9/30/05

Section III: Lentiviral Infection

Introduction:

This section contains protocols for large scale (6 cm plates) and high throughput (96-well and 384-well) lentiviral infections to achieve stable shRNA-mediated target gene knockdown.

Lentiviral infection consists of the following steps:

Day 0-1	Seed cells
Day 1	Add lentivirus to cells in growth media containing polybrene
	(optional for 96-well infections: centrifuge cells to promote infection)
Day 1-2	Remove media and replace with fresh growth media
Day 2+	(optional) Select for infected cells with media containing puromycin
Day 4+	Assay infected cells

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

Part 1: Lentiviral Infection in 6 cm plates

I. Materials

6 cm tissue culture plates (appropriate for cell-based assay)
Human or mouse cell line and appropriate growth media
Reagents required for cell-based assay
Polybrene (Hexadimethrine bromide; Sigma #H9268) or Protamine sulfate (MP Biomedicals #194729)
(Optional) Puromycin Dihydrochloride (Sigma #P8833)

II. Instructions

A. Optimization of lentiviral infection

Lentiviral infections should be optimized for each cell line and cell-based assay. For example, the following parameters should be tested before starting large-scale infections to determine the optimal conditions for a given experiment:

- Cell seeding density
- Amount of lentivirus
- Puromycin concentration
- Timecourse

B. Infection protocol

1. Seed cells at appropriate density in 6 mL media in 6 cm plates.

    a. Adherent cells: seed 1 day prior to infection. Incubate overnight (37 °C, 5% CO_2).

    b. Suspension cells: seed day of infection in media containing polybrene* (see table in step 2a).

2. Add virus to cells:

    a. (Adherent cells) Remove growth media and add fresh media containing polybrene* (see table). Alternatively, remove a portion of the growth media and supplement with media containing polybrene. Adjust volumes and polybrene concentration to achieve the correct final polybrene concentration.

Reagent	Per 6 cm plate
Media containing polybrene*	to 6 mL
Final polybrene concentration	8 µg/mL
Virus (added in step 2b)	
High MOI	≥0.5 mL
Low MOI	≤0.1 mL

** Protamine sulfate may be substituted if polybrene is toxic to cells.*

    b. Add virus to cells (see table in step 2a).

3. Viral infection:

    a. Incubate cells overnight (37 °C, 5% CO_2).

    b. Change media 24 hours post-infection. Remove media and replace with 6 mL fresh growth media. If puromycin selection is desired, use fresh growth media containing puromycin. *Note: Puromycin concentration should be optimized for each cell line; typical concentrations range from 2-5 µg/mL.*

4. Incubate cells (37 °C, 5% CO_2), replacing growth media (with puromycin, if desired) as needed every few days. Incubation periods are highly dependent on the post-infection assay. Puromycin selection requires at least 48 hours. The following recommendations are general guidelines only, and should be optimized for a given cell line and assay.

Post-infection assay	Incubation time post-infection	Incubation time with puromycin selection
mRNA knockdown (qPCR)	3+ days	2+ days
Protein knockdown (Western)	4+ days	3+ days
Phenotypic assay	4+ days	3+ days

5. Assay infected cells.

Part 2: Lentiviral Infection in 96-well or 384-well plates (high throughput)

I. Materials

96-well or 384-well tissue culture plates
Human or mouse cell line and appropriate growth media
Polybrene (Hexadimethrine bromide; Sigma H 9268) or Protamine sulfate (MP Biomedicals #194729)
(Optional) Puromycin Dihydrochloride (Sigma #P8833)

II. Instructions

A. Optimization of lentiviral infection

Lentiviral infections should be optimized for each cell line and cell-based assay. For example, the following parameters should be tested before starting large-scale infections to determine the optimal conditions for a given experiment:

- Cell seeding density
- Amount of lentivirus
- Puromycin concentration
- Timecourse

B. Infection protocol

1. Seed cells at appropriate density in 96-well (100 µL per well) or 384-well (50 µL per well) tissue culture plates.

    a. Adherent cells: seed 1 day prior to infection. 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).
    Note: allowing cells to settle at room temperature can reduce well-to-well variability and edge effects in 96-well plates.

    b. Suspension cells: seed day of infection in media containing polybrene* (see table in step 2a).

2. Add virus to cells.

    a. (Adherent cells) Remove growth media and add fresh media containing polybrene* (see table). Alternatively, remove a portion of the growth media and supplement with media containing polybrene to achieve a final polybrene concentration of 8 µg/mL (following addition of virus).

Reagent	per well, 96-well plate	per well, 384-well plate
Media containing polybrene*	to 100 µL	to 50 µL
Final polybrene concentration	8 µg/mL	8 µg/mL
Virus (added in step 2b)		
High MOI	5 to 20 µL	2 to 5 µL
Low MOI**	≤1 to 3 µL	N.D.

** Protamine sulfate may be substituted if polybrene is toxic to cells.*
*** Low MOI infections may require dilution of virus stock prior to addition to cells.*

    b. Add virus to cells (see table in step 2a).
    Note: The indicated range of viral volume for high and low MOI infections assume typical viral yields from the 96-well viral preparation method described in Section II.

3. Option 1: Spin infection

    a. Spin cells at 2250 RPM in plate for 90 minutes at 37 °C. Centrifugation can improve viral infection and decreases the length of exposure of cells to polybrene and virus.
    Note: Centrifugation is not recommended for 6-well plates or larger, as cells may not be fully covered with media during the spin.

    b. Change media immediately following spin infection. Remove media and replace with 100 µL (96-well plates) or 50 µL (384 well plates) fresh growth media.

    c. Incubate cells overnight (37 °C, 5% CO_2).

d. If puromycin selection is desired, remove media 24 hours post-infection and replace with 100 µL (96-well plates) or 50 µL (384 well plates) fresh growth media containing puromycin.
Note: Puromycin concentration should be optimized for each cell line; typical concentrations range from 2-5 µg/mL.

Option 2: No-spin infection

a. Incubate cells overnight (37 °C, 5% CO_2).

b. Change media 24 hours post-infection. Remove media and replace with 100 µL (96-well plates) or 50 µL (384 well plates) fresh growth media. If puromycin selection is desired, use fresh growth media containing puromycin.
Note: Puromycin concentration should be optimized for each cell line; typical concentrations range from 2-5 µg/mL.

4. Incubate cells, replacing growth media (with puromycin, if desired) as needed every few days. Incubation periods are highly dependent on the post-infection assay. Puromycin selection requires at least 48 hours. The following recommendations are general guidelines only, and should be optimized for a given cell line and assay.

Post-infection assay	Incubation time post-infection	Incubation time with puromycin selection
Viral titer (Puromycin selection/cell viability)	3+ days	2+ days
mRNA knockdown (qPCR)	3+ days	2+ days
Protein knockdown (Western)	4+ days	3+ days
Phenotypic assay	4+ days	3+ days

5. Assay infected cells.