GLADSTONE INSTITUTES | STEM CELL CORE
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sgRNA Testing in iPSCs	Revision Number: 1
	Effective Date: 3rd March 2025
	Written/Revised By: Sam Hu

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Version Number	Summary of Changes	Revised by/Date
01	Original Document	Sam Hu/3rd Mar 2025

1. PURPOSE

Describe the process of testing sgRNA in iPSCs for CRISPR-Cas9 gene editing using Lonza nucleofection kit and 4-D Nucleofector

2. SUPPLIES

Lonza nucleofection kit (Lonza V4XP-3032)

- Lonza P3 primary cell solution
- Lonza supplement 1

4-D Lonza nucleofector
Lonza nucleocuvette strip (Lonza V4XP-3032)
sgRNA (Synthego)
SpCas9-NLS 'HiFi' protein - R691A
iPSCs
GFR Matrigel (356231, Corning)
Accutase ((07920, Stem Cell Technologies)
iPSC culture media
ROCKi (S1049, Selleck Chemicals)
DNA extraction kit (Qiagen)
NucleoSpin Gel and PCR Clean-up kit (Qiagen)

3. 4-D Nucleofector X Unit Settings

3.1. Pulse code CA137 (Protocol also recommends CB150)

4. SCOPE

This procedure applies to testing cutting efficiency of sgRNA

5. PROCEDURE

5.1. Design sgRNA

5.1.1. Use CRISPOR to design gRNA by inputting desired DNA sequence http://crispor.tefor.net/crispor.py

5.1.2. After designing and ordering sgRNA (we design sgRNA using CRISPOR and order sgRNA from Synthego), reconstitute sgRNA (stock concentration: 1.5nmol) in **15ul**

of **nuclease-free water provided by synthego** for a final concentration of 100pmol (3.2ug/ul) (reconstitute gRNA inside the bio safety cabinet, use sterile conditions)

5.1.2.1. Make aliquots of 1.2 ul and store in -80C.

Important Note: Be sure to pipet up and down several times to mix sgRNA well

5.2. Design primers to amplify the target region for ICE analysis

5.3. Day 0 (1 day prior to nucleofection)

5.3.1. Perform a 1:1 clump passaging of iPSC without Rocki into T25 flask. 50-60% confluency on the day of nucleofection is ideal

Note: iPSC should be healthy looking with no differentiation, minimal cell death, must be proliferating and not over-confluent.

5.4. Day 1

5.4.1. Two hours prior to transfection change media with ROCKi (10uM final concentration)

5.4.2. Coat 12 well plate with GFR Matrigel (1 well per sgRNA). Make sure to have a well for negative control. **If a positive control is desired, the nucleofection kit includes a vial of GFP plasmids that can take replace gRNA**.

5.5. Prepare nucleofection master mix

5.5.1. Master mix with gRNA

Reagent	**Volume (ul)**
P3 primary cell solution	**16**
Supplement 1	**4**
sgRNA (3.2ug/ul)	**1**
Cas9 (6.4ug/ul)	**0.8 (5ug)**
Total Volume	**21.8**

5.5.2. Negative control master mix without gRNA (1 negative control is required per experiment)

Reagent	**Volume (ul)**
P3 primary cell solution	**16**
Supplement 1	**4**
Cas9 (6.4ug/ul)	**0.8 (5ug)**
Total Volume	**20.8**

Important Note: Incubate master mix at room temperature for 15-20 minutes to allow RNP (ribonucleoprotein) complex to form.

5.6. Prepare iPSCs for nucleofection (cells can be counted before master mix preparation but make sure that you centrifuge the cells right before adding master mix. Always store cell suspension in ice)

5.6.1. Prepare single cell suspension of iPSCs using accutase

5.6.2. Count 250,000 cells per reaction with **hemocytometer** and transfer into an Eppendorf and centrifuge at 300g for 5 minutes.

Note: Cell number accuracy is very important here. See **5.7** for instructions on counting cells with hemocytometer

5.6.3. Discard the supernatant carefully using pipette and resuspend cell pellet (250,000 cells) in nucleofection Master mix with sgRNA and negative control without sgRNA.

5.6.4. Transfer cell suspension into an unused well of nucleocuvette strip

5.7. Cell Counting with Hemocytometer

5.7.1. After single cell dissociating, centrifuging, and removing supernatant, resuspend cell pellet in 1mL of media with a P1000

5.7.2. Add an additional 3ml of media with a serological and pipet up and down, then add 4ul of RocKi

5.7.3. In a 1.5ml Eppendorf, add 90ul of media and 10ul of cell suspension. Pipet up and down several times to mix well (first dilution 1:10)

5.7.4. In a second 1.5ml Eppendorf transfer 10ul from the first Eppendorf and add 10ul of trypan blue. Pipet up and down several times to mix well (second dilution 1:2)

5.7.5. Total dilution: 1:20

5.7.6. Count cells by injecting 10ul from the second Eppendorf into hemocytometer

5.7.7. Count only the cells in the 4 corner boxes and the center box. Do not count any cells on the border

5.7.8. Cells per mL = $(\frac{number\ of\ cells\ counted}{number\ of\ boxes\ counted})$ x dilution factor x 10,000

Example) 28 cells counted in 5 boxes and the dilution factor is 20x

$(\frac{28}{5})$ x 20 x 10000 = 1,120,000 cells per ml

5.7.9. $(C1)(V1) = (C2)(V2)$

Example) 250,000 cells are needed and 1,120,000 cells per ml counted.

$((1120000)(ml) = (250000)(unknown\ ml)$

unknown ml = $250000/1120000$ = ~0.22 ml or ~220ul = 250k cells

5.8. Nucleofection

5.8.1. Turn on nucleofector

5.8.2. Select **X** when choosing device and select **strip** when choosing vessel

5.8.3. Select the wells for the experiment

5.8.4. Enter solution P3

5.8.5. Enter pulse code (CA137)

5.8.6. Insert strip and hit start. Make sure the strip is inserted correctly (see last page)

5.9. Post nucleofection

5.9.1. If haven't done so already, remove Matrigel from coated 12 well plate and add 1 ml of media with Rocki.

5.9.2. Collect cells from nucleocuvette and seed into a well of 12 well plate.

5.9.3. Shake the plate gently to spread the cells out evenly then place it in the incubator

5.10. Day 2-3

5.10.1. After 48 hours of seeding, replace media without Rocki

5.10.2. After 72 hours of seeding, collect cells for DNA Extraction

6. DNA Extraction

6.1.1. Collect cells using accutase in a 15ml conical or 1.5ml centrifuge tube

6.1.2. Centrifuge for 5 minutes at 300g
6.1.3. Remove supernatant, leaving only the cell pellet
6.1.4. Extract DNA using Qiagen kit

7. PCR and Gel Electrophoresis

7.1.1. Amplify the target sequence using primers designed earlier and run gel electrophoresis
7.1.2. Confirm band is present (or not) and it is the correct band size
7.1.3. Send for Sanger sequencing after purifying the PCR product using NucleoSpin
Make sure to sequence the negative control as well, which will be required for ICE analysis.

7.2. Perform ICE analysis

7.2.1. Once sequencing results come back, upload sequencing file. AB onto Synthego**:**
https://ice.synthego.com/#/
Make sure to include the negative control file as well