The RNAi Consortium (TRC)
Broad Institute

TRC Laboratory Protocols
Protocol Title: **Lentiviral Infection**
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Brief Description:

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

Table of Contents:

Workflow Timeline:

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

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

Part 1: Lentiviral Infection in 6 cm plates

1. 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 H 9268) or Protamine sulfate (MP Biomedicals #194729)
(Optional) Puromycin, blasticidin.

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/blasticidin concentration
- Timecourse

B. Infection protocol

1. Seed cells at appropriate density in 6 mL in 6 cm plates.
    a. Adherent cells: seed 1 day prior to infection.
    b. Suspension cells: seed day of infection in media containing polybrene*.

2. Add virus to cells:
    a. (Adherent cells) Remove growth media and add fresh media containing polybrene*. 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.

    b. Add virus to cells.

Reagent	Per 6 cm plate
Media containing polybrene*	to 6 mL
Final polybrene concentration	8 µg/mL
Virus	
High MOI	≥0.5 mL
Low MOI	≤0.1 mL

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

3. Viral infection:
    a. Incubate cells overnight.
    b. Change media 24 hours post-infection. Remove media and replace with 6 mL fresh growth media. If selection is desired, use fresh growth media containing the selection drug (e.g. puromycin, blasticidin).

    Note: Selection drug concentration should be optimized for each cell line; typical concentrations range from 2-5 µg/mL.

4. Incubate cells, replacing growth media (with selection drug, if desired) as needed every few days. Incubation periods are highly dependent on the post-infection assay. Puromycin selection requires at least 48 hours, whereas blasticidin needs at least 72 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 drug selection
mRNA knockdown (qPCR)	3+ days	2+ days (puro) 3+ days (blast)
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)

1. 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, blasticidin.

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
- Polybrene or Protamine sulfate concentration
- Puromycin/blasticidin 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.

    Note: allowing cells to settle at room temperature can reduce well-to-well variability and edge effects in 96-well plates. Some difficult cell lines prefer seeding 48hrs prior to infection

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

2. Add virus to cells.
(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 from 4-8 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	4-8 µg/mL	4-8 µg/mL
Virus (added in part 2b)		
High MOI	1 to 10 µL	0.5 to 5 µL
Low MOI**	≤1 to 3 µL	N.D.

** Protamine sulfate may be substituted if polybrene is toxic to cells. Some cell lines may be infected without either one; this should be tested if your cells or assay are sensitive.*

*** Low MOI infections may require dilution of virus stock prior to addition to cells.*

a. Add virus to cells (see table in part 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 30 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. *Optional* – For sensitive cell types, change media immediately or four hours 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.

   d. If 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 selection drug (e.g. puromycin or blasticidin).

      Note: Puromycin/blasticidin 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.

   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 selection is desired, use fresh growth media containing selection drug.

      Note: Puromycin/blasticidin concentration should be optimized for each cell line; typical concentrations range from 2-5 µg/mL.

4. Incubate cells, replacing growth media (with selection drug, if desired) as needed every few days. Incubation periods are highly dependent on the post-infection assay. Puromycin selection requires at least 48 hours, whereas blasticidin requires at least 72 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 selection
Viral titer (drug selection/cell viability)	3+ days	2+ days (puro) 3+ days (blast)
mRNA knockdown (qPCR)	3+ days	2+ days (puro)
Protein knockdown (Western)	4+ days	3+ days
Phenotypic assay	4+ days	3+ days

5. Assay infected cells.

Revision Notes: