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Endogenous Editing of iPSCs

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We use this protocol and it's working

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Abstract

This is a consensus protocol for CRISPR RNP editing of human iPSCs, based on RNP protocol from the Stephen Floor lab and iPSC-specific adaptations from the Lorenz Studer lab (written communication), as well as Rosalie's methods for sgRNA and HDR template design.

Materials

sgRNA oligos (order from Synthego)

HDR template (order 100-200 bp ultramers from IDT)

Nucleofector kit (i.e. P3 for iPSCs)--order from Lonza

Lonza 4D nucleofector (Mullins lab TC room has one)

mTeSR1 media

CloneR supplement

Emricasan Caspase Inhibitor (CAS Number: 254750-02-2)

Rock Inhibitor (Y-27632)

2BAct/trans-ISRIB

E8Flex medium for clonal selection

sgRNA Design via Benchling

- 1 Via Benchling, select new new CRISPR→GUIDES
- 2 Download the genomic sequence of your gene--be sure to select proper organism and transcript isoform.
- 3 Select a region of interest and Benchling will propose sgRNAs with on-target and off-target scores.
- 3.1 generally, it's best to select sgRNAs that cut as close as possible to your guide, even if they have lower scores. I would take anything with scores about 30, and order the top 3 closest guides.
- 4 Be sure to record your guide scores and whether the guide cuts to the left or to the right of your edit.

ssDNA HDR Design via Benchling

- 5 Via Benchling, select new CRISPR→HR Template Page 1 of 4
- 6 You will be prompted to select a gene, and then to introduce a mutation into your gene manually
- 7 Then Benchling will prompt you to add a sgRNA (pick your favorite from the above).
- 8 An HDR template will be proposed--I usually use 75 bp on each side of your template. Then Benchling will propose silent mutations that remove the PAM sequence such that your sgRNAs will not cut successfully edited loci. If this is not possible (because editing PAM would necessarily change the amino acid sequence), Benchling will propose making a few other silent mutations in the vicinity. I usually accept its options.
- 9 Note: if you want to introduce a restriction digest site to assay editing, you can do this also at this point. There are nice tools for this such as **SilentMutator**.
- 10 Next, you need to decide whether to order the sense or the antisense strand for your ssDNA HDR template. If the cut is to the left of the mutation, use a sense (+ strand) oligo. If cut is to the right of the oligo, use an antisense (- strand) oligo. This is proposed to



improve editing due to the asymmetric geometry of the HDR process ([ref](#)). I haven't super rigorously tested this but figured why not.

Nucleofecting sgRNAs

11 Once your sgRNAs have arrived, you can nucleofect them into the cells of your choice to measure cutting efficiency.

12 Before nucleofection, resuspend your sgRNAs to a concentration of 200uM in TE buffer.

13 Assemble RNPS:

13.1 Thaw Cas9, sgRNAs and HDR template on ice

In an RNase-free PCR tube, add 100pmole of sgRNA to PCR tubes. Usually, stock sgRNA is at 200uM, add 5uL.

For ssDNA HDRT, resuspend template at 100 μ M (typically this means adding 40 μ L ddH₂O to DNA ultramer tube from IDT) and add 100 pmole (1 μ L) per reaction. Mix with sgRNA.

Slowly pipette in 50 pmol of Cas9 (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.)

Incubate this RNP cocktail at 37C for 10-20 minutes prior to nucleofection to let complexes form

14 Prepare cells:

14.1 Culture iPSCs in the presence of Rock Inhibitor (RI) for 24 hours prior to nucleofection.

For each nucleofection, pipette 500k WTC11 cells in media containing RI using a P200 or larger into a 1.5 mL tube.

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

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.

Prepare supplemented mTeSR1 media for cell recovery post-nucleofection: mTeSR1 + 10 μ M Rock Inhibitor + 5

μ M Emricasan + 0.7 μ M trans-ISIB or 2BAc + 1X CloneR supplement.

