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Protocol: No-spin infection optimization and screening for adherent cells

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1. Brief Description

In pooled screens, a large population of cells is infected with a lentiviral pool containing different clones. Cells are individually perturbed by one clone and then grown as a population. After transduction and puromycin selection, cells are split into two or more arms, depending on the experimental design.

A spinfection, or centrifuging after the addition of virus, (see **Protocol: Optimization of Lentiviral Transduction Using Spinfection**) typically requires a low virus volume per the number of cells infected. However, this alternative no-spin protocol eliminates the need to centrifuge, as well as potentially reducing handling time and over-confluency issues that can arise with a spinfection.

Successful, high-quality genome-wide screens require a period of assay development to test and optimize the screening conditions. This protocol provides guidelines to experiments specific to no-spin pooled screening with the barcoded lentivirus libraries. The amount of virus required to obtain a targeted infection rate of 30-50% for a given cell line is highly variable. Accurate determination of viral volume for a target cell density is required to allow subsequent large scale infection of screening cell lines at the targeted infection efficiency.

2. Materials and Reagents

The following materials are required:

- ~5 million cells
- Growth media (FBS, supplements)
- PBS and Trypsin

- Cell counter
- 6-well plates
- Polybrene solution (Sigma-Aldrich, Cat#H9268), make a stock of 8 mg/mL in water
- Puromycin solution, 10 mg/mL (Sigma-Aldrich, Cat#P9620, 10 mL)
- Pipettes, 15 mL conicals
- 1 mL pRosetta or pRosettav2 virus
- 1 mL high-titer library virus or 2 mL low-titer library virus

The following materials are optional, but highly recommended:

- Flow cytometer
- 96-well V-bottomed plates
- Flow buffer (PBS, 2% FBS, 5 uM EDTA)

The pRosetta (PuroR, BlastR, GFP) or pRosettav2 (PuroR, BlastR, HygroR, GFP) and library lentiviruses are available from GPP (gpp-reagents@broadinstitute.org).

3. Assay Development for No-Spin Lentiviral Infection

STEP 1: Cell Density Optimization

Determine an optimal seeding density for the no-spin infection.

Day 1, PM:

1. Seed cells in a 6-well plate
 - a. Collect cells and count.
 - b. Seed cells in six wells of a 6-well plate using the following densities:

# of cells/6-well**	Total vol. in each 6-well (mL)
5.00e4	1.1 mL
1.00e5	
2.50e5	
5.00e5	
1.00e6	
1.50e6	

***Note: This range of values is for cell lines that are not especially large or small in size and double about once per day. Numbers can be adjusted.*

- c. Add media up to 1.1 mL.

Day 2, AM (17 hr post-seeding):

2. Change media
 - a. Aspirate media from each well.
 - b. Add 2.75 mL fresh media to each well.

Day 3, PM (48 hr post-seeding):

3. Check and record the confluency of each well

# of cells seeded Day 1	% confluence at Day 3
5.00e4	
1.00e5	
2.50e5	
5.00e5	
1.00e6	
1.50e6	

- **Choose the seeding density that reaches 90-95% confluent.**

Examples of appropriate seeding densities:

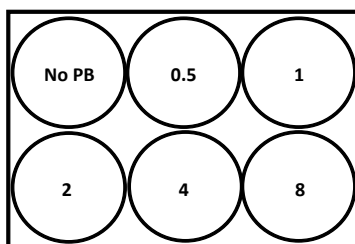
- A375: 2.5e5 cells/well
- HT29: 1.0e6 cells/well
- 293T: 2.0e5 cells/well

STEP 2: Polybrene and Selection Drug Concentration Optimization

Determine the optimal polybrene concentration for the infection by assessing cell toxicity & infectability via several methods.

Day 1, PM:

1. Make a cell and pRosetta master mix and seed 6-well
- Multiply the chosen seeding density from **STEP 1** by 6 to calculate the total number of cells required.
 - Collect and count cells.
 - Re-seed remaining parental cells for later flow cytometry assay (step 7).
 - Dilute the required number of cells in a total volume of 2.34 mL of media.
 - Add 960 uL of pRosetta lentivirus to the cell suspension.
 - Pipette 550 uL of the master mix to six wells of a 6-well plate.
 - Label each well as shown below:



2. Make polybrene serial dilutions
- To 1.1 mL of media add 2.2 uL of an 8 mg/mL polybrene stock.

- b. Serially dilute the polybrene media by half four times (e.g. 550 uL of polybrene media to 550 uL media).
- c. Add 550 uL of each polybrene media to the corresponding 6-wells (final concentrations 8, 4, 2, 1, 0.5 ug/mL).
- d. Add 550 uL of media to the no polybrene control well.

Day 2, AM (17 hr post-seeding):

3. Aspirate media and add 2.75 mL fresh media to each well

Day 3, PM (48 hr post-seeding):

4. Visually assess each well
 - a. Observe the confluency and amount of cell death and/or debris.
 - b. Note the polybrene concentration that results in the most cell death.
5. Collect the cells from each well, ensuring that every condition is kept separate
 - a. Count the cells and record yields.
 - b. **Save the cells in suspension if performing flow cytometry and to perform selection drug dosing.**
6. Optional, but highly recommended: assay the percentage of GFP-positive cells via flow cytometry
 - a. Collect the previously seeded, non-infected parental cells.
 - b. Take 200 uL of each cell suspension and seed into a V-bottomed 96-well plate -- **do not discard the remaining cells; they will be used for selection drug dosing.**
 - c. Add 100 uL of flow buffer (PBS, 2% FBS, 5 uM EDTA) to each well and mix by pipetting up and down.
 - d. Assay the 6 conditions from step 1 along with the non-infected parental cells via flow cytometry.
 - e. Use the non-infected parental cells to set the appropriate gates.
 - f. Record the % GFP-positive cells for each condition, indicating the general infectability at each polybrene concentration.
- **Determine the polybrene concentration for the cells that best balances high infectability (% GFP-positive) with minimal cell toxicity. If flow-cytometry was not performed, choose polybrene concentration based on cell toxicity alone. Then, recommended to make a 1000X working polybrene stock.**
7. Seeding for selection drug dosing
 - a. Calculate the number of cells needed for selection drug dosing by dividing the chosen seeding density from **STEP 1** by half and multiplying by six.
 - b. Continue the experiment with the cells from **STEP 2 Part 6** that yielded closest to 30% GFP+ cells. If this cell yield is less than the number needed for selection drug dosing, combine the two cell populations closest to 30% GFP+.

- c. In a 6-well plate, seed half the number of cells chosen in **STEP 1** into each well and add 5 mL of media.
- d. Incubate plate overnight at 37C.

Day 4, AM:

8. Drug Addition

- a. Refer to the “Drug Addition” section of **Protocol: Puromycin and blasticidin titration**, disregarding all previous sections. The days of these two assays will be shifted, but the timeline remains the same.
- b. Choose the correct selection drug based on the screening library. For most libraries available from GPP, this will be puromycin.
- c. Follow the remainder of the protocol as stated.

STEP 3: Viral titration

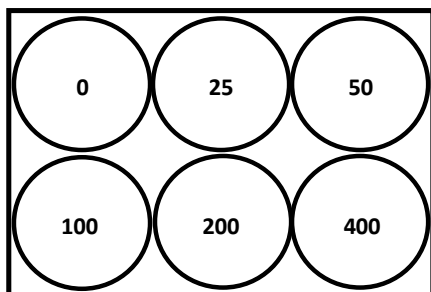
Infect cells with a range of virus volumes (for example a genome-wide CRISPR library) and determine the infection efficiencies after puromycin selection.

Day 1, PM:

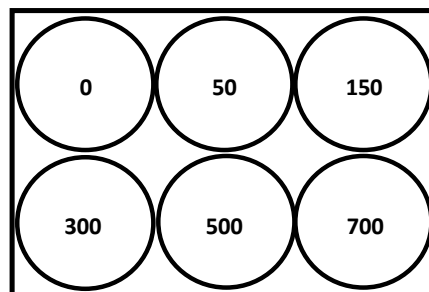
1. Make a cell and polybrene master mix and seed 6-well

- a. Multiply the chosen seeding density from **STEP 1** by 6 to calculate the total number of cells required.
- b. Collect and count cells.
- c. Dilute the required number of cells in a total volume of 3.30 mL of media.
- d. Add 2X polybrene (concentration chosen in **STEP 2**) to the cell suspension.
- e. Add 550 uL of the cell and polybrene master mix to six wells of a 6-well plate.
- f. Label the 6-well plate with the chosen virus volumes, as shown below:
 - For high-titer viruses: 0-25-50-100-200-400 uL
 - For low-titer viruses: 0-50-150-300-500-700 uL

High-titer:



Low-titer:



- g. Add the corresponding virus volume to each well.
- h. Add media up to 1.1 mL per well.

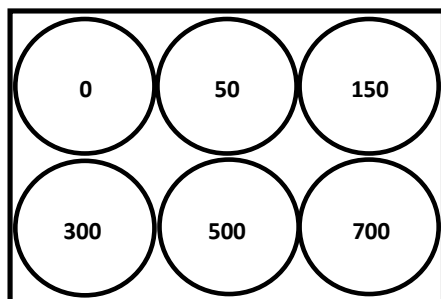
Day 2, AM (17 hr post-seeding):

2. Aspirate media and add 2.75 mL fresh media to each well

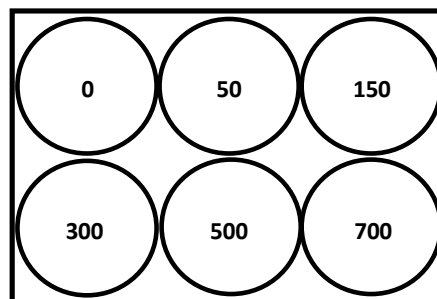
Day 3, PM (48 hr post-seeding):

3. Seed an in-line titer plate
 - a. Label *two new* 6-well plates, each with the virus volumes. Label one plate as No Puro and the other as Plus Puro (see the low-titer example below):

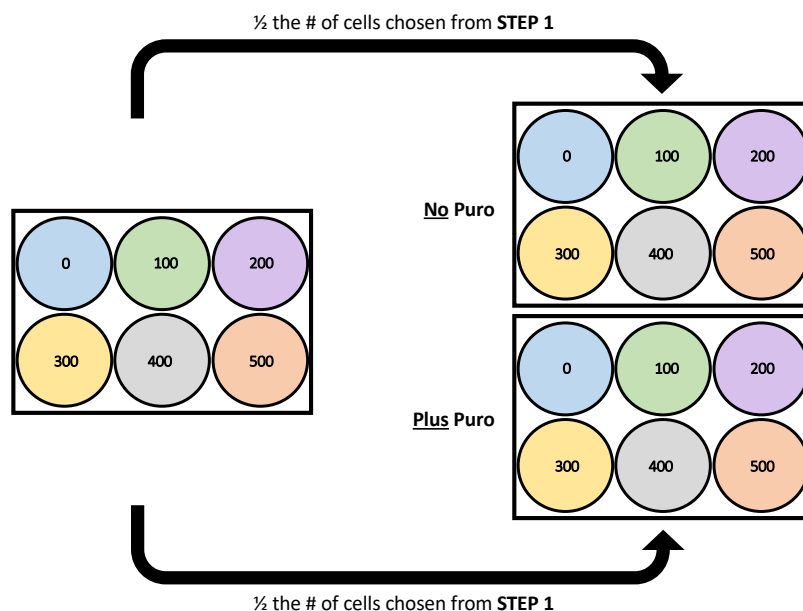
No Puro:



Plus Puro:



- b. Count the cells from each well.
- c. From each well, seed half the number of cells chosen in **STEP 1** into the corresponding new 6-wells. Each new 6-well plate now has a single well seeded from each original virus volume (see the next page):



- d. Add media up to 2 mL in each well

4. Add puromycin to cells

- To 12 mL of media add 2X puromycin (concentration previously determined in **Protocol: Puromycin and blasticidin titration**).
- Add 2 mL of puromycin media to each well of the *Plus Puro* plate.
- Add 2 mL of media to each well of the No Puro plate.

Day 4-6:

5. When fully selected, determine infection efficiency

- When the 0 uL virus plus puro well is completely dead, selection is complete and titer is ready to read out.
- Count the cells in each well and record yields.
- Assess the infection rate at each virus volume by comparing plus puro : no puro.

➤ **The amount of virus needed to achieve a 30-50% infection rate should be used.**

4. Screen With No-Spin Infection

After calculating the number of cells to be infected for the desired screen, determine the number of T175 flasks and total virus volume required. **The final number of cells to infect may be slightly higher than the original number calculated after rounding up the infection to a whole number of flasks.** After calculating the infection parameters, perform the no-spin infection with the library of choice.

Prior to starting:

1. Scale up for the screen (per replicate)

- Scale up the chosen seeding density from **STEP 1** to a T175 by multiplying by 18.2 ($175 \text{ cm}^2 / 9.6 \text{ cm}^2 = 18.2$).
 - This is the T175 infection seeding density.

$$\boxed{} \times \boxed{18.2} = \boxed{}$$

Seeding density from STEP 1 T175 infection density

- Divide the number of cells to be infected per replicate by the T175 infection-seeding density and round up to the nearest whole number.
 - This is the **number of T175s** needed per replicate.

$$\boxed{} \div \boxed{} \xrightarrow{\text{Round up}} \boxed{}$$

of cells to be infected per rep. T175 infection density # of T175s per rep.

- c. Multiply the **number of T175s** needed per replicate by the T175 infection-seeding density.

➤ This is the **final number of cells** to be infected per replicate.

$$\boxed{} \times \boxed{} = \boxed{}$$

of T175s per rep. T175 infection density Final # of cells per rep.

- d. Scale up the chosen virus volume from **STEP 3** by multiplying by 18.2 and divide by 1000.

