CELLECTA

USER MANUAL

Packaging, Titering, and Transduction of Lentiviral Constructs

v10a — Last update: 18 January 2024

Cellecta, Inc.

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1. Lentiviral Construct Packaging, Titering, and Transduction

The protocols in the Lentiviral Construct Packaging, Titering, and Transduction User Manual can be used with nearly all pre-made and custom lentiviral shRNA, sgRNA/Cas9, sgRNA-only, Cas9-only, cDNA, barcode, and reporter constructs available from Cellecta. For specific information on the particular constructs that you have purchased, please review the product information you received by email upon delivery (either by link or attachment):

References and Product Citations for all Cellecta products can be found on the Cellecta website:
https://cellecta.com/pages/citations-publications

Please read the entire user manual before proceeding with your experiment. Also, please note that, when working with pseudoviral particles, you should follow the recommended guidelines for working with Biosafety Level 2 (BSL-2) materials.

Click the ? Download as PDF link located at the bottom of the left menu to download the PDF version of this user manual.

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Last modified: 2 November 2020

2. Materials Provided

- Lentiviral expression vector or construct from Cellecta: cDNA or InDOXible vector, or other
- Recommended:
  - LentiPrep™ Reagent Set (Cat.# LTSET-G or LTSET-R, includes the 3 items listed below)
  - Ready-to-Use Lentiviral Packaging Plasmid Mix (Cellecta, Cat.# CPCP-K2A)
  - LentiTrans™ Transduction Reagent, 1000x (Cellecta, Cat.# LTDR1)
  - LentiFuge™ Viral Concentration Reagent (Cellecta, Cat.# LFVC1)

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2.1. Additional Materials for Production of Lentivirus

- 293T Cell Line (e.g. 293T/17, ATCC, Cat.# CRL-11268™ or 293FT, Thermo Fisher, Cat.# R70007)
- Ready-to-use Lentiviral Packaging Plasmid Mix (Cellecta, Cat.# CPCP-K2A). Libraries can be packaged into lentiviral particles with nearly any 2nd or 3rd generation HIV-based lentiviral packaging mix. Cellecta's 2nd generation lentiviral packaging mix contains two plasmids: psPAX2 and pMD2.G, pre-mixed in a ratio optimized for production of lentivirus.
- Dulbecco's Modified Eagle Medium (D-MEM) (1X) (Corning cellgro™, Cat.# 15-013-CV)

> **NOTE:** ADD FRESH GLUTAMINE (1X) at the time a sealed bottle of D-MEM is opened, even if the label indicates glutamine has already been added. Glutamine in solution at 4°C has a half-life of 1-2 months, so glutamine(+) D-MEM purchased "off-the-shelf" from a supplier is to be regarded as glutamine(-). In our experience, the addition of glutamine increases titer approximately 2-fold. If D-MEM comes supplemented with stable L-Alanyl-L-Glutamine dipeptide, addition of fresh glutamine is not necessary.

- Glutamine (L-Alanyl-L-Glutamine, Dipeptide L-glutamine) (Corning glutagro™, Cat.# 25-015-CI)
- Fetal Bovine Serum (Recommended: Corning, Cat.# 35-010-CV)
- Trypsin-EDTA, 1X (Corning, Cat.# 25-052-CI)
- Tissue Culture Plates
  - 150 × 25mm (15-cm) Tissue Culture Plates (Corning, Cat.# 430599)
  - 100 × 20mm (10-cm) Tissue Culture Plates (Corning, Cat.# 430167)
- Related Tissue Culture Supplies
- Lipofectamine® Transfection Reagent (Thermo Fisher, Cat.# 18324020)
- PLUS Reagent (Thermo Fisher, Cat.# 11514015)
- DNase I, RNase-free (Epicentre-Illumina, Cat.# D9905K)
- HEPES pH 7.2-7.6, 1M solution (Corning, Cat.# 25-060-CI)
- $MgCl_2$, 1M solution
- Nalgene™ Rapid-Flow™ Sterile 500 ml, 0.2 µm PES filter units (Thermo Fisher Cat.# 569-0020)
- LentiFuge™ Viral Concentration Reagent (Cellecta, Cat.# LFVC1) *(Optional, for concentration of virus)*
- D-PBS, 1X (Corning, Cat.# 21-031-CV)

Other than the specific reagents and instruments listed above, the protocols assume the user has access to standard materials (e.g., polypropylene tubes, pipette tips), equipment (table top centrifuges, pipettes, scales), and common reagents (e.g., TE buffer, ethanol) and buffers used in a typical life science laboratory.

