BROAD INSTITUTE | GENETIC PERTURBATION PLATFORM

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Protocol: Relative Viral Titering with a Resazurin [alamarBlue®] Cell Viability Assay

Brief Description:

This protocol describes a 96-well format relative titering method for lentiviral stocks, based on transduction at low MOI, selection for transduced cells (with puromycin or blasticidin, varying by vectors), and a cell viability assay to quantify survival. The result is a relative titer estimate compared to a standard curve of a serial dilution of a control virus.

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Cell-line Choices:

Ideal cell lines for titering are those easy to infect, easy to kill by the selection drug (0% survival of uninfected cells), easy to handle (stickin*g to the bottom of plates well).* A549 human lung carcinoma cells are best suited for titering vectors carrying puromycin selection marker, whereas Hepa mouse hepatoma cells for those with blasticidin selection marker.

Workflow Timeline:

The relative titering assay consists of the following steps:

Day 0	Seed A549 cells (for puromycin resistant virus) or Hepa cells (blasticidin resistant virus)
Day 1	Prepare virus dilutions (standard curve and "unknowns") Add diluted virus to cells in media containing polybrene Centrifuge cells to promote infection (Optional) Remove media and replace with fresh media
Day 3	*48 hours after virus addition:* spike in fresh media containing puromycin, or blasticidin
Day 5 (Day 6)	*48 hours after puromycin addition (72 hours after blasticidin addition):* Remove media; replace with fresh media containing 10% resazurin *2.5-4 hours after resazurin addition*: Read resazurin fluorescence on plate reader

All lentiviral procedures should be carried out in accordance with biosafety requirements of the host institution.

Materials & Instrumentation

96-well black/clear bottom tissue culture plates (Corning #3904)
A549 cells (human lung carcinoma)
Hepa 1-6 cells (Murine liver)
Growth media (DMEM + 10% FBS + P/S):
500 ml DMEM (Dulbecco's Modification of Eagle's Medium; e.g. Mediatech #10-013-CV)
50 ml FBS (Fetal Bovine Serum; e.g. Mediatech #35-015-CV)
5 ml 100x Pen/Strep (10,000 IU/ml penicillin, 10,000 μg/ml streptomycin; e.g. Mediatech #30-002-CI)
96-well polypropylene storage plates (Corning/Costar #3357)
Polybrene (Hexadimethrine bromide; Sigma #H9268)
Puromycin Dihydrochloride (Sigma #P8833)
Blasticidin S HCl (Invitrogen #A11139-03)
Resazurin (e.g. alamarBlue®, Biosource #DAL 1025)
Fluorescence plate reader (Ex: 530-560nm, Em: 590nm)

Instructions

1. Seed A549 cells or Hepa cells at 30,000 cells/ml (100 µl/well) in growth media (DMEM + 10% FBS + P/S) in 96-well black walls/clear bottom tissue culture plates. Allow seeded plates to sit undisturbed at room temperature for at least 1 hour before transferring to a tissue culture incubator overnight (37°C, 5% CO_2).

   Allow cells to settle at room temperature for 1hr in order to reduce well-to-well variability and edge effects in microtiter plates.

   After ~24 hours, the cells should be ~20-30% confluent.

2. Prepare a serial dilution series of a control virus stock, with sufficient volumes for multiple replicate wells per dilution (5 µl will be used per transduction well).
   a. Arrayed virus in 96-well

Dilution	Virus Stock or previous dilution (µl)	Media (µl)	Total Volume (µl)	Volume removed (µl)	Volume remaining (µl)	µl virus per 5 µl volume
1	400	100	500	250	250	4
2	250	250	500	250	250	2
3	250	250	500	250	250	1
4	250	250	500	250	250	0.5
5	250	250	500	250	250	0.25
6	250	250	500	250	250	0.125
7	250	250	500	250	250	0.0625
8	250	250	500	250	250	0.03125
9	250	250	500	250	250	0.015625
10	250	250	500	250	250	0.0078125
11	250	250	500	250	250	0.00390625
12	250	250	500	250	250	0.001953125
13	250	250	500	250	250	0.000976563
14	250	250	500	0	500	0.000488281
Blank	0	250	250	0	250	0

Prepare a dilution of “unknown” virus samples. For example, for a 1:10 dilution, transfer 45 µl fresh media to a 96 well storage plate, add 5 µl virus stock per well, and mix well by pipetting.

