**LX-miR Library Screening**

**Materials:**

* LX-miR plasmid library
* Lentiviral packaging plasmids
* HEK293T cells
* 2x HBS-LT
* TE 1/10
* 2M CaCl2
* 0.6M sodium butyrate
* 40% PEG solution
* Polybrene
* Crystal violet
* Cas9-expressing cell line of interest
* Blasticin selection medium

**Lentiviral Production:**

1. Plate 4 x 106 cells/plate of 293T cells in 100-mm tissue culture plate in 6 mL
2. Allow cells to reach 80% confluency
3. For each 100-mm plate, prepare the following using sterile tubes:
   * 1. **Tube #1 Tube #2**
     2. 0.5 mL 2X HBS-LT 440 µL TE/10(minus vol DNA solution)
     3. - DNA sol’n
     4. - 60 µL, 2M CaCl2

DNA Sol’n: 15 µg vector, 15 µg pCHGP, 5 µg pCMV-rev, 5 µg pCMV-G (or other lentiviral packaging plasmids)

1. Gently add – with bubbling – the contents from Tube #2 into Tube #1, mixing gently (can also add CaCl2 to DNA with bubbling)
2. Let sit 30 min at RT
3. Mix contents well by pipetting or vortex
4. Remove 1 mL from tissue culture plate
5. Slowly add – dropwise – 1 mL of the DNA solution into the cell culture dish, while gently swirling the dish
6. Incubate 4 h, 37°C + 5% CO2
7. Replace old medium with 6-mL fresh medium
8. Add 60 µL, 0.6 M sodium butyrate to cell culture dish
9. Incubate 48 h, 37°C + 5% CO2

**PEG Concentration of Virus**

1. Collect supernatant from 293T plates
2. Centrifuge supernatant 400 x g, 5 min
3. Filter supernatant through 0.45 µm filter
4. Add 40% PEG to make a 10% total PEG solution (To make math easier, can add extra medium)
5. Store at 4˚C overnight
6. Spin down at 2,000 g for 30 min
7. Resuspend in leftover medium
8. Store virus in 50 µL aliquots
9. Store aliquots in -80°C.

**Titration of** **Virus\***

1. Seed 12-well plate with enough cells for 80% confluency the next day in 1 mL culture medium
2. Culture cells overnight, 37°C + 5% CO2
3. Remove medium
4. Dilute virus in 500 µL medium
5. Add the respective concentration of virus as diagrammed (for cell lines with low transduction efficiency, less diluted concentrations may be necessary)

|  |  |  |  |
| --- | --- | --- | --- |
| No Virus |  | 10-4 |  |
| 10-5 |  | 10-6 |  |
| 10-7 |  | 10-8 |  |

1. Add 4 ug/mL polybene to each well
2. Incubate 24 h, 37°C + 5% CO2
3. Change medium and incubate overnight
4. Add 1 mL selection medium to each well
5. Incubate at 37°C + 5% CO2 until cells are dead in both “No Virus” control wells, changing medium and adding fresh selection medium every 2-4 days
6. Aspirate medium
7. Shake plate to remove excess medium
8. Add 500 uL crystal violet into each well
9. Let sit at RT, 5 min
10. Discard dye
11. Shake plate to remove excess dye
12. Wash each well with 1 mL PBS
13. To determine the titer, average the colony counts in the duplicate wells for two concentrations for which distinct colonies can be seen
14. Calculate MOI and the viral particle concentration

\* To ensure an accurate titer, it is recommended you titer the virus on the cell line which will be used for the screen

**Transduction and Screening:**

1. Calculate the number of Cas9-expressing cells necessary for at least 200x coverage of the library with a low (0.2) MOI
2. Plate enough cells to ensure 200x coverage
3. Culture cells overnight, 37°C + 5% CO2
4. Remove half of the medium
5. Add the volume of virus necessary for MOI of 0.2 + 4 µg/mL polybrene
6. Incubate for 24 hours
7. Change medium
8. Incubate for 24 hours
9. Add blasticidin selection medium
10. Perform positive or negative selection screen