CRISPR/Cas9-based knock-out in human primary T cells (24-well setup)

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Abstract

This protocol has been optimized for Thermo Fisher's Neon Electroporation System and is based on the report Reprogramming human T cell function and specificity with non-viral genome targeting. We have used TrueCut™ Cas9 Protein v2 and reached very comparable KO efficiencies to those previously shown by others.

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Guidelines

To reduce variation per electroporation sample and to account for practical issues of electroporation (e.g. sparks), we have also found that a 24-well plate setup where we seeded cells from three independent electroporation reactions (within 10-ul tip) into a single well with 500 ul to 1 ml culture media in it had been the most feasible approach. This setup is especially helpful during initial optimization experiments where the goal is to screen many conditions. Pooling three independent reactions into a single sample provides a virtual buffer against variation across electroporation samples and yields enough treated cells for common assaying approaches, such as Western Blotting. We always recommend optimizing or screening using 10-ul tip setup and then scaling the reactions up to 100-ul once the optimal settings are known.

Materials

TrueCut™ Cas9 Protein v2 A36497 by Thermo Fisher Scientific

TrueGuide™ tracrRNA A35506 by Thermo Fisher Scientific

Neon™ Transfection System 10 µL Kit MPK1096 by Thermo Fisher Scientific

Protocol

Cas9 RNP preparation

Step 1.

RNase Zap everything before you start

Cas9 RNP preparation

Step 2.
Resuspend the crRNA (2 nmol) in 100 ul (making 20 uM) and tracRNA (20 nmol) in 1 ml RNA storage buffer (making 20 uM). Aliquot and keep at -80°C.

Cas9 RNP preparation

Step 3.
Need 0.375ul crRNA and 0.375 ul tracRNA per well (this way, we'll use 7.5pmol sgRNA per 200K cells as Neon protocol suggests).

Cas9 RNP preparation

Step 4.
Mix 1.5 ul crRNA and 1.5 ul tracRNA (for 4 rxns) in a PCR tube.

Cas9 RNP preparation

Step 5.
Keep the RNA mix at 95°C for 5 minutes

Cas9 RNP preparation

Step 6.
Then keep the RNA mix at at 37°C for 25 mins.

Cas9 RNP preparation

Step 7.
For Cas9, Neon suggests 1250 ng Cas9 protein per 200K cells. Our Cas9 is at 5 mg/ml concentration. So, we will need 0.25 ul Cas9/200K cells.

Cas9 RNP preparation

Step 8.
After incubation of the sgRNA, slowly add 1ul Cas9 (for 4 reactions) in the PCR tube, mix and incubate at 37°C for 15 mins.

Cell preparation and electroporation

Step 9.
Need 200K cells for one electroporation event. The cells should be in 9 ul T buffer (9 ul T buffer reaction), so that the total volume (9 ul cells + 1 ul RNP mix) will be 10ul for the Neon 10 tip.

Cell preparation and electroporation

Step 10.

Debead and count the activated cells.

Cell preparation and electroporation

Step 11.
We need 200,000*3*24 (14.4 million cells).

Cell preparation and electroporation

Step 12.
To be safe, assume 4 reactions well so we will need 200,000*4*24 (19 million) cells.

Cell preparation and electroporation

Step 13.
19 million cells are actually good for 96 wells.

Cell preparation and electroporation

Step 14.
So, we need 96*9 = 864 ul T buffer

Cell preparation and electroporation

Step 15.
After debeading and counting the cells, spin them down at 200 x g for 7 mins.

Cell preparation and electroporation

Step 16.
Aspirate the media as much as possible.

Cell preparation and electroporation

Step 17.
Resuspend the pellet in 864 ul T buffer.

Cell preparation and electroporation

Step 18.
Add 36 ul of cell mix to each PCR tube and mix well.

Cell preparation and electroporation

Step 19.
The Cas9 RNP and cells are ready for electroporation

Cell preparation and electroporation

Step 20.

Electroporation at 1600 V 10 ms 3 pulses

EQUIPMENT
Equipment brand:
Thermo Fisher Scientific Neon™ Transfection System
SKU:
MPK5000S
Specifications:

Cell preparation and electroporation

Step 21.

Seed the electroporated cells on the prepared 24-well-plate with warm T cell media.

Profiling

Step 22.

The time to profile CRISPR/Cas9 treated cell depends on the particular assay of interest but in general, cells can be profiled via flow cytometer for surface proteins and via Western Blot for internal proteins 3 days after electroporation.