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2.2. Additional Materials for Transduction of Cells

The materials below are based on the transduction protocol in this manual which has been optimized for HEK293 and K-562 cells. Depending on the characteristics of your specific cells, the choice of media or other cell culture specifics may be different.

- LentiTrans™ Transduction Reagent (Cellecta, Cat.# LTDR1)
- Puromycin (Sigma-Aldrich, Cat.# P9620-10ML)
- Dulbecco's Modified Eagle Medium (D-MEM) (1X) (Corning cellgro™, Cat.# 15-013-CV)
- Glutamine (L-Alanyl-L-Glutamine, Dipeptide L-glutamine) (Corning glutagro™, Cat.# 25-015-CI)
- Fetal Bovine Serum (Recommended: Corning, Cat.# 35-010-CV)
- Trypsin-EDTA, 1X (Corning, Cat.# 25-052-CI)
- Tissue Culture Plates and Related Tissue Culture Supplies
- HEPES pH 7.2-7.6, 1M solution (Corning, Cat.# 25-060-CI)
- $MgCl_2$, 1M solution

Other than the specific reagents and instruments listed above, the protocols assume the user has access to standard materials (e.g., polypropylene tubes, pipette tips), equipment (table top centrifuges, pipettes, scales), and common reagents (e.g., TE buffer, ethanol) and buffers used in a typical life science laboratory.

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3. Recommended Experiments to Characterize Target Cells

Depending on your experiments, it may be useful to characterize specific growth and sensitivity parameters of the cell model you will transduce with your lentiviral constructs. Cell-type specific data, such as cell doubling time, antibiotic efficacy, and sensitivity to Polybrene (Hexadimethrine bromide) will enable you to proceed confidently with your experiments.

Also, if you have not used lentiviral vectors in your target cells before and are interested in maximizing expression of a cDNA—for example the Cas9 gene—we suggest checking which promoters will work best to express the selection and/or marker genes. The efficacy of pol II promoters (such as CMV, UbiC, and EF1) that drive expression of cDNA vary significantly across cell types. Cellecta sells pre-packaged control viruses expressing different marker genes from various promoters that can be used to determine which promoter will work the best in your cells.

Visit the Cellecta Vector Information web page for a list of our most common lentiviral vectors. If interested in using a vector not listed, please contact us at sales@cellecta.com. Cellecta's pre-made constructs and libraries can be re-cloned in almost any of Cellecta's standard or custom vectors.

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3.1. Cell Doubling Time

The doubling time is the time it takes your cells to double in number. It is useful to know the doubling time of your cells so that you can plate the appropriate number for transduction with a lentiviral library or construct.

1. Start with cells that have already been growing for a few weeks, rather than using cells that have just been thawed from a frozen state. To calculate the doubling time, trypsinize your cells as if you were going to split them.

2. Count them using a hemacytometer or cell counter, and keep track of the number that you replate onto the cell culture plates. The starting number of cells (at the beginning) is ***Xb***.

3. Propagate the cells as you normally do, replacing media as necessary.

4. The next time they are ready to be split, trypsinize them as usual and count them again using a hemacytometer or cell counter. The number of cells at the end is referred to as ***Xe***.

> **NOTE:** The cells should be in the log phase of growth to calculate doubling time properly, so it is important to not let the cells become confluent.

To calculate the doubling time, use the following formula:

> *Doubling Time* = [***T*** × (ln2)] / [ln (***Xe*** / ***Xb***)]
> where ***T*** = Time in any units

Example

> Let's say that on Day 0, you count 2×10^6 cells. Three (3) days later, you count 16×10^6 cells.

