Lentiviral Packaging System

Optimized Plasmid Mix and Suitable Cell Line

March 2013

Product Name: Lentiviral Packaging Mix
Catalog number: pLV-PACK-500/1000
Size: 500/1,000 μg
Storage Temperature: -20°C

Product Name: Lenti-LTR Packaging Mix
Catalog number: LTR-PACK-450/900
Size: 450/900 μg
Storage Temperature: -20°C

Product Name: Lenti-293T Cell Line
Catalog number: celine-01
Size: 1 ml/vial
Storage Temperature: -198°C

BIOSETTIA
A Better Solution in Life Science
www.biosettia.com
info@biosettia.com

Product Description

Production of lentiviral stocks requires packaging of the lentiviral genomic RNA transcribed from plasmid DNA with the helper vectors encoding HIV-1 *gag*, *pol*, and *rev* gene products and vesicular stomatitis virus G (VSV-G) protein. In general, at least two helper plasmids are required, with one plasmid expressing the Gag-Pol polyprotein and an accessory protein Rev and the other expressing VSV-G as envelop protein to increase cell tropism. Nevertheless, Gag-Pol and Rev proteins can be expressed from separated plasmids as well. Therefore, a three-plasmid system (lentiviral vector plus two helper plasmids) or a four-plasmid system (lentiviral vector plus three helper plasmids) can be used to generate lentiviruses.

Before you choose the packaging vector mix for generation of lentiviruses, you would like to know how the lentiviral genome is expressed. There are at least two ways of lentiviral genome is transcribed: (1) the lentiviral genomic RNA is transcribed from a chimeric 5′ LTR in which the HIV-1 promoter is replaced with RSV or CMV promoter; (2) the lentiviral genomic RNA is generated from a wild-type 5′ LTR. Please note the wildtype 5′ LTR requires the gene of Trans-Activator of Transcription (Tat) to be present in the packaging vector in order to be highly active. We are providing two packaging systems for producing lentiviral stocks from the lentiviral vectors with either chimeric or wildtype 5' LTR.

The Lenti-293T is an HEK 293 derived cell line stably expresses the SV40 large T antigen and neomycin resistance gene. The Lenti-293T cell line has been tested as a suitable cell line for lentiviral production due to high transfection efficiency, high level of protein yield and high titer of lentiviral production.

Lentiviral Packaging Mix (Catalog# pLV-PACK-500/1000): the packaging mix for the lentiviral vectors with a chimeric 5' LTR

The optimized packaging vector mix provides HIV-1 gag, pol, and rev gene products and vesicular stomatitis virus G (VSV-G) protein encoded in three plasmids (Fig. 1). This packaging mix is good for all of Biosettia lentiviral vectors but cannot be used in packaging lentiviral vectors with a wildtype 5′ LTR due to lack of Tat gene in the packaging vectors.

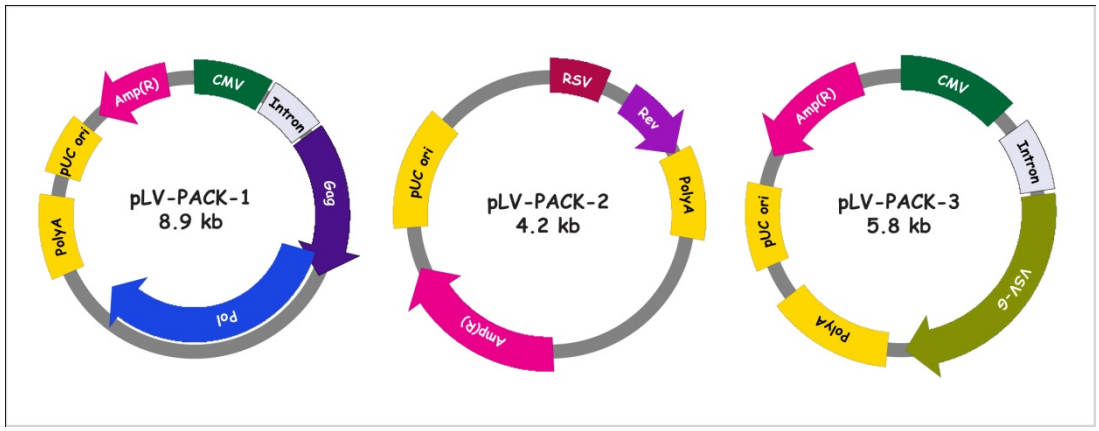

Figure 1. Map of the packaging vectors for the lentiviral vectors with a chimeric 5' LTR.