Note: Use an appropriate dilution such that the “unknown” samples fall within the linear range of the standard curve. Higher titer virus stocks will require a larger dilution factor, e.g. 1:40 dilution (195 µl fresh media + 5 µl virus stock).

b. Large scale of low-titer virus (ex: pLX_317, pXPR_023, pLX311-Cas9)

- Label 8 tubes. In tube #1, add 100ul of virus stock of unknown titer. In tubes #2-8, add 50 ul of media and make 2-fold dilution from tube #1.
- Prepare cells for transduction. Remove media and replace with 35 µl fresh growth media (DMEM + 10% FBS + P/S) supplemented with 10 µg/ml polybrene. (About 5 µl residual media is expected per well.)
- Add 10 µl diluted virus to each well of cells. Run at least one “standard curve” plate containing replicate wells of the serial dilution of control virus prepared in step 2.

c. Large scale of high-titer virus (ex: pLX_304, pXPR_003, pLKO)

- Label 8 tubes. In tube #1, add 40ul of virus stock of unknown titer and 60ul of media. In tubes #2-8, add 50 ul of media and make 2-fold dilution from tube #1.
- Prepare cells for transduction. Remove media and replace with 40 µl fresh growth media (DMEM + 10% FBS + P/S) supplemented with 10 µg/ml polybrene. (About 5 µl residual media is expected per well.)
- Add 5 µl diluted virus to each well of cells. Run at least one “standard curve” plate containing replicate wells of the serial dilution of control virus prepared in step 2.

3. Spin plates at 1,200 x g for 30 minutes at 37 °C.
4. (Optional) Remove media and replace with 100 µl fresh growth media (DMEM + 10% FBS + P/S).
   Note: A549/Hepa cells do not require an immediate post-infection media change with low MOI infections.

5. Incubate cells for 48 hours (37 °C, 5% CO_2).

6. Add 100 µL fresh growth media (DMEM + 10% FBS + P/S) supplemented with puromycin (achieving 1.5 µg/ml selection concentration) or blasticidin (achieving 3.5 µg/ml selection concentration).

   Note: The concentration of the selection drug and the selection duration should be optimized, as they depend on cell lines, number of the seeded cells and drug sensitivity of single integrants. The selection pressure should be enough to kill uninfected cells but not too high that it kills cells infected with a single integrant.

7. Incubate cells for appropriate selection duration (e.g. 2 days for A549/puromycin, 3 days for Hepa/blasticidin) (37 °C, 5% CO_2), after which, the “standard curve” plate should have a range of cell confluency, from ~100% live cells (highest amount of virus) to ~100% dead cells (no virus controls).

8. Remove media and replace with 50 µl fresh growth media (DMEM + 10% FBS + P/S) supplemented with 10% resazurin (alamarBlue).

Note: it is important to warm alamarBlue to room temperature before diluting in media.

9. Incubate cells for 2.5-4 hours (37 °C, 5% CO_2).

10. Determine fluorescence (Ex: 530-560nm, Em: 590nm) on a fluorescent plate reader at 1 hour; repeat as needed (return cells to incubator between reads). At different time points, there may be a different linear range of the assay as determined by the signal of the “standard curve” plate.

    Note: For consistency between plates, read the plates in the same order and relative timing (e.g. 1 minute apart) as for alamarBlue addition.

11. Generate a standard curve to determine the relative titer of “unknown” samples and the linear range and sensitivity of the assay.
    Example: