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PROTOCOL: OPTIMIZATION OF LENTIVIRAL TRANSDUCTION USING SPINFECTION

Table of Contents

1. Brief Description

Lentiviral transduction is an effective method for creating a stable cell line with a DNA cassette of interest integrated into its genomic DNA, e.g. an sgRNA or gene expression cassette. Transducing cells at a concentrated seeding density in 12-well plates while centrifuging ('spinfection'), is a method to achieve efficient transduction of a large number of cells. Cells are typically infected in the presence of polybrene, a polycation that neutralizes the charge repulsion between the virus and cell target surface and helps viral integration into the cell.

The ideal spinfection conditions can be highly variable across cell types and should be optimized, as cell lines vary in, e.g infectability, polybrene sensitivity and response to dense culture. A balance between seeding density, polybrene concentration and other spinfection variables will be determined to ensure successful transduction and minimal cost to cell viability. This protocol uses the pRosetta GFP-vector as a lentiviral control and as an optional, but highly recommended, simple assessment of the infectability of the cell line. Transduction efficiency is determined as the percentage of GFP-positive cells, via flow cytometry.

2. Materials and Reagents

The following materials are required:

- 30 million cells
- Growth media, PBS and trypsin
- Cell counter
- Trypan Blue (for suspension lines)
- 12-well plates
- Tissue culture flasks
- Polybrene (Sigma-Aldrich, Cat #H9268), make a stock of 8 mg/mL in water)
- Pipettes
- 15 mL, 50 mL tubes
- 2 mL of pRosetta virus (aka pLKO_TRC060) lentivirus (PuroR, BlastR, GFP), available from GPP (gpp-reagents@broadinstitute.org)

The following materials are optional, but highly recommended:

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

3. Optimization of Spinfection Conditions

STEP 1: Cell Density and Polybrene Matrix

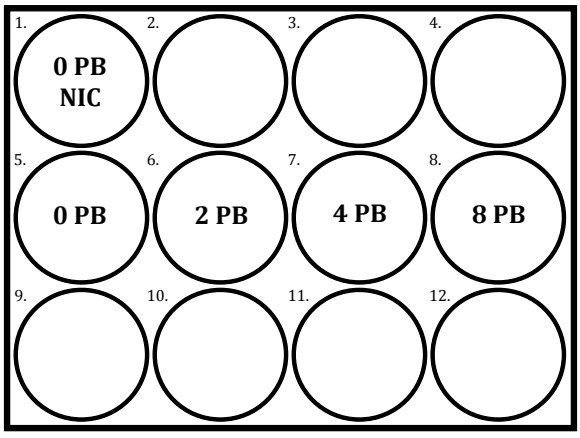
Perform a spinfection using a range of cell densities and polybrene concentrations.

Day 1:

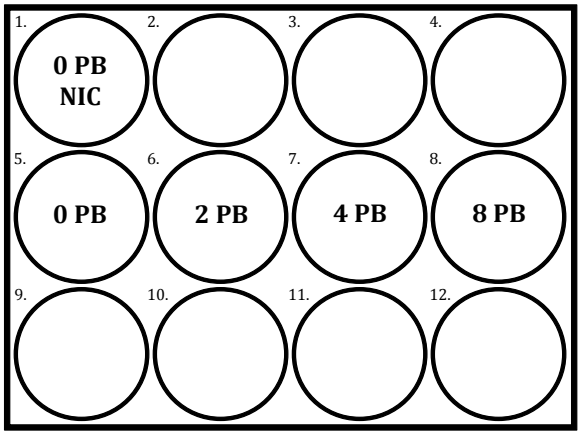
A: Prepare 12-well plates and master mixes

1. Label two 12-well plates as follows:

1.5E6



3E6



2. Collect the cells:
    - For adherent cells:
        i. Aspirate media and wash flask with warm PBS.
        ii. Add trypsin and allow cells to detach from flask.
        iii. Add warm media (2-3x the amount of trypsin) to the flask to quench the trypsin. Transfer the cell suspension into a conical.
        iv. Wash the flask with media and add the wash to the same conical.
        v. Mix the cell suspension thoroughly and count.
    - For suspension cells:
        i. Collect the cell suspension from the flask into a conical.
        ii. Spin down the cells at 335-524 g for 5 min and aspirate media supernatant.

iii. Re-suspend the cells thoroughly in fresh, warm media and count.