Lenti-LTR Packaging Mix (Catalog# LTR-PACK-450/900): The packaging mix for the lentiviral vectors with a wildtype 5' LTR

Two helper vectors are included in the optimized packaging mix. One vector provides HIV-1 gag, pol, rev, and tat gene products while the other vector encodes vesicular stomatitis virus G (VSV-G) served as envelope protein (Fig. 2).

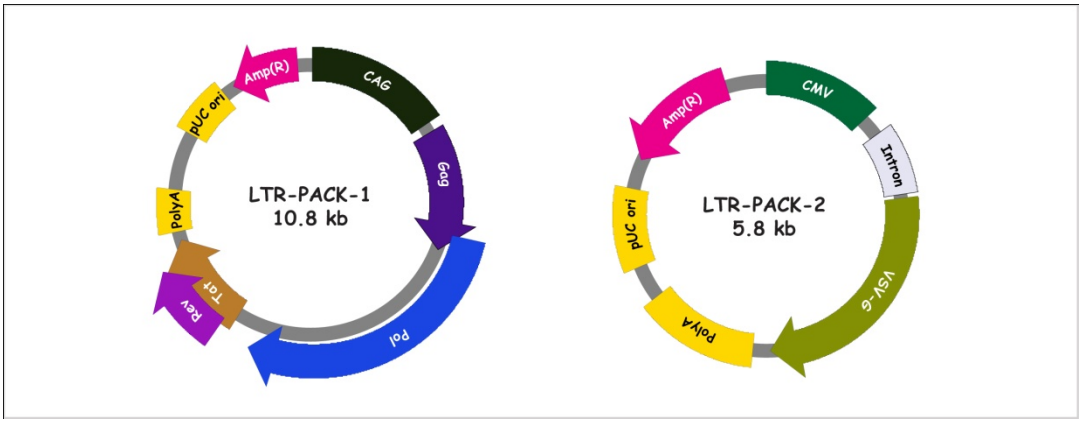

Figure 2. Map of the packaging vectors for the lentiviral vectors with a wildtype 5' LTR.

Protocol

Lenti-293T Cell Culture (Catalog# celine-01)

Lenti-293T growth medium: DMEM with high glucose (4.5 g/L), 4 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum (FBS, Invitrogen Cat# 16000-044), 1% Pen-Strep (optional), 0.1 mM MEM Non-Essential Amino Acids (NEAA, optional)

Thaw the vial of Lenti-293T cells in a 37°C water bath with gentle agitation; transfer the contents of the vial to a 15-cm tissue culture plate containing 45 ml of complete medium. Incubate the cells at 37°C incubator at 5% CO2 for 2-3 days to become 95-100% confluent.

Remove all medium from the 15-cm plate and add 2 ml of 0.25% trypsin/EDTA solution to the cells; incubate at 37°C incubator until all cells have detached (tilt the plate every 2-3 min); add 10 ml complete medium; gently resuspend cells with pipette; transfer 2 ml of the cell suspension to 15-cm plates containing 45 ml of complete medium. There will be 6 x 15-cm plates to generate lab cell stock.

Note: Always split cells every 2 days in 1:6 ratio.

Suggestion: make Lenti-293T cell stock by freezing the cells collected from the first subculturing 6x 15-cm plates.

Remove all medium from the 15-cm plate and add 2 ml of 0.25% trypsin/EDTA solution to the cells; incubate at 37°C incubator until all cells have detached (tilt the plate every 2-3 min); resuspend and collect all cells in a 50 ml conical tubes; centrifuge the cells at 300 x g for 5 min and resuspend the cells in 18-20 ml 90% FBS + 10% DMSO; aliquot 1ml cell suspension into each vial to make laboratory cell stocks.

Thaw a vial of Lenti-293T cell stock to start lentiviral packaging and virus production

Note: Always split cells every 2 days in 1:6 ratio. The earlier passages of Lenti-293T cells the better titer of lentiviruses will be generated. The Lenti-293T cells over 15 passages may produce less titer of viruses.

Lentiviral Production

The following procedure is provided as an example only. We produce lentiviral stocks in Lenti-293T cells using the transfection conditions summarized in a table below.

