May 01, 2019

Working

Cas9/sgRNA ribonucleoprotein nucleofection using Lonza 4D nucleofector with lower amount of RNP (final best version-tested)

Version 10

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dx.doi.org/10.17504/protocols.io.2ijgccn

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

------Referencing this paper for optimization of nucleofection using dsDNA HDRT: https://www.nature.com/articles/s41586-018-0326-5.epdf?author_access_token=hTOPYXbVI_zEiZj3d3PwlNRgN0jAjWel9jnR3ZoTv0PJkSBqpqUc27ivPcWBqjm5ofw-5kxKHrta_dUB6FvTY9t_KLUx2qfLfoD4thaDTsluDkI1J4N9CcP39muRRXSxxxHx8VX0Tw7o18py_Z30QA%3D%3D

STEPS MATERIALS

NAME	CATALOG #	VENDOR	CAS NUMBER	RRID
Amaxa SF Cell Line 4D-Nucleofector Kit S (96 RCT)	V4SC-2096	Lonza		
Lonza Nucleofector 4d	AAF-1002X	Lonza		

BEFORE STARTING

Grow cells to 80-90% confluency. Maintain cells very healthy before transfection by changing media frequently. Plate enough cells for 200K cells per nucleofection reaction.

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

Use SF cell line solution with added supplements as nucleofection solution for HEK293T cells. Don't leave cells in nucleofection solution for a long time as it might be toxic to the cells.

Stock of Cas9 ~ 40uM or higher. gRNA stock ~ 200uM. HDRT ~ 2ug/ul or higher.

Warm up trypsin, media and 1x PBS

1

Prepare Cas9, guide RNA and HDR template

2 -Thaw Cas9, sgRNAs and HDR template on ice
-Add 100pmole of sgRNA to PCR tubes. Usually, stock sgRNA is at 200uM, add 0.5uL.
-For ssDNA HDRT, add 100pmole of 100uM single-stranded donor DNA (1ul) to different PCR tubes
For dsDNA HDRT, 5ug of dsDNA repair template (concentration of 2ug/ul and above ideally so you only use about 2ul)

Prepare cells (part 1)

3 -Trypsinize cells: Leave cells in trypsin (2 mL for a 10cm plate) at 37C for 3-5 minutes.
Note: don't leave cells in trypsin for a long period of time.
-Add in warm media to neutralize trypsin (8 mL for a 10cm plate).
-Pellet cells at 500 x g for 5 mins.

4 Remove media containging trypsin and resuspend cells in an appropriate amount of warm DMEM (usually 7 mL DMEM for an 80-90% confluent 10cm plate).

5 -Count cells: Use the hemocytometer to count as it is more accurate and consistent in our experienece.
-Add 20ul of trypan blue with 20ul of media containing cells. Mix well.
NOTE: do not leave cells in trypan blue for more than 5 minutes as it is very toxic to the cells.
-Add about 15uL of the cell:trypan blue mixture to the hemocytometer. Count 5 squares and average them out.
-Record the cell concentration (cells/uL). In the meantime, put media containing cells in 37C.

Form the crRNA : tracrRNA duplex (if needed)

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

7 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.

8 Heat at 95C for 5 minutes. Alowing slow cooling to RT by leaving tubes on block before proceeding.

Prepare RNPs mix

9 Very slowly, add 50 pmol of Cas9 to PCR tubes previously prepared, containing 100 pmol of gRNA, forming 50pmol of RNP.
-For example, if Cas9 is at 40 uM and gRNA stock at 200 uM, add 1.25 uL of 40 uM Cas9 to 0.5 uL of 200 uM gRNA.

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

Prepare cells (part 2)

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

12 Spin 500xg for 6 minutes at RT to pellet cells softly.

13 Carefully remove media off of tubes. Add warm 1xPBS to wash cells and spin down again at 500xg for 6 minutes. This step is critical as trypsin and FBS commonly contain RNAses.

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

Nucleofection

15 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. Turn off the instrument and computer then turn them on again in the correct order. Use SF cell line program CM-130 for HEK293T cells.

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

Lonza Nucleofector 4d
by Lonza
Catalog #: AAF-1002X

16 After 10-20 mins, add RNP complexes to the previously prepared PCR tubes containing HDRTs and allow to incubate together at room temperature for at least 30s, but 10 mins for max efficiency.

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

18 Resuspend cells in 20 uL of nucleofector solution (SF cell line solution with added supplement for HEK293T) using a P200. These cuvette wells can take up to 25-26ul of total reaction mix. Calculate the total amount of cells+nucleofection buffer+cas9+gRNA+HDRT ahead of time. Nucleofection buffer can also be decreased to 18ul if needed.

19 Add the entire RNP+HDRT mix to the 20 µL resuspension and mix using a P200.

20 Add nucleofection mixes to the multiwell cuvette. Avoid bubbles. Cap.

21 Insert cuvette into nucleofector and zap using the configured program.

22 Add 80ul warm media into each cuvette well immediately. Allow cells to sit in nucleofection strips for 15 minutes at 37C post-nucleofection. This is supposed to increase viability of cells.

23 Pipette mixture out with a P200 into your pre-warmed 12-well plate.

24 Allow cells at least 48 hours to settle and recover before downstream analysis.