CRISPR/Cas9 using the Cas9-GFP Cells

1. Cas9-GFP MSCVneo HA-ER-HoxB8 cells
    a. Derived by transduction of bone marrow from the Cas9-GFP transgenic mouse
    b. JAX: https://www.jax.org/strain/026175
    c. These cells have constitutive expression of Cas9 and GFP (bicistronic sequence)

Transduction

1. The cells are readily transducible (infectable) using retrovirus or lentivirus.
    a. I have typically used lentivirus produced by the Broad Institute.
    b. These are sgRNA in the pXPR_BRD003 backbone, also known as “lentiguide”.
    c. This is their most common backbone, and it contains a PURO-R cassette.
2. The Broad Institute supplies virus in ~65 ul aliquots.
    a. Their viral titer generally approximates 10^7/ml.
    b. Therefore, I will use between 30-60 ul (~500,000 viral particles) per transduction
3. Transduction can be carried out in many formats.
    a. Where possible, I like to use 12-well plates.
    b. However, we also routinely transduce cells in 96-well plate format.

Transduction of Cas9-GFP ER-HoxB8 cells in 12-well plates

1. Count the cells & resuspend at 500,000/ml.
    a. Add polybrene to a final concentration of 8.8 ug/ml.
    b. Aliquot 500 ul (250,000 cells) into each well of a 12-well plate.
2. Add 50 ul of virus to each well
    - Final polybrene conc. = 8 ug/ml
3. Spinfection
    a. 1000g x 60 minutes at RT.
4. Following spinfection, add 3 ml of fresh media to each well.
    - Final volume = ~4 ml and final polybrene concentration = 2 ug/ml
5. At 24-hours, I perform a half-media change to further dilute the polybrene
    a. Aspirate ~2 ml of media from the top of the wells.
    b. The cells remain undisturbed on the bottom.
    c. Add 2 ml of fresh media.
    d. This dilutes the polybrene out to a non-toxic concentration of ~1 ug/ml.
6. At 36-48 hours, I begin PURO selection.
    a. The cells are in ~4 ml of media.
    b. I prepare PURO at 50 ug/ml (10x)
    c. Add 440 ul of PURO to each well.
    d. Final PURO concentration ~ 5 ug/ml.
7. The cells should be fully selected within ~48-72 hours.

Transduction of Cas9-GFP ER-HoxB8 cells in 96-well plates

1. Count the cells & resuspend at 1,000,000/ml.
    a. Add polybrene to a final concentration of 16 ug/ml.
    b. Aliquot 30 ul (30,000 cells) into each well of a 96-well plate.
2. Add 30 ul of virus to each well
    - Final polybrene conc. = 8 ug/ml
3. Spinfection
    a. 1000g x 60 minutes at RT.
4. Following spinfection, add 200 of fresh media to each well.
    - Final volume = ~260 ul and final polybrene concentration = 2 ug/ml
5. At 24-hours, I perform a half-media change to further dilute the polybrene
    a. Using a multi-channel pipet, remove the top 150 ul from each well
    b. The cells remain undisturbed on the bottom.
    c. Add 150 ul of fresh media.
    d. This dilutes the polybrene out to a non-toxic concentration of ~1 ug/ml.
6. At 36-48 hours, I begin PURO selection.
    a. The cells are in ~250 ul of media.
    b. I prepare PURO at 50 ug/ml (10x)
    c. Add 25 ul of PURO to each well.
    d. Final PURO concentration ~ 5 ug/ml.
7. The cells should be fully selected within ~48-72 hours.

Single cell cloning

1. Single cell cloning can be done by (a) FACS or (b) limiting dilution
   - Single cell cloning allows one to screen a series of clones to identify a knock-out
2. FACS
   - 96 well round bottom plates
   - Add 250 ul of media to each well
   - Using FACS, add 1 cell per well
3. Limiting dilution
   - Count the cells multiple times to get a good concentration of live cells
   - Using serial dilutions, dilute the cells to 12 cells/ml
   - In a 50 ml conical with 50 ml of media, add 600 cells (12 cells/ml)
   - Make a 1:3 dilution (15 ml of cells + 30 ml of media), now 4 cells/ml
   - Make a second 1:3 dilution, now 1.3 cells/ml
   - From each 50 ml conical, plate 250 ul per well using a multi-channel pipet
   - Now you will have three plates (3 cells per well, 1 cell per well, and 0.3 cells per well)
   - This will help account for counting errors, and usually one of the plates will have ~20 clones for expansion
4. The clones will grow out and can be visualized by day 10-12
   - I do not change the media during this 12-day period.
   - Visualize the clones by holding the plate up to the light and seeing the clones on the bottom of each well
   - Usually the cloning efficiency is 10-30%, so you will get ~10-30 clones per plate
   - Using a P200, remove the top half of the media and discard
   - Mix the clone up and down and transfer all of the cells to a 48-well plate with fresh media
   - Move from 48 to 24 to 12 to 6 well plate during the expansion process