> ***Xb*** = 2×10^6
> ***T*** = 3 days
> ***Xe*** = 16×10^6
>
> Doubling Time = [3 × (ln2)] / [ln(16,000,000 / 2,000,000)]
> = [3 × (0.69)] / [ln(8)]
> = 2.08 / 2.08 = **1 day**

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3.2. Calculating a Kill Curve

Most of the lentiviral vectors used in making Cellecta's pre-made libraries and constructs express a puromycin resistance gene. For other pre-made products as well as customized libraries and constructs, other selection markers such as blasticidin ($Blast^R$), hygromycin ($Hygro^R$), neomycin (Neo^R), or bleomycin ($Bleo^R$) may be substituted.

Regardless of the selection marker that the plasmids express, you need to know the concentration of antibiotic that kills untransduced cells within a given amount of time in order to successfully select transduced cells. We recommend the following methods for obtaining a "Kill Curve".

Puromycin Kill Curve

To create a Puromycin Kill Curve for your target cells, follow the protocol below.

1. Aliquot cells in a 12-well plate, at a density such that they are at 72 hours from confluency.
2. Add puromycin at concentrations of 0, 0.5, 1, 2, 5, and 10 µg/ml in six different wells.
3. Mix and place the cells at 37°C in a CO_2 incubator.
4. Grow cells under standard conditions for 72 hours.
5. Count viable cells, and determine the lowest concentration of drug that kills at least 95% of cells in 3-5 days.

Use this concentration at the puromycin selection step during your experiment.

Blasticidin Kill Curve

To calculate a Blasticidin Kill Curve, follow the same protocol above and use the same concentrations as for the Puromycin Kill Curve.

Hygromycin Kill Curve

If using hygromycin, follow the same protocol as for the Puromycin Kill Curve but use 0, 50, 100, 200, 400, and 800 µg/ml hygromycin in six different wells.

Neomycin Kill Curve

If you are using a construct with a neomycin selection marker, you need to test for resistance to geneticin (G418). Use the same protocol as for the Puromycin Kill Curve, but use a range of concentrations between 400-800 µg/ml.

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3.3. Check Toxicity of LentiTrans Transduction Reagent

The LentiTrans™ Transduction Reagent used during the transduction of target cells with lentivirus contains Polybrene, a transduction enhancement reagent. Some cell lines may be sensitive to this reagent. Before doing large-scale transduction, we recommend checking the toxicity of the LentiTrans Reagent using the procedure below:

1. In six wells of a 12-well plate, grow cells for 24 hours in complete culture medium without the LentiTrans Reagent, and with LentiTrans added at 0.25 µl/ml, 0.5 µl/ml and 1:1000 (1 µl/ml).

2. Replace old medium with LentiTrans-free complete culture medium and grow cells for an additional 72 hours.

3. Check for toxicity by counting viable cells.

For your experiments, use the highest concentration of LentiTrans Reagent that results in less than 10% cell toxicity compared to the no-LentiTrans culture (typically, a 1:1000 is recommended). For a few cell types, you may not be able to use LentiTrans Reagent.

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4. General Protocols for Lentiviral Packaging and Transduction

This section provides key protocols for packaging, transducing, and titering lentiviral plasmid libraries and constructs.

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4.1. General Lentiviral Packaging Protocol

The following protocol describes the general procedure for generation of pseudoviral packaged lentiviral constructs using ThermoFisher's Invitrogen Lipofectamine™ and PLUS Reagent (see **Additional Materials for Production of Lentivirus**). Other transfection reagents may be used, but the protocol should be adjusted to fit the manufacturer's protocol. This protocol can be used to package individual lentiviral plasmid constructs expressing shRNA, sgRNA, Cas9/dCas9, barcodes, cDNA, promoter reporters, and sgRNA, shRNA, or barcode libraries in 3rd generation lentiviral vectors.

- The yield of recombinant lentiviral particles produced under these optimized conditions is typically between 1×10^6 TU/ml and 5×10^6 TU/ml for individual lentiviral constructs with a viral transcript less than 7kb (i.e. the region from the beginning of 5'LTR to the end of 3'LTR).

Plate Size	Surface Area	Volume of Media	Approximate Virus Yield *
10 cm	55 cm^2	10 ml	2×10^7 TU
15 cm	150 cm^2	30 ml	6×10^7 TU

> * **NOTE: The yield of lentiviral particles will be *significantly lower* for lentiviral constructs and libraries with viral transcripts larger than 7kb (e.g., approximately 1×10^5 TU/ml for Cas9 constructs with viral transcripts around 8kb).**

- If using tissue culture plates or flasks of other sizes, please scale amounts in the protocol based on surface area.

2-3 Days Prior to Packaging

1. Start growing 293T cells in D-MEM medium plus glutamine* supplemented with 10% FBS without antibiotics, and expand until you have sufficient cells to package at the scale desired.

> * **NOTE:** ADD FRESH GLUTAMINE (1X) to Dulbecco's Modified Eagle Medium (D-MEM) at the time a sealed bottle of D-MEM is opened, even if the label indicates glutamine has already been added. Glutamine in solution at 4°C has a half-life of 1-2 months, so glutamine(+) D-MEM purchased "off-the-shelf" from a supplier is to be regarded as glutamine(-). If D-MEM comes supplemented with stable L-Alanyl-L-Glutamine dipeptide, addition of fresh glutamine is not necessary. In our experience, the addition of glutamine increases titer approximately 2-fold.

Day 0 – Plate Cells

1. Twenty four (24) hours prior to transfection, plate 12.5 × 10^6 293T cells per **15-cm plate** (or 150 cm^2 flask) and use 30 ml of media per plate. If you are using **10-cm plates**, plate 4 × 10^6 cells and use 10 ml of media per plate. Disperse the cells and ensure even distribution.

2. Incubate at 37°C in a CO_2 incubator for 24 hours.

> **NOTE:** The goal is to have the 293T cells reach ~80% confluency by Day 1. You may want to calculate the number of cells seeded empirically since cell counts can vary.

Day 1 – Transfection into 293T Cells

1. Using the volumes in the table below, mix Ready-to-use Lentiviral Packaging Plasmid Mix and your Plasmid Lentiviral construct in a sterile, appropriately-sized polypropylene tube. Add the D-MEM medium without serum or antibiotics to the plasmid mixture, then mix. Add the PLUS Reagent, mix, and incubate at room temperature for 15 minutes.

1 × 10-cm plate	1 × 15-cm plate	4 × 15-cm plates	8 × 15-cm plates	16 × 15-cm plates	24 × 15-cm plates	Component
20 µl	60 µl	240 µl	480 µl	960 µl	1440 µl	Packaging Plasmid Mix (0.5 µg/µl)
2 µl	6 µl	24 µl	48 µl	96 µl	144 µl	Plasmid / Library (1 µg/µl)* *see **NOTE***
1,000 µl	1,200 µl	4,800 µl	9,600 µl	19,200 µl	28,800 µl	D-MEM, no FBS, no antibiotics
20 µl	60 µl	240 µl	480 µl	960 µl	1,440 µl	PLUS Reagent
1,042 µl	**1,326 µl**	**5,304 µl**	**10,608 µl**	**21,216 µl**	**31,824 µl**	**Total volume**

> **NOTE:** The volume of plasmid DNA assumes the DNA is suspended at a 1 µg/µl concentration. For plasmid DNA at other concentrations, adjust the volume accordingly (e.g., for 0.5 µg/µl, use twice the indicated µl).

2. Add Lipofectamine Reagent to the D-MEM medium without serum or antibiotics in order to make a master mix according to the table below. Mix gently.

1 × 10-cm plate	1 × 15-cm plate	4 × 15-cm plates	8 × 15-cm plates	16 × 15-cm plates	24 × 15-cm plates	Component
1,000 µl	1,200 µl	4,800 µl	9,600 µl	19,200 µl	28,800 µl	D-MEM, no FBS, no antibiotics
30 µl	90 µl	360 µl	720 µl	1,440 µl	2,160 µl	Lipofectamine Reagent
1,030 µl	**1,290 µl**	**5,160 µl**	**10,320 µl**	**20,640 µl**	**30,960 µl**	**Total volume**

3. Add the diluted Lipofectamine (from Step 2) to the DNA / PLUS Reagent complex (from Step 1 above), mix gently by flicking the tube or vortexing and incubate at room temperature for 15 minutes.

4. Add 2.5 ml of the resulting DNA / PLUS Reagent / Lipofectamine complex to each plate (from the previous step **Day 0 – Plate Cells**), and mix complexes into the medium with gentle rotation. Take care not to dislodge cells from the plate.

5. Incubate at 37°C in the CO_2 incubator for 24 hours.

Day 2 – DNase I Treatment

1. At 24 hours post-transfection, replace the medium containing complexes with 30 ml (for 15-cm plates) or 10 ml (for 10-cm plates) of fresh D-MEM medium supplemented with 10% FBS, DNase I (1 U/ml), $MgCl_2$ (5 mM), and 20mM HEPES, pH 7.4.

2. Continue incubation in the CO_2 incubator at 37°C overnight.

> **NOTE:** Overnight DNase I treatment before harvesting virus does not negatively affect lentiviral titer or infectivity and helps prevent undesirable carryover of plasmid DNA into the virus prep.

> ***IMPORTANT:*** Failure to change the media the day after transfection results in large carryover of plasmid (free and/or Lipofectamine-bound) into your lentiviral prep. This may cause problems with most downstream molecular biology applications, especially whenever there is a PCR step involved such as during NGS sample preparation after a pooled library screen.

Day 3 – Collect Lentiviral Supernatant

1. At 48 hours post-transfection, collect the entire virus-containing medium from each plate and filter the supernatant (~30 ml per 15-cm plate or ~10 ml per 10-cm plate) through a Nalgene 0.2 µm PES filter (a low protein-binding filter) to remove debris and floating packaging cells. Failure to filter supernatant could result in carry-over of cells into your lentiviral prep.

> **NOTE:** Usually, the peak of virus production is achieved at 48 hours post-transfection. Supernatant can also be collected again at 72 hours post-transfection—replace the collected 48-hour supernatant with 30 ml (for 15-cm plates) or 10 ml (for 10-cm plates) of fresh D-MEM medium supplemented with 10% FBS and 20mM HEPES pH 7.4, and continue incubation in the CO_2 incubator at 37°C for 24 hours. Then, repeat Step 1 above for 72 hours post-transfection.

NOTE: Freezing and thawing lentivirus results in some loss of titer with each cycle.

Concentration of Lentivirus (Optional)

The following procedure was optimized to concentrate virus harvested at 48 hours with high recovery. Although concentrating virus is optional, it is recommended if any of the following conditions applies:

- Very high titer virus stock is needed to achieve desired MOI in hard-to-transduce target cells.
- The virus needs to be suspended in a different media (besides D-MEM/10%FBS) that is optimal for sensitive target cells.
- If packaging an shRNA, sgRNA, or barcode library for screening, because the concentration minimizes problems that might arise from genomic DNA carryover.

Virus Concentration Protocol

1. Aliquot lentiviral supernatant in clear, sterile centrifuge tubes.

2. *(Recommended)* Add Cellecta's LentiFuge™ Viral Concentration Reagent (see **Additional Materials for Production of Lentivirus**) according to the protocol described in the LentiFuge User Manual.

3. Centrifuge at 15,000 × g for at least 1 hour at 4°C. Mark the tubes to identify the location where the pellet will be. At the end of centrifugation, you may or may not be able to see a pellet—assume it is at the location of the mark.

4. Immediately discard the supernatant by aspirating.

5. Place the tubes on ice, resuspend the pellet (which may not be visible) in PBS, PBS/10%FBS, or PBS/1%BSA, make aliquots, and freeze at -80°C. 100-fold concentration is recommended (e.g., resuspend in 1 ml PBS if starting from 100 ml supernatant).

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4.2. General Lentiviral Transduction Protocol

This section provides the general protocol for transduction of mammalian cells with VSV-G packaged lentiviral particles. This protocol was developed and optimized using HEK293 and K-562 cells, and has been successfully used with many other common cell types. However, each cell is different and, depending on the characteristics of your specific cells, some optimization may be necessary.

Lentiviral transductions are performed by mixing cells and virus in culture media. For both adherent and suspension cells, transductions are initiated in suspension and carried out overnight. Adherent cells are allowed to adhere to substrate during transduction and are transduced at a cell density that allows for 2-3 population doublings before reaching confluence. Suspension cells are typically transduced at a higher density than standard growth density, and then they are diluted to standard growth density 18-24 hours after transduction. Do not let cells become too dense or let the medium become yellow at any point.

Before transduction, seed and expand cells from frozen stocks. Cells should be actively growing.

> ! **CAUTION:** Only open the tube containing the lentiviral particles in the laminar flow hood. Please refer to the **Safety Guidelines** and check with your institution regarding the use of lentiviruses.

