Transfection of EnGen® Spy Cas9 HF1 (NEB #M0667) into adherent cells using the Lipofectamine® RNAiMAX System

Overview

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EnGen Spy Cas9 HF1 contains Simian virus 40 (SV40) T antigen nuclear localization sequence (NLS) on the N- and C- termini of the protein and may be used *in vivo* to create targeted genome modifications. There are several ways in which to introduce Cas9-single guide RNA complexes into cells. Here, we present a method for the introduction of Cas9 RNP complexes into HEK293 FT cells using the Thermo Fisher Lipofectamine® RNAiMAX Transfection Reagent. This is a 'reverse transfection' method that uses a final concentration of 10 nM RNP per transfection in a 96-well culture plate.

Required Materials:

Cell Culture and Transfection

- HEK293 cells (or other cell line) at 70-90% confluency in a T-75 flask
- EnGen® Spy Cas9 HF1 (NEB #M0667)
- sgRNA containing the targeting sequence in the region of interest
  - sgRNAs can be generated using the EnGen sgRNA Synthesis Kit, *S. pyogenes* (NEB #E3322S)
  - sgRNAs must contain the target sequences (20 nucleotides) adjacent to the Protospacer Adjacent Motif (PAM, NGG) in the target DNA. See the EnGen sgRNA Synthesis Kit manual for further details.
- Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher)
- Sterile 1X PBS without Ca^{2+} and Mg^{2+}
- DMEM with Glutamax (or appropriate growth medium) with 10% FBS
- Optimem Reduced Serum Medium (ThermoFisher)
- 96-well culture plate

DNA Extraction and Genome Editing Analysis

- EnGen Mutation Detection Kit (NEB #E3321S)
- Epicentre QuickExtract™ DNA Extraction Solution (Epicentre #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
- Transfection conditions may be highly variable. It is recommended to optimize your conditions for each cell type and Cas9 target you may have. This protocol follows conditions that have been optimized for a particular target and use of HEK293 cells

Protocol:

RNP Complex Formation

1. Make a 3 µM working solution of sgRNA by diluting the stock with nuclease-free water.
2. Make a 3 µM working solution of Spy Cas9 HF1 by diluting with 1X NEBuffer r3.1 or Optimem.
3. Form the RNP complexes as follows below:

COMPONENT	SINGLE REACTION	x3.3 (TRIPLICATES)
sgRNA (3 µM)	0.5 µl	1.65 µl
EnGen Spy Cas9 HF1 (3 µM)	0.5 µl	1.65 µl
Optimem	11.5 µl	37.95 µl
Total	12.5 µl	41.25 µl

4. Gently mix the reaction and incubate at room temperature for 10 minutes.
5. Form the liposome complexes as follows below. You can make a master mix of the RNAiMAX and Optimem and add this directly to the RNP tube from above.

COMPONENT	SINGLE REACTION	x3.3 (TRIPLICATES)
RNP (120 nM)	12.5 µl	41.25 µl
RNAiMAX	1.2 µl	3.96 µl
Optimem	11.3 µl	37.29 µl
Total	25 µl	82.5 µl

6. Gently mix the reaction and incubate at room temperature for 10 minutes.
7. Form the liposome complexes as follows below. You can make a master mix of the RNAiMAX and Optimem and add this directly to the RNP tube from above.
8. Gently mix the reaction and incubate at room temperature for 20 minutes.

Trypsinize and Prepare HEK293 Cells

1. Seed the cells so that they will be around 70-90% confluent on the day of transfection.
2. During the RNP/liposome incubation, trypsinize the cells, washing once to remove any traces of trypsin. Resuspend the cells in 10 ml of media and count.
3. Calculate the dilution and volume needed to get the cells to 3.2 x 10^5 cells per ml. You will need 125 µl of cells per well.

Transfect Cells with Liposome Complexes

1. From each tube of RNP/liposome complex, aliquot 25 µl into 3 wells of a 96-well plate.
2. Add 125 µl of cells (3.2 x 10^5 cells/ml) to each well containing RNP/liposome complex and pipette up and down gently a few times.
3. Incubate the cells in a humidified 37°C, 5% CO_2 incubator for 48-72 hours.

Harvest DNA and Amplify Target Region

1. Gently aspirate the media from the cells and wash twice with 100 µl 1X PBS.
2. Add 75 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. Dilute the DNA 1:10 in nuclease-free water.
4. Follow the protocol detailed in the EnGen Mutation Detection Kit (NEB #E3321) manual.