Protocol

Titration of Lentivirus Vectors

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The titer of a lentivirus vector is often expressed in transducing units per milliliter. This is a functional titer that reflects the lentivirus' ability to transduce a particular cell line under specific conditions. Transduction of other cell lines is likely to be different and will require optimization. 293T cells are used for production of lentivirus stocks, and they can be easily transduced with vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped lentivirus vectors. Consequently, this cell line is commonly used to determine the functional titer of lentivirus vector stocks produced with this envelope. For lentivirus vectors encoding fluorescent proteins under the control of promoters functional in these cells, titration can be performed using the limiting dilution method or a flow cytometry-based method. For lentivirus vectors lacking a fluorescent marker, or for those carrying promoters that may not be functional in 293T cells, titer can be determined either by real-time polymerase chain reaction (PCR) quantification of viral genomes in genomic DNA from transduced cells or a p24 ELISA-based assay.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

293T cells
Complete growth medium <R>
DMEM
Fetal bovine serum (FBS)
Lentivirus vector stock (see Protocol: **Production of High-Titer Retrovirus and Lentivirus Vectors** [Sena-Esteves and Gao 2018a])
Paraformaldehyde (4%) (optional; see Step 15)
Penicillin/streptomycin (P/S) solution (100×)
Phosphate-buffered saline (PBS)
Polybrene (hexadimethrine bromide) (8 mg/mL)
QuickTiter Lentivirus Titer Kit (lentivirus-associated HIV p24) (Cell Biolabs)

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Trypsin–EDTA (0.05%)
UltraRapid Lentiviral Titer Kit (System Biosciences) (optional; see Step 23)

Equipment

Culture dishes, 12 well
Flow cytometer
Hemocytometer
Humidified cell culture incubator (37°C, 5% CO_2)
Inverted fluorescence microscope with appropriate filters for fluorescent protein
Micropipettes
Microplate reader capable of reading at 450 nm
Pipette barrier tips, sterile (20, 200, and 1000 µL)
Tubes, sterile (1.5 mL)

METHOD

Titer Determination by Limiting Dilution

This protocol applies only to vectors expressing fluorescent proteins under promoters functional in 293T cells.

1. The day before titration, plate 3×10^5 293T cells per well in a 12-well dish in 1 mL of Complete growth medium. Ensure uniform distribution of cells in the wells by gently rocking the plates back and forth and then sideways. Prepare one dish for each lentivirus vector stock to titer. Incubate the cells in a cell culture incubator overnight at 37°C in a 5% CO_2 atmosphere.
2. On day 1, replace the Complete growth medium in all wells with 0.5 mL of Complete growth medium supplemented with 8 µg/mL polybrene (1:1000 dilution of 8 mg/mL polybrene stock solution), prewarmed to 37°C.
3. Prepare serial dilutions of lentivirus vector stocks in Complete growth medium. For nonconcentrated stocks, prepare 1:10, $1:10^2$, and $1:10^3$ dilutions. For concentrated stocks, prepare $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$ dilutions. Add 5 µL of vector dilution per well in triplicate (three wells per dilution). For nonconcentrated stocks, include a set of wells receiving 5 µL of nondiluted stock. Return the dishes to the 37°C incubator.
4. On day 2, add 0.5 mL of Complete growth medium prewarmed to 37°C. Incubate the dishes for 2 more days.
5. On day 4, carefully remove the Complete growth medium from the wells and replace it with 1 mL of PBS.
6. Using an inverted fluorescence microscope equipped with appropriate filters to visualize the fluorescent protein, count the total number of colonies per field of view using a 10× objective. Choose a vector dilution that gives an average of one to 20 clones per field of view. Count five random fields per well for that particular dilution (total of three wells × five fields/well = 15 fields of view).

   Calculate the average number of clones/field of view. Calculate the titer using

$$\text{Titer(TU/mL)} = (N \times C \times D)/0.005,$$

   where N is the average number of clones/field of view, C is the well area divided by the field of view area, and D is the fold dilution of vector stock.

