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Section 1: Functional titer and transduction optimization of lentiviral vectors

The number of viral particles used and the transduction efficiency will determine the average number of lentiviral integrations into the target cell genome. The following protocol is designed to evaluate functional titer of the virus produced. Antibiotic selection may be used to remove untransduced cells. A kill curve should be performed as described in the product manual.

Increasing transduction efficiency:
Optimizing transduction conditions can extend the utility of viral particles and limit cell toxicity. Several variables influence transduction efficiency including components of the media, duration of transduction, cell type, cell health and plating density. It is possible to optimize many of these variables prior to the experiment.

- Serum is a known inhibitor of transduction and should be minimized (0 - 2%) in transduction media. For cells sensitive to low serum conditions either reduce the transduction time in low serum media or increase the transduction time in complete media.
- Transduction volume should be kept to a minimum. Media should barely cover cells.
- Extending transduction incubation times may increase efficiency. However, it may be necessary to increase the volume of media applied to the cells for transduction to limit the effects of evaporation.
- Hexadimethrine bromide (Polybrene) is a cationic lipid known to enhance viral particle binding to the surface of many cells types. A range of concentration (0 - 10 µg/ml) should be tested to determine the highest transduction efficiency that can be achieved with minimal cell toxicity.
- Cell density may influence transduction efficiency. Plate cells at a range of densities to determine its effect on your cell line. Rapidly dividing cells are often transduced more efficiently.

Determining Functional Titer

Functional titer must be determined using the experimental cell line to ensure optimal transduction. The functional titer is the number of viral particles, or transducing units (TU), able to transduce the target cell line per volume and is measured in TU/ml. Cell type, media components and viral production efficiency influence functional titer. It should therefore be calculated for every batch of virus produced and every cell line.

Once a baseline titer is known, this protocol can be used to further optimize transduction efficiency. To do so, follow this procedure and alter variables known to influence transduction efficiency.

- The following protocol evaluates titer by manually counting positive colonies.
- Transduction optimization should be done with empty vector control viral particles.
- HEK293T cells are readily transduced under standard conditions and are included in the protocol as a positive control for transduction.

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Materials

- Experimental cells and HEK293T cells
- Complete media for HEK293T cells and experimental cell line
- Serum free media for each cell line
- 24-well tissue culture plate
- Lentiviral particles (Harvested or purchased)
- Sterile Microcentrifuge tubes
- Polybrene
- Appropriate antibiotic for selection
- **For Inducible vectors only**: Doxycycline (1.0-2.0 µg/ml as determined in kill curve)

Equipment

- Automatic pipette /Pipette-aid (for tissue culture)
- Pipette (for dilutions and handling of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

Titering Protocol

The following protocol represents the standard procedure followed for determining functional titers in your target cell lines and HEK293T (positive control) cells. Optimal cell numbers, serum and polybrene concentrations, times, and culture conditions are likely to be different for the experimental cell line.

1. Plate your target cells and HEK293T cells 18-24 hours prior to transduction in a 24 well plate. Plate at a density of 7 x 10^4 cells per well in 12 wells for each cell line with complete media (see **Figure 1**). Incubate overnight with 5% CO_2 at 37°C. It is important to seed enough cells so that the cell confluency ranges between 30 and 40% at the time of transduction.
2. Prepare a serial dilution series with serum free media and viral supernatant as shown in **Table 1** and **Figure 1**.
    a. Serial dilutions can be set up in a sterile 96-well plate or in sterile micro centrifuge tubes. The number of wells or tubes needed depends on the expected titer of the viral particles (generally 5-8 wells/tubes). The higher the expected titer, the more wells/tubes needed for the dilutions.
    b. Make Dilution Media by taking serum-free cell culture media and adding Polybrene to a final concentration of 5-8 µg/ml.
    c. Add 80 µl of Dilution Media to Tube/Well 1 and then 160 µl of Dilution Media to each remaining tube (Tubes/Wells 2-5).
    d. Add 20 µl of viral particles to Tube/Well 1 and mix well by gently pipetting up and down (10 - 15 times) without creating bubbles, and discard the tip.
    e. Transfer 40 µl from Tube/Well 1 to Tube/Well 2. Mix well and discard the tip.
    f. Transfer 40 µl from tube 2 to tube 3. Mix well and discard the tip.

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g. Repeat the procedure for the remaining tubes.
h. Incubate at room temperature for 10-15 minutes.

3. Remove media from each well.
4. Add 200 µl of culture media containing 1% serum to each well containing cells.
5. Add 25 µl from each viral dilution to two wells for each cell line (225 µl final volume) for a total of 10 wells per cell line. The remaining 4 wells (without viral particles) should be evaluated as negative controls.
6. Rock plate gently a few times to mix.
7. Incubate overnight with 5% CO_2 at 37°C.
8. Replace the viral supernatant with complete media containing the appropriate antibiotic and allow cells to grow for 72-96 hours.
    a. **Note**: Add 1.0-2.0µg/ml doxycycline for inducible vectors ONLY
9. Colony counting: (**Note***:* Counting 50-200 colonies in a well is sufficient to provide accurate titers.)
    a. Antibiotic titering by selection and colony counting:
        i. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal concentration determined in "kill curve"
        ii. Continue feeding and observe the cells for approximately 7 days until you see single colonies surviving the selection. The negative control should have no surviving cells.
        iii. Use a microscope to count the number of surviving colonies.
    b. Fluorescent colony counting
        i. Replace the viral supernatant with complete media (including doxycycline for inducible vectors) and allow growth for 48 hours.
        ii. Count the number of colonies expressing the fluorophore. A colony consisting of multiple cells should be counted as a single transduction event.

10. Use the calculation below and **Table 1** to determine functional titer. (Alternate methods for calculating are described in Appendix 3.)

$$(Number\ of\ colonies\ ) \times (Dilution\ factor) \div (volume\ added\ to\ cells\ (ml)) = TU/\text{ml}$$

Example:
If the average number of colonies counted in well A5 and B5 is 70 the titer is calculated as follows:
70 colonies X 3125 ÷ 0.025 ml = 8.75 X 10^6 TU/ml

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Dilution table and schematic for titration protocol

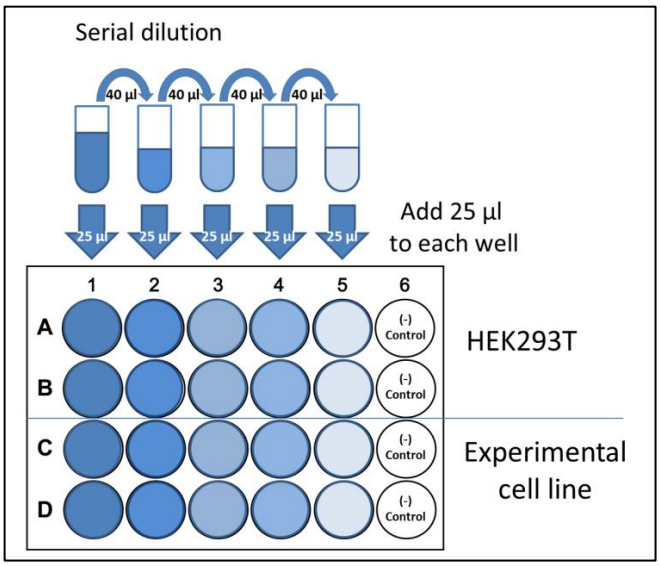

Figure 1. Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

Table 1. Dilution factors for calculating viral titer

Tube/Well	Viral particles	Dilution medium	Dilution factor
1	20 µl (from virus aliquot)	80 µl	5
2	40 µl (from Tube 1)	160 µl	25
3	40 µl (from Tube 2)	160 µl	125
4	40 µl (from Tube 3)	160 µl	625
5	40 µl (from Tube 4)	160 µl	3125
6	0 µl		n/a

Example:
Typical unconcentrated virus production will yield 1-5 x 10^5 TU/ml. The expected number of fluorescent colonies for a viral titer of 5 x 10^5 TU/ml would yield the following number of fluorescent colonies in titering assay:

Tube	1	2	3	4	5	6
Dilution	1/5	1/5	1/5	1/5	1/5	n/a
Diluted titer TU/ml	100,000	20,000	4,000	800	160	0
ml transduced cells	0.025	0.025	0.025	0.025	0.025	0
Fluorescent colonies expected	2500	500	100	20	4	0

Counting 50-200 colonies is sufficient for an accurate measure of titer.

Section 2: Determining Multiplicity of Infection (MOI)

After the functional titer has been determined in the experimental cell line, the volume of virus required for a particular multiplicity of infection (MOI) can be calculated. The MOI is the number of transducing units per cell in a culture. The necessary MOI needed is dependent on the cell line being used and can vary widely.

Calculating volume of viral particles for a given MOI

Calculate the total number of transducing units (TU_{total}) that would be added to a well for a given MOI with the following equation:

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TU_{total} = (MOI x Cell Number)/Viral titer (TU/µl)

Where:

- MOI = the desired MOI in the well (units are TU/cell)
- Cell number = number of cells in the well at the time of transduction

For example, if the experiment requires:

- MOI of 10 (highest MOI)
- Cell density of 10,000 cells per well at time of transduction
- Viral Titer is 1 x 10^7 TU/ml

Then, TU_{total} per well is calculated:

TU_{total} = (10 TU/cell) x (10,000 cells/well)] / 1 x 10^4 TU/µl = 10 µl of viral stock/well.

Therefore, the volume of viral particles with a titer of 1x10^7 TU/ml required for an MOI of 10 is 10 µl per well.