Prepare a matrigel-coated 24-well plate containing 2 wells with 0.5 mL of supplemented media per nucleofection.

Pre-warm at 37C.

15 Nucleofection:

- 15.1 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 (For iPSCs use program: CA-137 for 4D Lonza nucleofector in P3 Primary nucleofector solution).
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. After centrifugation, cell pellets are soft so carefully remove PBS from cells. Resuspend cells in 20 μ L of nucleofector solution using a P200. These cuvette wells can take up to 25-26 μ L 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 18 μ L if needed.
Add the entire RNP+HDRT mix to the 20 μ L resuspension and mix using a P200.
Add nucleofection mixes to the multiwell cuvette. Avoid bubbles. Cap.
Insert cuvette into nucleofector and zap using the configured program.
For iPSCs use program: CA-137 for 4D Lonza nucleofector in P3 Primary nucleofector solution. (ensure you use Rock inhibitor as needed- during centrifugation of cells and plating of cells)
After pulsing, Add 80 μ L 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. Pipette mixture out with a P200, splitting one cuvette's contents into two wells of your pre-warmed 24-well plate.
- 16 Once your nucleofection is complete, allow two days for your cells to recover.
- 16.1 For iPSCs, HDR has been reported to be higher efficiency when cells are recovered at 32C instead of 37 ([see ref](#)). Maintain cells at 32C for 24 hours then return to 37C.
- 17 Extract genomic DNA from your nucleofected cells.
- 18 Run a PCR with a genotyping primer set (suggested design tool NCBblast: generally speaking you should pick a 500 bp product that is roughly centered around your mutation) using the extracted gDNA.



- 19 Purify your PCR product and send the product for sequencing.
- 20 Once your sequencing data is available, analyze the results with Synthego's **ICE analysis tool**.
- 21 The sgRNA with the highest ICE score is the best guide to use.

HDR with RNPs containing sgRNAs and ssDNA HDR templates

- 22 Once you have selected an sgRNA, you can repeat the nucleofection procedure described above, this time incorporating HDRT into your RNP mix.
- 23 Proceed as before; after 48 hours extract genomic DNA from some cells, freeze down some cells, and dilute others as below.
- 24 PCR the gDNA, purify the PCR product, and send the product for sequencing. Page 3 of 4
- 25 Use Synthego's **Knock-in efficiency tool** to analyze the KI in the pool of cells. High-efficiency knock-in will be around 30-50% of cells, while other times it is much lower. Use the pool KI efficiency to see if it is worth single-cell cloning.
- 26 If the KI efficiency is high enough, serial dilute cells in a 96 well plate, or sort. Rosalie prefers sorting; generally survival post sorting for WTC11s ranges 20-50%.

Clonal Selection

- 27 Sort cells into matrigel-coated plates 96-well plates containing 100 μ L/well 4x supplemented mTeSR1 medium (mTeSR1 + 10 μ M Rock Inhibitor + 5 μ M Emricasan + 0.7 μ M trans-ISRIB or 2BAct + 1X CloneR supplement)
- 28 After 2 days, switch to E8flex medium with CloneR supplement only.
- 29 In E8flex, can get away with changing media every 3 days; wait 10 days-2 weeks until you can clearly see colonies.
- 30 At this point I typically split wells with established colonies onto 2 new matrigel-coated 96-well plates. At this stage you can consolidate colonies from multiple plates onto a



single 96-well plate. Then I like to do genotyping, PCR, PCR-clean-up, and sequencing in 96-well plate format using kits such as **Zymo Research Quick-DNA 96 kit**.

31 Use Synthego's **Knock-in efficiency tool** to screen for heterozygous and homozygous clones.

32 When successful clones have been identified, expand and freeze down aliquots ASAP.

33 It is good practice to assess candidate new clonal cell lines for karyotypic abnormalities-
-i.e. with **hPSC genetic analysis kit**, and for pluripotency, i.e. with **Human Pluripotent Stem Cell 3-Color Immunocytochemistry Kit**.