Electroporation of EnGen® Spy Cas9 NLS RNP (ribonucleoprotein) into adherent cells using the Neon® Electroporation System

Overview

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Cas9 nuclease may be used in vivo to create targeted genome modifications. There are several ways in which to introduce Cas9-guide RNA complexes into cells. Here we present a method for the introduction of Cas9 RNP's into HEK293 FT cells using the Thermo Fisher Neon Electroporation System. This method uses a guide RNA to protein ratio of 2:1. Actual pmols of RNA and protein used may be optimized.

Required Materials:

Cell Culture and Transfection

- HEK293 cells (other cell lines may need optimization) at 70-90% confluency in a T-75 flask.
- EnGen® Spy Cas9 Nuclease NLS, (M0646T or M0646M)
- sgRNA containing the targeting sequence in the region of interest
  - sgRNAs can be generated using the EnGen sgRNA Synthesis Kit, *S. pyogenes* (E3322S)
  - sgRNAs must contain the target sequences (20 nucleotides) adjacent to the Protospacer Adjacent Motif (PAM, NGG) in the target DNA. (1,2) See the EnGen sgRNA Synthesis Kit manual for further details.
- Thermo Fisher Neon Transfection System 10 µl kit (MPK1025))
- Sterile 1X PBS without Ca^{2+} and Mg^{2+}
- Trypsin to release cells
- DMEM with Glutamax (or appropriate growth medium) with 10% FBS
- 24-well culture plate, or desired plate

DNA Extraction and Genome Editing Analysis

- EnGen Mutation Detection Kit (E3321)
- Epicentre QuickExtract™ DNA Extraction Solution (QE09050)

Before You Start:

- We strongly recommend wearing gloves and using nuclease-free tubes and reagents to avoid RNase contamination. Further recommendations for avoiding ribonuclease contamination can be found here: https://www.neb.com/tools-and-resources/usage-guidelines/avoiding-ribonuclease-contamination
- Please refer to the Neon Transfection System manual for proper usage of the equipment.

- The Neon 10 µl Transfection System draws 10 µl of cells and transfection material into an electroporation pipette tip. This tip may be used twice for two sequential electroporations. Therefore, the volumes in the protocol below allow for duplicate reactions set up in the same tube, with an overage of 5 µl. Volumes can be adjusted according to the user's needs.

Protocol:

Electroporation

1. Seed the cells so that they will be around 70-90% confluent on the day of transfection.
2. Set up the RNP formation reaction as follows below. (Resuspension Buffer R is included with the Neon transfection kit. It is not necessary to use the 10X buffer included with the EnGen Cas9 NLS at this step)

Component	14.5 µl reaction
Resuspension Buffer R	10.0 µl
EnGen Spy Cas9 NLS (20 uM)	2.5 µl
sgRNA (50 uM)	2.0 µl

3. Gently mix the Resuspension Buffer R, EnGenCas9 NLS, and gRNA and incubate at room temperature for 20 minutes.
4. During the incubation, trypsinize the cells, washing once to remove any traces of trypsin. Resuspend the cells in 5-10 ml of media. Dilute 20 µl of the cells with 20 µl of trypan blue. Determine the cell number and viability using a hemacytometer.
5. Calculate the number of cells you will need for the entire experiment (1-2 x 10^5 cells per duplicate transfection) and move those to a sterile microfuge tube. Pellet for 5 min at 500 x g. Wash the cells once with 1X PBS and repeat the centrifugation.
6. Calculate the volume of Resuspension Buffer R you will need to resuspend the cells (10.5 µl per transfection). Resuspend the cells in your calculated volume.
7. Prepare a 24-well plate by adding 500 µl growth medium to the appropriate number of wells.
8. Add 10.5 µl of cells to each 14.5 µl RNP reaction and pipette gently.
9. Aspirate 10 µl of the RNP/cells mix into a 10 µl Neon tip. Electroporate the cells under the following conditions: 1700V, 20 ms, 1 pulse.
10. Immediately transfer the cells to the prepared 24-well plate.
11. Incubate the cells in a humidified 37C, 5% C02 incubator for 48-72 hours.

Harvest DNA and Amplify Target Region

1. Gently aspirate the media from the cells and wash twice with 250 µl 1X PBS.
2. Add 50 µl of Epicentre QuickExtract™ DNA Extraction Solution and shake/vortex for 5 minutes. Transfer the solution to a PCR plate or tubes and place in a thermocycler, running the following program:

    65°C for 15 min
    95°C for 15 min
    Hold at 4°C

3. Analysis of editing can be done following the protocol detailed in the EnGen Mutation Detection Kit (E3321) manual.