Protocol for determining optimal MOI

This protocol provides a basic outline of the transduction process. The following should be optimized prior to transduction:

- Transduction media: % Serum, Polybrene µg/ml
- Time exposed to transduction media: hours or overnight
- Selection media: µg/ml appropriate antibiotic

Materials

- Experimental cells
- Complete media for experimental cell line
- Serum free media for each cell line
- 24-well tissue culture plate
- Lentiviral particles
- Sterile Microcentrifuge tubes
- Polybrene
- Doxycycline (for inducible vectors ONLY)

Equipment

- Automatic pipette /Pipette-aid (for tissue culture)
- Pipette (for dilutions and handling of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

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- Fluorescent microscope with appropriate filter

Protocol

The following protocol represents the standard procedure followed for determining optimal MOI in HEK293T cells. Cell numbers, serum and polybrene concentrations, times, and culture conditions are likely to be different for the experimental cell line. The optimal conditions for a target cell line can be determined using the protocol for functional titer and transduction optimization (Section 1).

1. Plate cells 18-24 hours prior to transduction in a 24 well plate with complete media. Plate at a density of cells so that the cell confluency ranges between 30 and 40% at the time of transduction.
2. Incubate overnight with 5% CO_2 at 37°C.
3. Prepare viral particles:
    a. Set up 8 sterile microcentrifuge tubes and label two tubes each with MOI. For example: 1, 2, 5, and 10.
    b. Add 50 µl of medium containing 1% serum and appropriate level of Polybrene.
    c. Add the volume of viral stock that corresponds to the MOI (use the calculation above for determining volume for the desired MOI).
    d. Bring volume in each tube up to 100 µl with medium containing 1% serum and appropriate level of Polybrene.
    e. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
    f. Incubate for 10 minutes at room temperature.
4. While viral particles are incubating, remove media from cells in each well.
5. Add to each well 125 µl of 1% serum media containing NO Polybrene.
6. After the 10-minute incubation, transfer all (100 µl) of virus from tubes to the corresponding wells (225 µl final volume) for a total of 8 wells (two well for each MOI). The remaining wells (without viral particles) should be evaluated as negative controls.
7. Rock plate gently a few times to distribute the viral particles across the well.
8. Incubate overnight with 5% CO_2 at 37°C (12-24 hours).
9. Replace the viral supernatant with complete media
    a. **For inducible vectors ONLY**, include 1.0 - 2.0 µg/ml doxycycline for one set of MOIs (induced) and no doxycycline for the other set (non-induced)).
10. Incubate cells in culture for 72-96 hours.
11. Using a fluorescent microscope, assess fluorescent expression in the wells.
    a. **For inducible vectors**: Choose an MOI that results in a high level of induction (bright fluorescent expression in the induced set) and low level of leakiness (no or faint fluorescent expression in the non-induced set).

    Note: levels of fluorescent protein expression will vary greatly across a culture due to random integration of lentiviral vectors into regions of the chromosomes with varying levels of transcriptionally active and non-active states.

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Section 3: Transduction Guidelines & Protocols

This protocol provides a basic outline of the transduction process. The following should be optimized prior to transduction:

- Transduction media: % Serum, Polybrene µg/ml
- Time exposed to transduction media: hours or overnight
- Selection media: µg/ml antibiotic

Required materials

- Complete media for experimental cell line
- Selection media: complete media for experimental cell line supplemented with the appropriate antibiotic
- Transduction media containing viral particles (optimized for serum and Polybrene concentration)

Equipment

- Automatic pipette/Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Assay specific equipment

Protocol

Prepare cells

1. Plate cells such that they are actively dividing and 30 - 40% confluent at the time of transduction.
2. Feed cells with complete media 3 - 4 hours prior to transduction.
3. Make transduction media just prior to transduction.

Transduce cells

4. Exchange media with transduction media.
   Note: media should be serum free for maximum transduction efficiency. Alternatively, see section 1 for information on transduction optimization.
5. Incubate cells 12 - 24 hours in transduction media.
6. Replace transduction media with complete media (no selection reagent).

Note: to improve transduction for non-adherent cells, cells can be moved to a round bottom tube and incubated with rotation. Rotation allows the cells and viral particles to come into contact.

Antibiotic selection

7. Allow cells to grow for 48 hours.
8. Replace media with selection media.

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9. Continue feeding cells selection media until untransduced cells have been removed.

Induction (Applies only to inducible vectors)

10. Induce expression using 1.0 – 2.0 µg/ml of doxycycline. Allow cells to culture for 48-96 hours post induction. If cells need passaging during this incubation period, maintain the same concentrations of the appropriate selection antibiotic and doxycycline.

Analysis

11. Analyze target gene activity in population. Determine cellular phenotype or harvest cell for gene expression analysis according to your experimental design.