   To calculate the area of the field of view, determine the field of view diameter ($FOV_{Ø}$) by dividing the ocular lens field number (this is the number written on the side of the ocular piece; e.g., 22 corresponds to 22 mm) by the objective magnification (e.g., 10) and use the formula area = $\pi \times (FOV_{Ø}/2)^2$.

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Titer Determination by Flow Cytometry

7. Prepare 293T cells for titration as in Step 1. Prepare the necessary number of wells in 12-well dishes, plus three additional wells.
8. On day 1, count the total number of cells in three wells. Remove the Complete growth medium, wash the cells with 1 mL of PBS, add 0.5 mL of 0.05% trypsin–EDTA, and incubate for 5 min at room temperature. Add 0.5 mL of Complete growth medium and mix. Transfer the cell suspension to a 1.5-mL tube and determine the concentration of cells using a hemocytometer. Calculate the average total number of cells per well.
9. Proceed with the titration procedure as in Steps 2–4.
10. On day 4, remove the Complete growth medium and wash the cells with 1 mL of PBS. Add 0.5 mL of 0.05% trypsin–EDTA per well and incubate for 5 min at room temperature.
11. Add 0.5 mL of Complete growth medium to each well, mix, and transfer the cell suspension to 1.5-mL tubes.
12. Pellet the cells by centrifugation at 500*g* for 5 min at room temperature. Aspirate the Complete growth medium and resuspend the cell pellet in 1 mL of PBS.
13. Pellet the cells again by centrifugation at 500*g* for 5 min at room temperature. Remove the supernatant and resuspend the cells in 1–2 mL of PBS.
14. Determine the percentage of fluorescence-positive cells using a flow cytometer.

    If the analysis is not performed within 1 h, fix the cells in 4% paraformaldehyde in PBS for 30 min, wash with PBS, and store at 4°C in PBS.
15. Calculate the titer (TU/mL) using

$$\text{Titer(TU/mL)} = (N \times F \times D)/0.005,$$

    where N is the average number of cells per well on day 1, F is the percentage of fluorescent cells, and D is the fold dilution of vector stock.

    The accuracy of the titer depends on the amount of vector used falling within the linear range between vector input and percentage of fluorescent cells. If this percentage is >40%, repeat the assay with additional dilutions.

Titer Determination by PCR Quantification of Vector Genome Genomic DNA of Transduced Cells

16. Perform a titration assay as in Steps 1–4.

    Some protocols include a treatment with DNase I on day 2 to eliminate any plasmid DNA that may be carried over in the lentivirus vector supernatants (Kutner et al. 2009).
17. On day 4, remove the Complete growth medium and wash the cells with 1 mL of PBS. Add 0.5 mL of 0.05% trypsin–EDTA per well and incubate for 5 min at room temperature.
18. Add 0.5 mL of Complete growth medium to each well, mix, and transfer the cell suspension to 1.5-mL tubes.
19. Pellet the cells by centrifugation at 500*g* for 5 min at room temperature. Aspirate the Complete growth medium and resuspend the cell pellet in 1 mL of PBS.
20. Pellet the cells again by centrifugation at 500*g* for 5 min at room temperature.
21. Isolate the genomic DNA from transduced cells using a commercially available kit (e.g., DNeasy Tissue Kit, QIAGEN) and measure the concentration of genomic DNA.
22. Determine the lentivirus vector copy number per target cell diploid genome using real-time PCR quantification as described in Kutner et al. (2009). Alternatively, use the UltraRapid Lentiviral Titer Kit (System Biosciences), also based on real-time PCR. Follow the manufacturer's instructions and bypass Steps 18–24.

23. Calculate the lentivirus vector titer using

$$\text{Titer(TU/mL)} = (N \times C \times D)/0.005,$$

where N is the average number of cells per well on day 1, C is the lentivirus vector copy number per diploid genome, and D is the fold dilution of vector stock.

