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Creation of pooled CRISPR KO cell lines using Synthego sgRNA

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We use this protocol and it's working

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Abstract

This is the protocol we use for the creation of pooled CRISPR knockout cell lines using sgRNAs designed by and ordered from Synthego. We include here a protocol for validation of knockout via genomic DNA sequencing.

Materials

Synthego sgRNA kit (**https://design.synthego.com/#/**)
Cell line of your choice
S.p. HiFi Cas9 Nuclease V3, 500 µg (IDT 436397008)
Opti-MEM (Gibco 31985088)
Electroporation device for mammalian cells (NEPA21 Type II Electroporator)
PCR thermocycler
QuikExtract (Biosearch QE09050)
Primers for amplification of your target genomic DNA
GoTaq Green Master Mix (Promega M7122)
0.2 cm gap electroporation cuvette (Bio-Rad 1652086)
Zymo Gel DNA Recovery Kit (Zymo D4001)
DMEM (Cytiva SH30243.02)
FBS (Sigma F0926)
Penicillin-Streptomycin (Sigma P4333)

Preparation of sgRNA

1 Design sgRNAs using Synthego Knockout Guide Design (**https://design.synthego.com/#/**) by entering your species and gene. Synthego will output the top ranked guides for this gene; we use the top two ranked guides as a pool.

2 Resuspend sgRNA (1.5 nmol) in 50 µL TE buffer (provided in gRNA kit) to a final concentration of [M] 30 micromolar (µM) .

Creation of ribonucleoprotein (RNP) complex

3 Dilute Cas9 protein (IDT, 62 µM stock) to [M] 20 micromolar (µM) in TE buffer.

4 Calculate the amount of 30 µM sgRNAs and 20 µM Cas9 needed to create a ribonucleoprotein complex.
We combine sgRNAs and Cas9 at a 6:1 sgRNA:Cas9 ratio. We generally electroporate 500,000 cells and allow cells to recover in a 6-well plate, presuming ~50% cell death after electroporation; scale accordingly.

For 1-2×10^5 cells, plan to use 0.5 µL of 20 µM Cas9 and 2 µL of 30 µM sgRNA pool. We usually use 5×10^5 cells (~3.5 fold more), with 1.75 µl Cas9 and 7 µL sgRNA pool (3.5 µL of each sgRNA if using 2 sgRNAs).

5 Pool sgRNAs at an equal ratio.

Add the appropriate amount of 30 µM sgRNA pool (from step 4) to a new tube. Then add in your calculated amount of 20 µM Cas9 and mix. Bring to a total of 14 µL volume using TE buffer; mix well.

As described above, for electroporating 500,000 cells, add 7 µl sgRNA pool (3.5 µl of each
guide), 1.75 µl Cas9, and 5.25 µl TE buffer for total of 14 µl ribonucleoprotein complex.

6 Incubate at RT for 00:10:00 .

Electroporation of cells with Ribonucleoprotein complex

7 Resuspend the desired number of cells to be electroporated per reaction in a total of 86 µL Opti-MEM right before electroporation.

8 Add 14 µL ribonucleoprotein complex to cells and transfer the total (100 µL) volume to a 0.2 cm gap electroporation cuvette.

9 Electroporation parameters will need to be optimized for each cell line. We use a NEPA21 Type II electroporator; use an electroporation device appropriate for mammalian cells.

For A549 cells, we use the following parameters:
poring pulse: **150V**, 5 ms pulse length, 50 ms pulse interval, 2 pulses, 10% decay rate
transfer pulse: 20V, 50ms pulse length, 50ms pulse interval, 5 pulses, 40% decay rate

From cell line to cell line, the only changes we make are in the voltage for the poring pulse. For 3T3s, MEFs, and RPEs we use **180V**.

10 Immediately transfer electroporated cells to a 6-well plate or tube filled with complete DMEM (DMEM + 10%FBS, 1% Pen/Strep) and resuspend well. Let cells grow and recover (~48 hours).

Also plate 1 well of a 6-well plate with parental cells that have not been electroporated. Ensure that there is cell death in electroporated cell wells - this indicates sufficient electroporation.

It may be ideal to use a thinner pipette tip like a gel loading tip to completely remove all of the cells from the electroporation cuvette.

11 Pass cells and expand as needed. After 1 week, you should have about 1 confluent 10 cm dish. Collect cells and confirm knockout by immunoblotting, qPCR, and/or genomic DNA sequencing.

12 At this point, cells can be frozen or further expanded.

Genomic DNA extraction and sequencing

13 After about one week, for an initial well of a 6 well plate, cells will have expanded to one 10cm dish. Harvest cells by trypsinization and reserve 10% for sequencing. Wash cells with PBS. To the cell pellet, add 25 µL QuikExtract and resuspend well.

14 Incubate the QuikExtract/cell sample in a thermocycler as follows:
65°C 6 min

98°C 10 min
Store at 4°C or -20°C

15 Design and order the appropriate primers for amplification of ~250 bp genome sequence surrounding your sgRNA target site(s).

16 Use GoTaq Green Master Mix (Promega) and set up the following PCR reaction:
For a 25 µL reaction:
GoTaq (2X): 12.5 µL (final 1X)
F primer (10 µM): 2.5 µL (final 1 µM)
R primer (10 µM): 2.5 µL (final 1 µM)
DNA template (QuikExtract/cells): 2.5 µL
Add nuclease-free water up to 25 µL: 5 µL

17 PCR amplify using the parameters below. Choose an annealing temperature that is ~5°C lower than your ideal Tm. Use 1 min extension (at 72°C) per kb.

Step 1: 95°C, 5 min

Step 2: cycle the following 30X:
95°C, 30s
[Annealing Tm] 30s
72°C, 1 min

Step 3: 72°C, 5 min

Step 4: Hold at 4°C

18 Resolve all PCR products on a 2% agarose gel.

19 Gel extract PCR products using Zymo Gel DNA Recovery Kit.

20 Send PCR products for sequencing using the same primers used for amplification.

21 Validate knockout efficiency using Synthego ICE analysis program (**https://ice.editco.bio/#/**), following their instructions.

Protocol references

Synthego basic resuspension protocol/sgRNA info:
https://app.hubspot.com/documents/2418554/view/22928031?accessId=de65de

Synthego electroporation info:
https://app.hubspot.com/documents/2418554/view/22928034?accessId=6f36f8

GoTaq PCR info:
https://www.promega.com/-/media/files/resources/protocols/product-information-sheets/g/gotaq-green-master-mix-protocol.pdf?rev=87143910365a492eb4bd0933f903621a&sc_lang=en