Day 0—Inoculate Cells

1. Quickly thaw the lentiviral vector particles in a water bath at 37°C. Transfer the thawed particles to a laminar flow hood, gently mix by rotation, inversion, or gentle vortexing, and keep on ice. Unused viral particles can be aliquoted, refrozen at -80°C, and used again for subsequent experiments. There will be some loss of viral activity (usually 10-20%) with each refreeze.

2. Suspend sufficient cells for transduction in appropriate complete media supplemented with 1 µl/ml LentiTrans™ Transduction Reagent—if cells are sensitive to the LentiTrans Reagent, use a lower concentration or omit it. For HEK293 cells, we usually suspend at a density of 1×10^5 cells/ml in D-MEM supplemented with 10% FBS and LentiTrans Reagent. For K-562 cells, we usually suspend at a density 2×10^6 cells per ml in RPMI/10%FBS

supplemented with 20mM HEPES, pH 7.4 and LentiTrans Reagent.

3. Aliquot cells into wells or plates. For small scale transductions or titering assays, cells may be plated into multiwell microtiter plates (e.g., 0.5 ml/well for 24-well plates or 1 ml/well for 12-well plates). To transduce larger numbers of cells, use larger plates and scale up the volume accordingly

4. To each plate, add an appropriate amount of lentivirus. The amount of virus will depend on your viral titer and your experiment. Refer to the Transduction Guidelines or Assay Procedures section of the product manual for application specific recommendations.

5. Close the plate and mix by gentle agitation:
    - For adherent cells, place the plate into the CO_2 incubator and grow cells under standard conditions for 16-24 hours.
    - For suspension cells only, “spinoculate” by wrapping the perimeter with parafilm, placing the plate into the centrifuge with an appropriate balance, and spin the cultures at 1,200 × g at +25°C for 2 hours. Following centrifugation, remove plate(s) from centrifuge, carefully remove parafilm, and place in incubator. After 3 hours, “feed” cells with 0.5 ml additional complete medium per well (no LentiTrans Reagent).

Day 1—Change Media

Between 16 to 24 hours post-transduction, remove media and replace with fresh complete media without LentiTrans Reagent. For suspension cells, spin down and resuspend cells in complete media at 1-5 × 10^5 cells/ml. Place in incubator and grow for an additional 24-48 hours. Avoid confluency or too high a density of cells during and after transduction. If necessary, replate.

Day 3 or 4—Harvest or Split Cells

At about 72 hours after adding virus, you may expand cells as normal or harvest cells for an assay. To continue growing cells, split the cells 1:4 to 1:8 (or as appropriate, depending on the type of cells) as the culture approaches confluence, and add complete medium. As required by your experiments add antibiotics, other factors (tetracycline), etc., and expand as normal.

> NOTE: Typical doxycycline concentration for induction is 0.5 ug/ml. However, some cell lines can be more sensitive than others, so you may want to optimize the concentration.

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4.3. Assessing Transduction Efficiency by Flow Cytometry or Antibiotic Selection

For lentiviral constructs with a fluorescent marker or antibiotic resistance marker, transduction efficiency (i.e., % infected cells) can be determined from the fraction of fluorescent or antibiotic resistant cells in the population. To calculate the viral titer, it is also important to know the total number of cells at the time of transduction.

To determine viral titers, transductions should be set up as a serial dilution range of different amounts of virus in 12-well plates, 100,000 cells/well. For example, when titering standard virus from Cellecta, we recommend aliquoting 0, 0.1, 0.3, 1, 3.3 of concentrated stock to 5 different wells (or, for non-concentrated virus, scale up volumes ca. 100-fold). If you are using

cells that transduce with low efficiency (e.g., primary cells, some suspension cell lines, etc.) you may need to use 10 µl, or even 30ul, of concentrated virus stock for titering. Always be sure to include the initial well of cells only (no virus) as a negative control.

Option 1: Use flow cytometry with a fluorescent protein marker to determine % infected cells:

> **IMPORTANT:** Do not use fluorescence microscopy to assess the percentage of transduced cells. A significant number of transduced cells will be missed, so transduction efficiency will be underestimated.