Titration by p24 ELISA

An alternative to transduction-based assays is to determine the concentration of HIV-1 p24 levels in lentivirus vector stocks using an ELISA. The limitation with this approach is the contamination of lentivirus vector supernatants with free p24 generated by transfected cells during production. Most ELISA Kits quantify the total amount of p24 antigen in the supernatant. The QuickTiter Lentivirus Titer ELISA Kit (Cell Biolabs) quantifies p24 levels associated with the lentivirus virions using a proprietary reagent to separate lentivirus virions from free p24.

24. For nonconcentrated lentivirus vector stocks, prepare 1:10 and $1:10^2$ dilutions of vector stock in OptiMEM and measure in triplicate for each dilution. For concentrated lentivirus vector stocks, prepare $1:10^2$, $1:10^3$, and $1:10^4$ dilutions of vector stock in OptiMEM and measure in triplicate.
25. Use the QuickTiter Lentivirus Titer ELISA Kit to determine the concentration of virion-associated p24 levels in lentivirus vector stocks according to the manufacturer's instructions.

According to the manufacturer, there are 2000 molecules of p24 per lentivirus particle (LP), and 1 ng of p24 corresponds to 1.25×10^7 LPs.

DISCUSSION

The most accurate, but also the most time-consuming, method to measure the titer of the lentivirus preparation is real-time PCR quantification of vector genomes in target cells. This method is independent of promoter functionality in the cells used to determine the titer. However, this approach can work only in cells expressing the necessary cell-surface receptors for alternative envelope glycoproteins used to pseudotype lentivirus vectors. In these cases, two alternative approaches are available. One is to determine titers based on lentivirus particle-associated p24 levels, and the other is to generate a cell line engineered to overexpress the appropriate cell-surface receptor. It is important to consider that lentiviral titer is a functional measure (transducing units per milliliter) determined using a particular cell line under very specific conditions. Changes in target cell type and transduction conditions have a dramatic effect on transduction efficiency. Consequently, a lentivirus vector titer should be used as a guideline to set up empirical transduction experiments for new cell lines, primary cells, and in vivo gene delivery.

The vast majority of lentivirus vectors are produced with the VSV-G envelope protein, which is compatible with most commonly used cell lines, such as 293, 293T, HT-1080, and HeLa cells. In addition to VSV-G, lentivirus vectors have been pseudotyped (carrying an envelope protein other than the native HIV-1 envelope) with a variety of envelope proteins derived from other viruses, which equips the resulting lentivirus pseudotypes with an ability to transduce particular sets of target cells (Cockrell and Kafri 2007). However, titration of some of these vectors by transduction of reference cell lines can result in inaccurately low titers because the cell-surface receptors for the particular envelope protein may be expressed at only low levels or not at all. In this case, the titer can be calculated by measuring the level of p24 using an ELISA. However, it is important to bear in mind that this is not a functional titer.

RELATED INFORMATION

National Institutes of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules do not directly address work with lentivirus vectors. Therefore, the Recombinant DNA Advisory

Committee (RAC) has issued guidelines on how to conduct a risk assessment for lentivirus vector research. The major risks associated with lentivirus vector work are the potential for generation of replication-competent lentiviruses (RCLs) and oncogenesis. In the laboratory setting, BL2 or enhanced BL2 containment procedures are adequate for most applications not involving transgenes with high oncogenic potential. A risk assessment by the local Institutional Biosafety Committee (IBC) based on the lentivirus vector design and packaging system, nature of transgene insert, vector titer and total amount of vector produced, inherent biological containment of the animal host, and negative RCL testing will determine the appropriate procedures. Review and approval by the local IBC is mandatory before initiating any work with lentivirus vectors.

For information on screening lentivirus vector stocks for RCLs, see Protocol: **Monitoring Lentivirus Vector Stocks for Replication-Competent Viruses** (Sena-Esteves and Gao 2018b).

RECIPE

Complete Growth Medium

Reagent	Quantity (for 1 L)	Final concentration
DMEM	890 mL	
Fetal bovine serum (FBS)	100 mL	10%
Penicillin/streptomycin (P/S) solution (100×)	10 mL	1×

Store at 4°C.

REFERENCES

Cockrell AS, Kafri T. 2007. Gene delivery by lentivirus vectors. *Mol Biotechnol* **36:** 184–204.

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