Genome editing in iPSCs

Crispr design
We used the Feng Zhang lab CRISPR Design tool (http://crispr.mit.edu) to design the guide RNA. The guide RNA should bind as closely as possible to the target nucleotide (ideally between 2-10 bp).
To correct a mutation, the mutated DNA sequence should be used for the gRNA design.
To introduce a mutation, the wild-type DNA sequence should be used for the gRNA design.
For each selected gRNA, order two oligonucleotides - the sense and antisense of the guide sequence.
The BbsI restriction site overhangs (depends on the plasmid) are as follows:
Fw oligo: 5'-CACC[G]"guide_sense_sequence"-3'
Rv oligo: 5'-AAAC"guide_antisense_sequence"[C]-3'
Resuspend each oligo in distilled water to 100**µ**M.
Note: only add the G and the C in square brackets if the guide doesn't start with a G. This is necessary for optimal transcription initiation from the U6 promoter.

Typically, we design two gRNAs and test the cutting efficiency before the HDR experiment.

Design of ssDNA Oligo HDR donor template
We use a mix of up to 4 HDR donor ssDNA Oligos (100nt long) depending on the targeted mutation, the gRNA binding site and the PAM sequence.
We will design the donor Oligos

Cloning of guide RNA in the pSpCas9(BB)-2A-GFP vector (Addgene: px458)
Step1. Oligo phosphorylation and annealing:
To phosphorylate and anneal each pair of oligos, combine the following in a 0.2ml tube:
1 µl oligo Fw (100**µ**M in water)
1 µl oligo Rv (100**µ**M in water)
1 µl 10X T4 Ligation Buffer (NEB)
6.5 µl ddH2 O
0.5 µl T4 PNK (NEB)
Total volume = 10 µl total

Incubate
37°C 30 min
95°C 5 min
Ramp down to 25°C at 5°C/min

Step 2. Digestion and ligation:
1 µl pX330 or other backbone vector pSpCas9(BB)-2A-GFP (100ng/ul)
2 µl of diluted oligo duplex from step 1 (diluted 1:250 in water)
2 µl 10X FastDigest Buffer
1 µl DTT (10mM)
1 µl ATP (10mM)
1 µl FastDigest BbsI (ThermoFisher)
0.5 µl T7 DNA ligase (NEB)
11.5 µl ddH2O

Total volume = 20 µl

Incubate:
37°C 5 min
23°C 5 min
Cycle 6 times (total run time ~1h)

Step 3. Transformation:

- Add 5µl of ligation mix to 50 µl E.coli strain e.g One Shot competent cells
- Incubate on ice for 20 minutes
- Heat-shock the cells at 42°C for 45 seconds
- Cool on ice for 2 minutes
- Add 500µl of SOC media and incubate at 37°C with shaking for 30 minutes
- Plate 100µl of the transformed bacteria on LB-agar + 100µg/ml ampicillin plates.
- Incubate at 37°C overnight.

Step 4. E coli colony screening for gRNA
The next morning, pick 5 colonies and incubate overnight in 5ml LB + 100µg/ml ampicilline at 37°C with shaking. After 8 hours, isolate the DNA with a DNA miniprep kit and send for Sanger sequencing with the hU6_Fw primer (5'-ACTATCATATGCTTACCGTAAC-3').

iPSC culture
Reagents:

- Essential 8 (Life Technologies, A14666SA)
- Matrigel™ hESC-Qualified Matrix (Corning, cat. no. 354277)
- Gentle Dissociation Solution (Stem Cell Technologies 07174)
- Y-27632-HCl (Biorbyt, cat. no. orb154626)

Thawing hiPSC:

- hiPSC should be either generated in-house or can be obtained from available depositories e.g Wicell, Coriell or the Stanford CVI iPSC Biobank.
- Remove vial from liquid nitrogen, place in 37 °C water bath until only a sliver of ice remains. Transfer the vial content dropwise (~1 mL) to a 15 mL conical tube filled with 4 ml of pre-warmed E8 supplemented with 2.5µM Y-27632 (E8 + iRock)
- Centrifuge at 200 g for 4 min. Carefully aspirate the supernatant. Re-suspend the cell pellet in 2 mL of E8 + iRock and transfer to 1 well of a Matrigel-coated 6-well plate
- Change media every 24 h with fresh E8. Cells should be 70-80% confluent in 3-4 days

Passage of hiPSC:

- Ideally cells should have reached 70-80% confluence in 3-4 days (adjust split ratio accordingly, typically 1/6-1/12).
- Aspirate the E8 culture medium.
- Add 2 mL per well of Gentle Dissociation Solution, incubate for 6-8 min at RT (in hood)
- Whilst waiting, aspirate medium from Matrigel-coated plates and replace with 1 mL of E8Y.

- Aspirate Gentle Dissociation Solution from each well.
- Add 1 mL of E8 + iRock medium to the well. Gently detach the colonies by scraping with a serological pipette or a cell scraper. Add 6 mL of E8 + iRock for a 1:6 split.
- Mix gently and transfer 1 mL in each well of the 6-well plate (2ml per well total volume).

NOTE: We aim to keep the pluripotent cells in the logarithmic growth phase. Cells should not be allowed to become more than 90% confluent.

Transfection

The day before transfection, split the cells 1:2/1:3. Cell should be 50-60% confluent the next day:

- Remove the medium
- Wash once with PBS
- Add 2.0ml Gentle Dissociation Solution
- Incubate 5 min 37°C
- Gently pipette up and down to dissociate the cells
- Plate in a previously coated new well in E8 + iRock.

On the day of transfection:

- Replace media with fresh E8 media (1.0 mL/well).
- For each well of a 6-well plate, prepare 4 separate reactions
- Prepare the reactions by adding the reagents in the order shown:

(i) Set up the CRISPR-Cas9-gRNA rxn:

Mix A:
- 200 µL of Opti-MEM
- 10 µL of Lipofectamine Stem reagent

Mix B:
- 200 µL of Opti-MEM
- 1µg of CRISPR/Cas9 vector (pSpCas9(BB)-2A-GFP)

Combine mix A & B – mix well and incubate for 15min @ RT

(ii) A separate reaction is set up for the ssDNA donor oligos:

Mix C:
- 200 µL of Opti-MEM
- 7.5 µL of RNAi max reagent

Mix D:
- 200 µL of Opti-MEM
- 4 µg of ssDNA donor mix (if using 4 oligos, use 1µg each)

Combine mix C & D – mix well and incubate for 15min @ RT

- Add 400µl transfection mix of A+B and 400µl transfection mix of C+D to one well
- Place the cells back in the incubator
- After 4 hours, aspirate the transfection media from each well and replace with 2ml of fresh E8 supplemented with E8 + iRock

- 16-24h later, check the transfected cells under a fluorescent microscope for GFP^+. Typically, the transfection efficiency is 5%-30% depending on the iPSC line. We FACs sort the cells 24-36h post transfection.

FACS GFP^+ cells

- Aspirate the media
- Wash once with PBS
- Add 2.0 ml TrypLE express
- Incubate 5-6 min 37°C until cells have detached – mix gently with a P1000 pipette to break down the cells to single cells
- Add 4ml E8 + iRock
- Transfer to a 15ml tube
- Centrifuge 5 min @200g room temperature
- Discard the supernatant and re-suspend the cells in 0.4 ml E8 + iRock
- Filter the suspension through a 35-µm mesh Corning™ Falcon™ Test Tube with Cell Strainer Snap Cap
- Prepare a 15ml collection tube containing 6ml E8 + iRock
- Sort GFP-expressing cells using FACS sorter with a 100-µm nozzle – typically we sort 12,000 cells
- Plate sorted cells in 6-well plates at a density of 2000 cells/well in E8 + 2.5µM iRcok

Isolation of iPSC Clones

Usually 8-10 days after sorting, single iPSC colonies are large enough to be picked. We usually pick 30-40 clones that are clearly isolated from 3-6 wells of a 6-well plate.

- Aspirate media and add 3ml of fresh E8 + iROCK media per well at least 2h before picking.
- Manually pick individual iPSC clones (we use a P200 pipette set at 100µl) using a stereo-microscope located inside a cell culture hood, and transfer each clone to a 1.5ml tube.
- Pipet up and down 2-3 times to partially dissociate the clone and transfer 90 µl of cell suspension into a separate well of a 24-well plate (Matrigel-coated) containing 500µL of E8+iRock. Save the remaining 10µl of cells suspension for HDR screening by direct PCR (the samples can be stored at -20°C).
- Allow the cells to attach for 24–48 h and then add 500µl E8 media. Feed the cells with fresh E8 media every other day.

Genomic DNA isolation

Protocol adapted from *Phire Animal Tissue Direct PCR Kit* (Thermo Fisher; Cat#: F140WH).

- For each clone, dilute 0.5µl of DNA Release Additive in 19.5µl Dilution Buffer. We make a master mix for all the clones. Add 20µl of the master mix to each tube containing the ~10 µl of residual cell suspension from the clone picking step.
- Mix well and incubate at room temperature for 10 minutes.
- Then heat the samples at 98°C for 2 minutes.
- Add 25µl of molecular grade water and centrifuge at top speed for 1 min at room temperature to allow for cell debris sedimentation. Use 3µl of the resulting solution as a template in the PCR reaction.

Direct PCR
Perform PCR using *PrimeSTAR GXL DNA Polymerase* (Clontech) and primers that amplify a region of 500nt around the target nucleotide. For each sample combine the following:

13.5µl ddH_2O (for 3µl template DNA)
5µl PrimeSTAR GXL Buffer 5X
2µl dNTP (2.5mM each)
0.5 µl 10µM Fw Primer
0.5 µl 10µM Rv Primer
0.5 µl PrimeSTAR GXL DNA Polymerase
3.0 µl of cell sample
Total volume = 25 µl

Set up the following program on the thermocycler:

2 min 98°C

10 sec 98°C
15 sec 62°C
20 sec 68°C
(repeat X40)

2 min 68°C
hold 4°C

Sanger Sequencing
First, run 5µl of the PCR reaction on a 1% agarose gel to verify genomic DNA amplification.
Once verified, the unpurified PCR samples (~20 µl) are sent out for sequencing using either the forward or the reverse primer.