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Large Scale Infection for Pooled Screens of shRNA libraries

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This protocol can be further optimized if cells being used are limited, or known to be sensitive to viral infection, polybrene, or centrifugation. Appendix C is added to outline a process for further optimization.

General information:

1. Puromycin selection conditions for your cell line must be calculated in advance. (See Appendix A)
2. The amount of virus (**N**) to infect ~30-50% of the cells is predetermined (See Appendix B)
3. For each set of infections, use a 12 well plate.
4. Cells, virus, and polybrene are mixed and aliquoted into the 12 wells on a 12 well plate, so that each infection well should finally contain in a total volume of 2 mls media
    - 3e6cells + **N** virus + 4ug/mL polybrene
5. A concentration of 1.5e6 cells/mL in 240mL media is required for ten replicates. (This can be proportionally scaled down if fewer replicates are to be performed)
    - For suspension cells: this equals approx. _____ T175 flasks at _____ % confluency.
    - For adherent cells: this equals approx. _____ T175 flasks at _____ % confluency. Freshly trypsinized adherent cells can be used for infection with virus packaged with VSV-G.
6. An in-line infection rate measurement will be performed during large scale infection

In-line infection rate measurement of the large-scale infection

In a 6-well plate, 150,000 of the infected cells and the uninfected cells are cultured in the presence and absence of Puromycin. After effective Puromycin selection is achieved, the infection rate is calculated:

$$\left[\frac{\text{(\# Infected with puro)}}{\text{(\# Infected without puro)}} - \frac{\text{(\# Uninfected with Puromycin)}}{\text{(\# Uninfected without Puromycin)}}\right] = \text{\% Infection}$$

Protocol:

1. Determine number of cells in flasks using coulter counter
    - 7-15um and 10-30um size range have been used

2. Make a *master mix* containing only cells and polybrene. (*note: we make this master mix because we want a total of 10 replicates*. Scale appropriately for the number of replicates needed)
    - For 250mL *master mix*:
        - Dilute 3.75e8 cells to 1.5e6 cells/mL in 250mL media
        - Add 4ug/mL polybrene (125uL of 8mg/mL polybrene stock)
    - **Do not add virus to the *master mix* at this point**

3. Aliquot *master mix* 24mL/tube in 10 tubes.
4. Add 12*__N__ virus to each of the 10 tubes containing *master mix*.

5. For each tube, add 2mL/well of *master mix* containing virus in a 12 well plate.

6. Repeat for remaining tubes.
    - At the end, there should be a total of ten 12 well plates.

7. Spin infection: 2000rpm for 2hrs at 37C (temperature setting is 30C on centrifuge)

8. STOP HERE: Go to **part A for suspension cells** or **B for adherent cells.**

A. For suspension cells.
1. Pool each of the 12 wells for one plate into one 50mL conical tube.
2. Repeat for all 10 plates.
3. Centrifuge 1200rpm for 5 minutes.
4. Carefully remove supernatant
5. Resuspend pellet into 200mL R10 into a T175 flask.
6. From 1 replicate remove 800 ul (~150,000 cells) and plate into each of 2 wells of the 6 well plate containing 1.2 ml media for in-line infection rate measurement. (With Infection samples)And put aside for later.
7. Incubate @37C

    24 post infection, set up in-line infection rate plate.
    - In a 6 well dish, plate 150,000 uninfected cells in 2 mls media in duplicate.
    - Plate 150,000 infected cells from one replicate in duplicate into 2mls final volume.
    - Add 2 mls media only to one well of each Uninfected and Infected cells
    - Add 2 mls media+ 2X Puromycin to one well of each Uninfected and Infected cells, so that the final Puromycin concentration is at 1X working concentration

- After effective Puromycin selection is achieved, the infection rate is calculated (see below)

8. Also 24 hours post infection, add Puromycin to the T175 flasks to a final 1X working concentration
9. 72 hours post infection, 50% of the cells can be passaged into new T175 in selection media, the remaining 50% cells can be spun down, resuspended with 0.5ml PBS, stored at -20C for genomic DNA preparation. (EARLY TIME POINT SAMPLE)
10. Continue passaging with puro selection (30mL of high density cells into 200mL new media). The other 170ml of cells can be spun down, resuspended with 0.5ml PBS, stored at -20C for genomic DNA preparation.

B. For adherent cells.
1. After the 2 hr spin infection, remove sup from each well
2. Add 2mL R10 into each well.
3. Repeat for all 10 plates.
4. Incubate plate @37C
5. The next day, for each plate, trypsinize and pool each infection into a 50mL tube.
6. Repeat for all 10 plates

