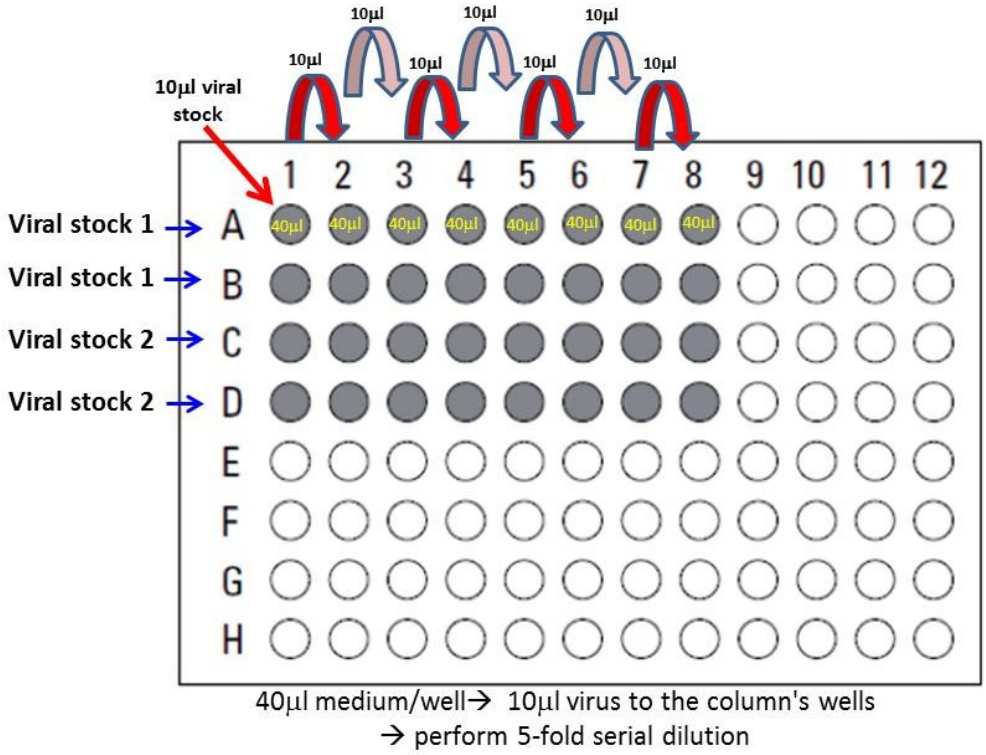
THE UNIVERSITY OF TEXAS
MD Anderson Cancer Center
Making Cancer History®

Titering of virus in a 96-well plate format

1. The day before transduction, seed a 96-well tissue culture plate with HKK293T cells at 2.5~3´10^4 cells/well in 100ml of growth medium i.e. DMEM with 10%FBS and 1% P/S;

   [prepare 2.5~3´10^5 cells/ml → 100ml/well → leave the plate for 1 hr to let the cells attach]

2. 24 hrs later, make 5-fold serial dilution of viral stock in a round bottom 96-well plate using serum-free media as shown below:



   [mix the dilution by pipetting contents of well up and down for 10~15 times →discard pipette tips]

3. Gently remove the culture medium from each well, add 30 ml of diluted virus to each well→ Spin down at 2000rpm (room temperature) for 2 hrs → incubate the plate at 37°C for 4~6 hrs;

4. 4~6 hrs later, add 170 ml of growth medium to each well (total 200 ml/well), continue to incubate the cells at 37°C for **3** days;

5. **72** hrs later, count the GFP expressing cells or colonies of cells with GFP expression by fluorescence microscopy;

6. Calculate the Transducing Units per mL (TU/mL) using the following formula:
   # of GFP colonies counted ´ dilution factor ´ 33.3 = # TU/mL (Note: 1000/3=33.3)

THE UNIVERSITY OF TEXAS
MD Anderson Cancer Center
Making Cancer History®

Lentiviral Titer by Limiting Dilution-Colony Counting

Materials

Solutions

1. Puromycin (2mg/ml);
2. 0.1% Crystal Violet Solution (Dissolve 100mg Crystal Violet into 95 ml of MilliQ water plus 5ml of Ethanol);
3. 4% paraformaldehyde;

Lentiviral Particles

Packaged lentivirus stored at -80 ºC;

Media and Cells

DMEM; HeLa Cells;

Methods

4. Seed 1.5×10^5 cells to each well of 6-well plate in DMEM medium containing 10% FBS without no antibiotics;
5. Incubate the cells overnight at 37 ºC, 5% CO_2;
6. On the next day, thaw lentivirus on ice;
7. Remove culture medium from each well and add 975 µl fresh medium without FBS and antibiotics;
8. Perform 5-fold serial dilution of lentivirus;
9. Gently add 25 µl of diluted lentivirus to the cells in each well of 6-well plate;
10. Mix by gently turn the plate from one side to another side;
11. Optional: Centrifuge at 2000 RPM for 2 hours at room temperature;
12. Incubate the cells at 37 ºC, 5% CO_2 for 6 hours;
13. Add 2 ml of DMEM containing 10% FBS and antibiotics;

14. Incubate at 37 ºC, 5% CO_2 for 42 hours;
15. Change medium with fresh DMEM containing 2 ug/ml puromycin, 10% FBS and antibiotics;
16. Continue to incubate at 37 ºC, 5% CO_2 for 4~6 days, and replace culture medium every other day with puromycin-containing medium;
17. Observe the cells every day to monitor the death of cells that are sensitive to puromycin;
18. Remove the culture medium from each well;
19. Gently add 1 ml of PBS to wash the cells;
20. Fix cells with 1ml of 4% paraformaldehyde for 15 min at room temperature;
21. Wash one time with 1 ml of PBS;
22. Stain cells with 1 ml of crystal violet solution at room temperature for 20 minutes;
23. Remove crystal violet solution;
24. Wash cells with PBS for three times (3 ml for each time);
25. Count the blue-stained colonies using a microscope at a magnification of 40×;
26. Calculate the lentiviral titer using the formula below: Titer = colony number per well× dilution fold × 40 (TU/ml)

THE UNIVERSITY OF TEXAS
MD Anderson Cancer Center
Making Cancer History®

Protocol for Virus titer by flow cytometry

Day 1, prepare HEK-293 T cells

1. Digest 293T cells (at log growth phase) and seed the cells into 6-well plate with $5x10^5$ cells/well;
2. Incubate the cells at 37 ºC/5% CO_2 overnight (the cells will become about 50-60% confluence on next day);

Note: Prepare an extra plate for cell counting on next day;

Day 2, prepare virus infection

1. Determine the cell number for transfection: detach the cells by trypsin/EDTA treatment and count the cells;
   Cell Number (N) in each well used for infection = total number from 6 wells/6;
2. Take the virus from -80 ºC and perform 10-fold serial dilution of the virus by adding 5 µl of virus to 45 µl of culture medium without antibiotics (perform duplication for each dilution), mix well by gentle pipetting up and down;
3. Remove the medium from 6-well plate gently and add 0.5 ml of fresh medium without antibiotics to each well;
4. Label the wells carefully with dilution fold and transfer 20 µl of diluted virus to each well correspondingly;
5. Swirl the plate gently to ensure all cells covered by virus media;
6. Spin the plate at 2000 rpm (~894×g, Sorvall Legend XTR centrifuge, Thermo Scientific), 25 ºC for 1 hour; (this step is omitted for HGW-control virus)
7. Incubate the cells at 37 ºC, 5% CO2 for 5 hours;
8. Add 0.5 ml of culture media without antibiotics to each well;
9. Continue to incubate the cells at 37 ºC, 5% CO2 overnight;

Day 3, add fresh culture media

1. Add 2 ml of fresh culture media to each well;
2. Incubate the cells at 37 ºC, 5% CO2 for 48 hours (i.e., 72 hours post transduction at the end of incubation);

Day 4, detach the cells and perform flow cytometry analysis;

1. Remove the culture media carefully without detaching the cells;
2. Wash the cells with 1 ml of PBS;
3. Add 0.5 ml of Trypsin/EDTA to each well;
4. Detach the cells by incubation at 37 ^{o}C, 5% CO2 for about 2 minutes;
5. Add 1 ml of culture media containing FBS to each well and mix by gently pipetting up and down;
6. Transfer cells from each well to a correspondingly labeled 15-ml falcon tube;
7. Centrifuge at 1000 rpm (~224×g, Sorvall Legend XTR centrifuge, Thermo Scientific), room temperature, 3 minutes;
8. Remove supernatant carefully and wash the cells with 2 ml of PBS;
9. Spin at 1000 rpm and remove PBS;
10. Re-suspend the cells in 0.4 ml of fresh PBS;
11. Transfer the cells to FACS tubes;
12. Perform FACS analysis using non-infected cells as a negative control;

Calculate the titer

1. Choose the cells with 10%~20% GFP/RFP positive for titer calculation;
2. Calculate the titer using the following equation:

$$\text{Titer (TU/ml)} = (\mathbf{N} \times \mathbf{P})/(\mathbf{V} \times \mathbf{D})$$

Note:
N = Cell Number in each well used for infection on Day 2;
P = percentage of GFP/RFP positive cells (should be 10%~20%);
V = virus volume used for infection in each well; the V (ml) = 20 (µl) $\times 10^{-3}$ in this protocol;
D = dilution fold;
TU = transduction unit;