➤ This is virus volume per T175.

$$\boxed{} \times \boxed{18.2} \div \boxed{1000} = \boxed{}$$

Virus volume from STEP 3 (uL) Virus vol. per T175 (mL)

- e. Multiply the virus volume per T175 by the **number of T175s** needed per replicate.

➤ This is the **virus volume** needed per replicate.

$$\boxed{} \times \boxed{} = \boxed{}$$

Virus vol. per T175 (mL) # of T175s per rep. Virus vol. per rep. (mL)

- f. Multiply the **number of T175s** needed per replicate by 20 mL.

➤ This is the **total volume** needed per replicate.

$$\boxed{} \times \boxed{20 \text{ mL}} = \boxed{}$$

of T175s per rep. Total volume per rep. (mL)

- g. Determine the **volume of polybrene** needed per replicate based on the **total volume** per replicate and the chosen concentration from **STEP 2**.

$$\boxed{} \xrightarrow[\text{From STEP 2: 1000X polybrene}]{\text{Calculate}} \boxed{}$$

Total volume per rep. (mL) Vol. polybrene per rep. (uL)

Day 1, PM:

2. Infect with the library virus of choice

- Collect and count the parental cells expanded up for the screen.
- Multiply the **final number of cells** (these will now be in suspension), **volume of polybrene**, and **total virus volume** per replicate x #of replicates and make a master mix (this will be part of the **total volume**).

$$\left[\begin{array}{c} \boxed{} \\ \text{Final \# of cells per rep.} \end{array} + \begin{array}{c} \boxed{} \\ \text{Vol. polybrene per rep. (uL)} \end{array} + \begin{array}{c} \boxed{} \\ \text{Virus vol. per rep. (mL)} \end{array} \right] \times \boxed{} \text{ \# of replicates}$$

Master Mix (mL)

- Multiply the **total volume** needed per replicate x #of replicates, then subtract the master mix volume. This will calculate the remaining volume of media to add.

$$\left[\begin{array}{c} \boxed{} \\ \text{Total volume per rep. (mL)} \end{array} \times \boxed{} \text{ \# of replicates} \right] - \boxed{} \text{ Master Mix (mL)} = \boxed{} \text{ Vol. media to add}$$

- Then pipette 20 mL of the **total volume** into each T175.

3. Seed a non-infected control (NIC) well

- From the collected parental cells, dilute the number of cells chosen in **STEP 1** in a total volume of 550 uL media.
- Add the cell suspension to one well of a 6-well plate labeled NIC.
- Add 2X polybrene (concentration determined in **STEP 2**) to 550 uL of media
- Add the media to the NIC well.

Day 2, AM (17 hr post-seeding):

4. Change media

- Aspirate media.
- Add 2.75 mL fresh media to the NIC well.
- Add 50 mL fresh media to each T175.

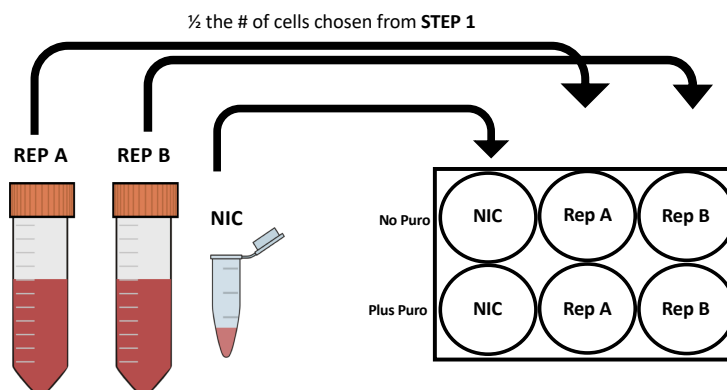
Day 3, PM (48 hr post-seeding):

5. Pool the replicates

- Collect and pool the cells from the appropriate number of T175 flasks to set the replicates (i.e. 30-flask infection, 3 replicate screen → pool 10 flasks per replicate).
- Collect the cells from the NIC well.
- Count the cells from each pooled replicate and from the NIC well.

6. Seed an in-line titer plate

- Label 6-well plate(s) with the replicate numbers and NIC in duplicate, one well as No Puro and one well as Plus Puro.
- Seed half the number of cells determined in **STEP 1** from each replicate into the corresponding wells.
- Seed half the number of cells determined in **STEP 1** from the NIC into the corresponding wells.



- Add up to 2 mL of media in each well.

7. Seed the remaining cells from each replicate into the appropriate flasks for selection

8. Add puromycin

- Add puromycin at the concentration previously determined (**Protocol: Puromycin and blasticidin titration**) to the flasks.
- Add 2 mL of a 2X puromycin media to each *Plus Puro* well of the in-line plate.