3. Dilute the cells in media to the following concentrations:
   - **a.** 1.5E6 cells/mL in 6 mL total (low-density)
   - **b.** 3E6 cells/mL in 6 mL total (high-density)
4. Make the following media, 8 mg/mL polybrene stock and pRosetta virus solutions:
   - **c.** 3 mL of media
   - **d.** 2.7 mL media + 0.3 mL virus
   - **e.** 2.7 mL media + 0.3 mL virus + 1.5 uL polybrene (final = [2 ug/mL])
   - **f.** 2.7 mL media + 0.3 mL virus + 3 uL polybrene (final = [4 ug/mL])
   - **g.** 2.7 mL media + 0.3 mL virus + 6 uL polybrene (final = [8 ug/mL])

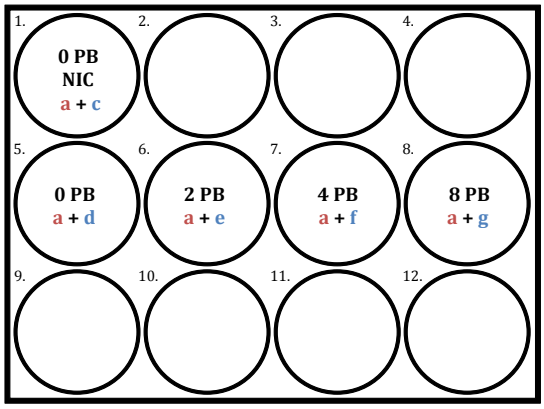
B: Seed cells in 12-well plates

1. Add 1 mL of **cell suspension** and 1 mL of **media/PB/virus solution** to the corresponding labeled wells, as follows:

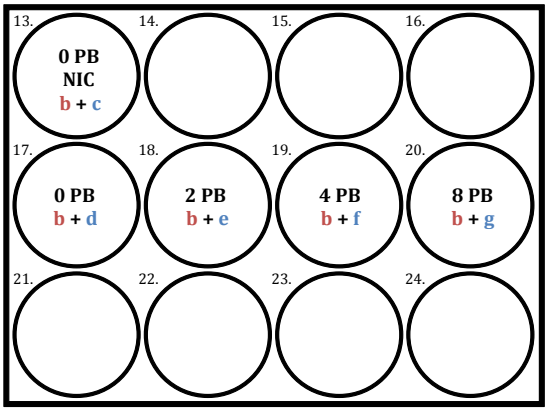
   Note: the wells labeled '0 PB, NIC' are the no polybrene, no-infection controls.

   Each well will have a total of 2 mL.

1.5E6



3E6



C: Centrifuge the plates

1. Spin the plates at 931 g for 2 hours at 30°C.

D: Add media to plates and incubate

1. After 2 hours of spinning, add 2 mL of media (without polybrene) dropwise on top of each well.
2. Place in 37°C incubator overnight.

Day 2:

E: Inspect cells visually

1. The next day (16-24 hrs later), remove the plate from the incubator and inspect the cells under a microscope.
2. Take note of the following things:
    - If the cells clumped together and lifted off the plate
    - The confluency of the wells
    - How adhered the cells are to the plate (suspension cells will be loosely adherent)
    - The amount of dead cells (typically shriveled and floating)

General Assessment:

- If in all conditions:
    - The cells clumped together and lifted off the plate, they *may* be sensitive to a high confluency and/or centrifugation. Be sure to gently break up the clumps before counting in **STEP 1, Day 2, Part G**.
    - There appear to be a lot of loosely adherent cells, they *may* be sensitive to density.
    - There appear to be a lot of dead cells, they *may* be sensitive to centrifugation.