1. About 72 hours after adding virus to the cells, spin down and resuspend cells in plate in 1X D-PBS.

2. If trypsin is used, block it with FBS/media, then centrifuge.

3. Determine the percentage of transduced (RFP/GFP/BFP-positive) cells by flow cytometry. For detection of Cellecta's RFP-, GFP-, and BFP-positive cells use the following settings:

Flow Cytometry Settings for TagRFP	
Excitation:	561nm (yellow laser) [530nm laser is still acceptable]
Emission:	590/20nm band-pass filter, or similar
Flow Cytometry Settings for TagGFP2	
Excitation:	488nm (blue laser)
Emission:	530/20nm band-pass filter, or similar
Flow Cytometry Settings for TagBFP	
Excitation:	405nm (violet laser)
Emission:	470/20nm band-pass filter, or similar

4. Use the formula below to calculate the percentage of transduced cells:

> (Fluorescent cells / Total number of cells) x 100 = **% infected cells** (use this for the titer calculation)

Option 2: Use antibiotic selection to determine % infected cells:

1. About 72 hours after adding virus to cells following the standard transduction protocol, split each transduction dilution (including the no-virus control) into 2 separate twin wells using 1:8 split ratio.

2. Add an appropriate amount of antibiotic to one twin well for each viral dilution (including the no-virus control).

3. Incubate all cultures for 2-5 additional days to allow antibiotic time to kill >99% cells in the no-virus control. Do not let the cultures reach confluence (split if needed).

4. After the antibiotic has had time to kill the cells, remove media from cells and replace with fresh media containing a 1/10 total volume alamarBlue stock solution. Include a media-only sample (no cells) for a background alamarBlue reading.

5. Incubate 1 hour at 37°C. Read the fluorescence intensity of the alamarBlue staining using a plate reader. Use the alamarBlue fluorescence values in the following calculation to determine the percent transduced cells in each antibiotic/non-antibiotic pair:

> ([antibiotic-selected cells – media-only] / [non-selected {no antibiotic} cells – media-only]) x 100 = **% infected cells** (use for titer calculation)

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4.4. Lentiviral Titer Calculation

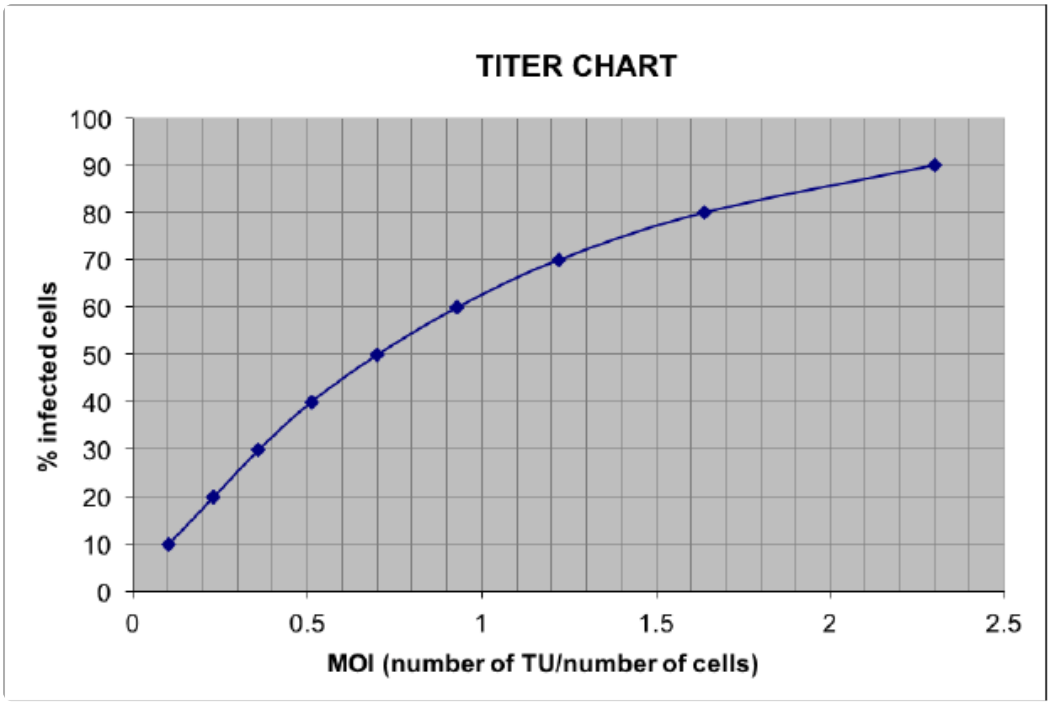
This section describes how to calculate the titer of packaged lentiviral particles. To calculate the number of transducible viral particles in the viral stock, you need to know the number of cells transduced at a specific dilution factor of the stock virus. This can be assessed by counting RFP-positive cells on a flow cytometer or using staining to assess cells with antibiotic resistance (e.g., puromycin).

