time, aliquot and store at -20C).

If there are "x" nucleofection reactions, add (x+1)*2.5 uL 40 uM Cas9 to (x+1)*2.5 uL Cas9 buffer to make a mastermix of Cas9.

Prepare ribonucleoproteins RNPs mix (part 1)

Step 8.

Add 1.2 uL of 100 uM cr:tracrRNA duplex to 3.8 uL of Cas9 buffer (120 pmol or 3880 ng of guide duplex, MW32,327g/mol).

Prepare cells (part 2)

Step 9.

For each nucleofection, pipette 200k cells using a P200 or larger into a 1.5 mL tube.

Prepare cells (part 2)

Step 10.

Spin 100 x g for 10 minutes to pellet cells softly.

Prepare cells (part 2)

Step 11.

Carefully remove media off of tubes.

Prepare cells (part 2)

Step 12.

VERY IMPORTANT: Wash cells with warm 1X PBS and spin down again at 100 x g for 10 minutes. This step is critical as trypsin and FBS commonly contain RNAse activity.

Prepare cells (part 2)

Step 13.

Prepare a 12-well plate containing 1 mL of media per well. Pre-warm at 37C.

Prepare ribonucleoproteins (RNPs) mix (part 2)

Step 14.

Add Cas9 to sgRNA slowly while swirling pipette tip.

Prepare ribonucleoproteins (RNPs) mix (part 2)

Step 15.
Incubate at 37C for 10-20 minutes to let RNP form.

Nucleofection

Step 16.

Prepare and label wells on nucleofection cuvettes. To avoid cells staying in nucleofection solution for a long period of time in the subsequent steps, configure Lonza 4D ahead of time using the recommended cell-type program. Use SF cell line program CM-130 for HEK293T cells.

REAGENTS

Amaxa SF Cell Line 4D-Nucleofector Kit S (96 RCT) V4SC-2096 by Lonza

Lonza Nucleofector 4d AAF-1002X by Lonza

Nucleofection

Step 17.
After centrifugation, cell pellets are soft so carefully remove media from cells.

Nucleofection

Step 18.
Resuspend cells in 20 uL of nucleofector solution (SF cell line solution with added supplement for HEK293T) using a P200.

Nucleofection

Step 19.
Add the entire 10 uL RNP mix to the 20 µL resuspension and mix using a P200.

Nucleofection

Step 20.
If using a repair template, add 1uL of 100uM single-stranded donor DNA (100 pmoles) and mix well.

Nucleofection

Step 21.
Add nucleofection mixes to the multiwell cuvette, and cap.

Nucleofection

Step 22.
Insert cuvette into nucleofector and zap using the configured program.

Nucleofection

Step 23.
Allow cells to sit in nucleofection strips for 10 minutes post-nucleofection. This is supposed to increase efficiency.

Nucleofection

Step 24.
Add 80uL of pre-warmed media to each well. Pipette mixture out with a P200 into your pre-warmed 12-well plate.

Nucleofection

Step 25.
Allow cells 24 hours to settle and recover before attempted downstream analysis. Consider including un-zapped controls to test viability.