F: Collect the cells from the plates

1. Collect the cells from the plate:
    - For adherent cells:
        i. Label 10 conicals with the condition names from the plate.
        ii. Collect the media from each well into the appropriate conical.
        iii. Add 1 mL of PBS to each well and collect it into the appropriate conical.
        iv. Add 250 uL of trypsin to each well and let the cells detach from the plate.
        v. Add 750 uL of media to each well to quench the trypsin and transfer the cell suspension to the appropriate conical.
        vi. Add 1 mL of media to wash each well and transfer the wash to the appropriate conical.
        vii. Spin the conicals at 335-524 g for 5 min.
        viii. Aspirate the media and re-suspend the 'low-density' cells in 1 mL of fresh media and the 'high-density' cells in 2 mL of media.
    - For suspension cells:
        i. Label 10 conicals with the condition names from the plate.
        ii. Gently pipette the media in each well up and down to dislodge the cells and then collect the cell suspension into the appropriate conical.
        iii. Add 1 mL of media to wash each well and transfer the wash to the appropriate conical.
        iv. Spin the conicals at 335-524 g for 5 min.

v. Aspirate the media and re-suspend the low-density cells in 1 mL of fresh media and the high-density cells in 2 mL of media.

G: Count the cells and assess the recovery from the 12-well plates

1. Count the cells, using Trypan blue for suspension cells.
2. Record yields – *note more than 25% loss or cell death in any condition*.

General Assessment:

- Compare the recoveries between the (-)polybrene, (-)virus wells and the (-)polybrene, (+)virus wells (#1 vs. #5 and #13 vs. #17): if the numbers are lower with virus, lentivirus *may* be toxic to the cells.
- Compare the recoveries between the (-)polybrene wells and the (+)polybrene wells (#5 vs. #s 6, 7, 8 and #17 vs. #s 18, 19, 20): if the numbers are lower with polybrene, polybrene *may* be toxic to the cells.
- Compare the recoveries between the 'low' vs. 'high-density' wells (ex. #5 vs. #17): if more cells are lost from the 'high-density' wells, the cells *may* be sensitive to density.

H: Seed the cells into flasks

1. Choose appropriately sized flasks based on cell size and doubling time to ensure the cells will not become confluent within 2-4 days.
2. Label the 10 flasks with each well condition name.
3. Seed the cells from each conical into the corresponding labeled flask and add the appropriate volume of media.
4. Incubate the cells.

Day 4-6:

I: Monitor the cells post-spinfection and seeding

1. Check the cells under the microscope over the next 2-4 days and note any significant cell death and/or debris in any of the conditions.
2. Before the cells get confluent, stop the assay and continue with **STEP 2**.

STEP 2: **Infection Efficiency and Cell Viability Read Out**

Determine the infection efficiency and cell viability via several methods.

A: Visually assess the confluency and health of the cells

1. Check each flask under the microscope.
2. Take note of the following things:
    - Cell confluency
    - Morphological changes

- Amount of cell death and/or debris

B: Collect and count the cells from the flasks to obtain yields

Note: there must be cells left over from each flask to perform flow cytometry.

1. Collect the cells from each flask, ensuring that every condition is kept separate.
2. Count the cells and record yields.
3. Save the cells in suspension to perform flow cytometry.

C: Optional, but highly recommended: assay the percentage of GFP-positive cells via flow cytometry

1. Take 200 uL of each cell suspension and seed into a V-bottomed 96-well plate for flow cytometry.
2. Add 100 uL of flow buffer (PBS, 2% FBS, 5 uM EDTA) to each well and mix by pipetting up and down.
3. Assay the 10 conditions via flow cytometry, using the no-polybrene, no-infection control well to draw the appropriate gates for GFP-negative cells.
4. Record the % GFP-positive cells for each condition, indicating the percentage of infected cells.

General Assessment:

- The density and polybrene combination with the highest yield from manual counts (and the highest % viable cells from Trypan counts for suspension cells) and the highest percentage of GFP-positive cells will likely be the best to use for future spinfections.