- It is important to use an amount of virus sufficient to only transduce a fraction of the target cells so that you can accurately assess the number of transductions occuring. You cannot accurately assess the number of transductions if the whole population is transduced (i.e., fluorescent, or die out due to antibiotic selection). Ideally, it is best to aim for transducing less than half the cells so that most of the cells have been transduced with a single viral particle. For this reason, it is typical to titer several dilutions of the viral stock to obtain a culture with enough transduced cells for the calculation but not to the point where there are more viral particles than cells.

- To calculate the titer of a viral stock, it is necessary to have the following information:
    - The number of cells at transduction.
    - The percent of transduced cells after transduction (i.e., the Transduction Efficiency).
    - The volume of the viral stock used to transduce the cells.

Calculate the Transduction Units at Infection.

Lentiviral titer is measured as Transduction Units per ml (TU/ml). One TU produces one integration event in target cells. To calculate the viral titer, it is first necessary to determine the number of Transduction Units (TU) used to infect the cells. When the percentage of infected cells is at or below 20%, the number of integrations is approximately equal to the number of transduced cells. However, at higher transduction levels, the fraction of transduced cells with multiple integrations increases, so that the percentage of transduced cells relative to integration events per cell is no longer linear. Using the chart below, the number of integrations per cell, or **MOI** (Multiplicity Of Infection), can be accurately estimated for cultures with up to 75% transduced cells (i.e., MOIs in the range of ~0.2-1.5).



% Infected Cells	MOI
10	0.10
20	0.23
30	0.36
40	0.51
50	0.70
60	0.93
70	1.22
80	1.64
90	2.30

Estimation of percentage of cells infected based on Multiplicity Of Infection (MOI).

Calculate the Stock Titer

To calculate the titer of the original viral stock, apply the formula below:

> TU/ml = (# of cells at Transduction) × [MOI / (ml of Lentiviral Stock used at Transduction)]

- # of cells at Transduction = Total number of cells in the culture when viral particles were added
- MOI = Derived from the chart above based on the percentage of transduced cells.
- ml of Lentiviral Stock used for Transduction = The volume in ml of the virus added to the cells. Include any dilution of the viral stock.

Example Calculations:

> **IF:**
> The original # of cells at Transduction was 100,000, and
> The volume of virus stock used was 10 µl, and
> The observed % of transduced cells (RFP+ or antibiotic resistant) is 25%,
>
> **THEN:**
> The calculated MOI is 0.3 (from the chart), and
>
> The **TITER** is:
> 100,000 × 0.3 / 0.01 = 3,000,000 TU/ml

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5. Technical Support

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For the latest technical news and updates, visit Cellecta's blog at: https://cellecta.com/blogs/news

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6. Safety Guidelines

The HIV-based lentivector system is designed to maximize its biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter upstream of 5'LTR in the lentivector allows efficient Tat-independent production of lentiviral RNA, reducing the number of genes from HIV-1 that are used in this system.
- Number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (gag, pol, rev). The corresponding proteins are expressed from different plasmids lacking packaging signals and share no significant homology to any of the expression lentivectors, pVSV-G expression vector, or any other vector to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (gag, pol, rev) will be present in the packaged lentiviral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.
- Lentiviral particles will carry only a copy of your expression construct.

Despite the above safety features, use of HIV-based vectors falls within NIH Biosafety Level 2 criteria. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at:

> https://www.cdc.gov/biosafety/publications/bmbl5/index.htm

It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and follow standard microbiological practices, which include:

- Wear gloves and lab coat at all times when conducting the procedure.
- Always work with lentiviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area are to be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.

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7. Contact Us

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