   Set up in-line infection rate plate.
   - In a 6 well dish, plate 150,000 uninfected cells in 2 mls media in duplicate.
   - Plate 150,000 infected cells from one replicate in duplicate into 2mls final volume.
   - Add 2 mls media only to one well of each Uninfected and Infected cells
   - Add 2 mls media+ 2X Puromycin to one well of each Uninfected and Infected cells, so that the final Puromycin concentration is at 1X working concentration
   - After effective Puromycin selection is achieved, the infection rate is calculated (see below)
7. Resuspend each tube into 100mL of R10 into two T175 flasks.
8. Incubate plate @37C
9. 24 hours post infection, , add Puromycin to the T175 flasks to a final 1X working concentration
10. 72 hours post infection, cells are harvested and both flasks from the same replicate are pooled in 40 mls media in a 50 ml tube. Passage 50% of the cells by adding 10ml of cells into each of two new T175 in media with final 1X Puromycin working concentration. The remaining 50% cells (20 mls) can be spun down, resuspended with 0.5ml PBS, stored at -20C for genomic DNA preparation. (EARLY TIME POINT SAMPLE)
11. Every 3 or 7 days, ¼ of the harvest from each biological replicate (NOTE: minimum of 1E7 cells from each biological replicate) are passaged into 2 new T175 flasks and remainder of cells are spun down, resuspended with 0.5ml PBS and stored at -20C for genomic DNA preparation. Cells should be mixed just before placing into freezer to minimize clumping.

Appendix A: Standard Puro Kill Curve

Day 1: Seed 3x 96-well black clear bottom plates at 1000-2500 cells/mlfor fast growing cells, for slower growing cells 2500-4000 cells/well
(keep in mind, this is a long term experiment, you would not want your cells to be overconfluent by the end of the experiment)

Day 2: Step 1: Change media on the 96w black clear bottom plates so that they contain media w/ 4ug/ml polybrene. (We suggest you to have done a polybrene sensitivity test prior to this experiment.)

Step 2: Infect the plate with pooled library virus: (ul infection volume)

Virus dilutions (uL of Virus)											
0	0	1	1	2	2	4	4	8	8	16	0
0	0	1	1	2	2	4	4	8	8	16	0
0	0	1	1	2	2	4	4	8	8	16	0
0	0	1	1	2	2	4	4	8	8	16	0
0	0	1	1	2	2	4	4	8	8	16	0
0	0	1	1	2	2	4	4	8	8	16	0
0	0	1	1	2	2	4	4	8	8	16	0
0	0	1	1	2	2	4	4	8	8	16	0

Step 3: Spin the plates at 2000rpm @ 30°C for 120min.

Day 3: Step 1: Remove the media from the infected cell plates.
Step 2: Add 100ul media containing various Puromycin concentrations in the following fashion:

Puromycin dilutions (ug/mL)											
0	0	0	0	0	0	0	0	0	0	0	0
0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
1	1	1	1	1	1	1	1	1	1	1	1
2	2	2	2	2	2	2	2	2	2	2	2
3	3	3	3	3	3	3	3	3	3	3	3
4	4	4	4	4	4	4	4	4	4	4	4
5	5	5	5	5	5	5	5	5	5	5	5
7	7	7	7	7	7	7	7	7	7	7	7

Day 6: Step 1: Remove the Puromycin containing media from the cell plates.
Step 2: Add 50ul of 10% Alamar blue in media w/ NO PURO to each well of the cell plates.). Incubate at 37°C for 2.5-3hrs.
Step 3: Read the plates using recommended filter sets and protocol.
Step 4: Calculate Optimal Puro concentration for 100% lethality in non-infected cells and maximal viability in infected cells

Appendix B: optimal Volume of Pooled Viruse to get 30-50% infection Rate

Day1: Step 1: Plate non-infected cells 3E6 cells/well in 2ml media +polybrene in 6 wells of a 12 well plate

Step 2: Infect cells with titrated amounts virus (0, 25, 50, 75, 150, 400µl)

Step 3: Spin 2000 rpm for 120mins at 37C (temperature setting is 30C on centrifuge)

Step 4: Replace supernatant with 2ml fresh media without polybrene

Step 5: Incubate at 37 O/N

Day 2: Step 1

For adherent cells

a. Remove media and Wash twice with 1ml warm PBS per well
b. Add 200µl trypsin solution and allow cells to detach from plate
c. Add 2 ml of tissue culture medium, harvest cells
d. In quadruplicate, plate 60 ul of each titration infection into well of a 6 well plate. Each of the 4 plates should have a single well with each viral infection condition, in a total vol of 2ml
e. proceed to step 2.
f.

For suspenstion cells

a. resuspend cells
b. In quadruplicate, plate 45 ul of each titration infection into well of a 6 well plate. Each of the 4 6 well plates should have a single well with each viral infection condition, in a total vol of 2ml
c. proceed to step 2.

Step 2: For the first two plates add 2ml media only (No puro control)

Step 3: For second two plates add chosen amount of Puromycin from Appendix A using 2ml 2X puro mix

Day 4 to 6: Monitor cells, and when Non-Puromycin cells reach 100% confluence, count all wells using coulter counter

$$\left[\frac{\text{(\# Infected with puro)}}{\text{(\# Infected without puro)}} - \frac{\text{(\# Uninfected with Puromycin)}}{\text{(\# Uninfected without Puromycin)}}\right] = \text{\% Infection}$$

Appendix C: Optimization of Infection for sensitive and limited availability cell types

To be added soon