***Troubleshooting* (*also see the* Decision Tree):**

- If in **STEP 1, Day 2, Part E** the cells clumped and lifted off the plate and/or there was more than 25% loss AND in **STEP 2, Part B and C** there were poor yields and/or low GFP percentages, the cells are likely sensitive to a high confluency and/or centrifugation. Repeat the protocol trying one or more of the following options:
    - Continue with **STEP 1 Day 2 Part F**, 4-6 hours post-spin on Day 1.
    - A no-spin lentiviral transduction in flasks (see **Protocol: No-spin infection for adherent cell lines**).
    - A lower seeding density of 8E5 – 1E6 cells/12-well.

- Notes on viral toxicity:
    - If there is a significant loss of cell yield when comparing the (-)polybrene, (-)virus flasks to the (-)polybrene, (+)virus flasks, lentivirus is likely toxic:
        - Use a low range of virus volumes and a low seeding density when titrating with another virus.
        - If lentivirus remains toxic, seek an alternative method of delivery.

- Notes on comparing polybrene concentrations:
    - The lowest concentration that does not affect cell yield should be used.
    - If there is a significant loss in cell yield at all concentrations, polybrene is likely toxic. Repeat the protocol trying one or more of the following options:
        - Instead of adding additional media on top of the wells post-spin, gently aspirate the media from the wells. Then, dropwise and down the side of the well, add 2 mL of fresh media on top of the cells – *Note: do not aspirate media from suspension cells!*
        - Instead of incubating the cells overnight, continue with **STEP 1 Day 2 Part F**, 4-6 hours post-spin on Day 1.
        - A lower seeding density of 8E5 – 1E6/12-well and no polybrene during the spinfection.

- Notes on comparing low vs. high density:
    - If there are poor infection efficiencies at the 'low density' (<25%), the cells have very low-infectability. Repeat using an even lower seeding density of 8E5 – 1E6/12-well.
    - If there are poor infection efficiencies at the 'high density' (<25%), the cells have low-infectability. Use the 'low density' when infecting with another virus.
    - If there is not a significant loss in cell yield/infection efficiency with the 'high density,' it can be used with high-titer viruses – *Note: this is especially useful for some genome-wide libraries.*

5. Perform the Spinfection with Another Virus

The chosen spinfection conditions can now be applied to future lentiviral infections with other viruses besides the pRosetta control. For a list of available viruses please visit https://intranet.broadinstitute.org/gpp/db/ (Broad login required), or email gpp-reagents@broadinstitute.org.

- Most of these vectors have an antibiotic selection marker to confirm successful integration of the vector into the cell's genome. The puromycin or blasticidin dose for selection of the cells must be optimized prior to infection:

    ☐ Puromycin or blasticidin dosing (see **Protocol: Puromycin and blasticidin titration**)

- A commonly used vector is pXPR_111 (pLEX_311Cas9v2) for introducing Cas9 into cells. This vector produces very low titer virus, and it can therefore be challenging to obtain a high infection efficiency. Project Achilles has a high success rate with the following conditions:
    - 1.5E6 cells per 12-well

    - Polybrene concentration cell line dependent
    - 750 uL of pLEX_311Cas9v2 virus per 12-well
    - Infecting several wells at the same time to obtain more screenable cells faster (with one non-infection control well to assess infection efficiency and complete blasticidin selection)

- Before beginning a screen, a 6-well seeding density test and viral titration with the library virus should be performed first:

    - [ ] Cell Density and Doubling Rate assay (see **Protocol: Pooled Screen Viral Titration**)
    - [ ] Viral titration with library virus (see **Protocol: Pooled Screen Viral Titration**)

6. Example Spinfection Conditions

Here are some example spinfections conditions for reference:

HA1E – very fast doubling time, with high-titer virus
- 1.5E6 cells per 12-well
- 4 ug/mL polybrene
- Collected cells out of 12-wells 4-6 hours post-spinfection to avoid clumping and lifting off of plate

TC32 – sensitive to polybrene, moderate infectability, with high-titer virus
- Optimized 2E6 cells per 12-well
- 2 ug/mL polybrene
- Aspirated and replaced media after 2 hour spin

BE(2)C – low infectability, not polybrene sensitive, with low-titer virus
- 1.5E6 cells per 12-well
- 8 ug/mL polybrene

A549 – high-titer virus
- 3E6 cells per 12-well
- 4 ug/mL polybrene

7. Spinfection Decision Tree