	10-cm plate		6-well plate	
	pLV-PACK	LTR-PACK	pLV-PACK	LTR-PACK
Lentiviral vector	9 µg		1.5 µg	
Packaging vector mix	9 µg	9 µg	1.5 µg	1.5 µg
Total plasmid DNA	18 µg		3.0 µg	
Lipofectamine™ 2000	45 µl		7.5 µl	
Total Opti-MEM	3 ml		0.5 ml	
Lenti-293T cells / vol. of medium	1.0×10^7/5ml		1.7×10^6/1ml	

Note: The transfection reagent Lipofectamine™ 2000 (LF2000, Invitrogen) is preferred for transfection. The average lentiviral titers in our preparations are around 5 x 10^6 - 1 x 10^8 infection units per ml (IU/ml) when titered with H1299 cells.

Day 0: Seed 6.0×10^6 (1.0×10^6) Lenti-293T cells in a 10-cm plate (6-well plate), so that the cell density will be around 1.0×10^7 (1.7×10^6) at the time of transduction.

Note: one 15-cm confluent plate generally contains 4-6 x 10^7 Lenti-293T cells.

Day 1: Gently mix 45.0 (7.5) µl LF2000 and 1.5 (0.25) ml Opti-MEM medium and incubate at room temperature for 5 minutes. Meanwhile, gently mix 18.0 (3.0) µg in total of lentiviral vector and packaging vector mixture into 1.5 (0.25) ml Opti-MEM medium (Invitrogen).

Gently mix DNA and LF2000, incubate at room temperature for 20 minutes to allow DNA and lipid to form complexes. In the meantime, replace the overnight culture medium with 5.0 (1.0) ml DMEM + 10% FBS without antibiotics. Add the 3.0 (0.5) ml DNA-LF2000 complexes to Lenti-293T cells.

Note: We have noticed that fetal bovine serum purchased from different manufacturers may affect the attachment of Lenti-293T cells to the bottom of tissue culture plates, resulting in variation in the efficiency of lentiviral production. We recommend switching to a different brand of FBS if Lenti-293T cells detached from plates during lentiviral production. Invitrogen FBS (Cat# 16000-044) is highly recommended.

Day 2: Replace the media containing the DNA-LF2000 complexes with 10.0 (2.0) ml complete medium at 12-16 hours post-transfection.

Day4: Collect supernatants at 48 hours post-transfection and transfer media to a polypropylene storage tube. Spin the virus-containing media at 1300 rpm for 5 minutes to pellet any Lenti-293T cells that were carried over during collection. Carefully transfer the supernatant to a sterile polypropylene storage tube.

Note: Lentiviral stock may be stored at 4 °C for up to 5 days, but should be aliquoted and frozen at -80 °C for long-term storage.

Suggestion: To reduce the number of freeze and thaw cycles, aliquot lentiviral stock to smaller tubes before storage at -80 °C.

Titering the Lentiviral Stocks

It is important to titer the lentiviral stocks in the cell line of interest to produce consistent results using the equivalent multiplicity of infection (MOI) in experiments. Knowing MOI will help you to control the copy number of lentivirus integrated into the chromosomes of the cells of interest. While titering virus with drug selection, the titering procedure includes selection of stably transduced cells with antibiotics and counting the antibiotic-resistant cell colonies. Alternatively, a flow cytometry can be used to determine the viral titer by measuring the number of fluorescent cells. If you are using both methods to titer your lentiviruses, please keep in mind that the titers may be different due to sensitivities of FACS machine and cell lines resistant to drug selection.

A. Determination of lentiviral titers by antibiotics selection.

Day 0: Seed the cells of your choice in a 6-well plate so that the cell density that will be ~25-50% confluent at the time of transduction.

Day 1: Thaw lentiviral stock, gently mix virus and then prepare 2 ml 10-fold serial dilutions ranging from 10^{-3} to 10^{-7} in complete medium containing 5-8 μg/ml polybrene.

Remove the medium from previous day and add 2 ml fresh dilutions into each well.

Suggestion: Leave one well uninfected for mock control. Spin transduction in a desktop centrifuge (e.g. Sorvall RT6000) at 1,000 × g for 30-60 min at room temperature may be necessary if titering virus in cell lines other than 293T.

Day 2: Replace the medium containing virus and polybrene with 2 ml of complete medium.

Day 3-4: Replace medium with fresh medium containing antibiotic to select for stably transduced cells.

Note: At least 48 hours of transduction allows lentivirus to integrate into the host genome. The optimal antibiotic concentration varies from cell line to cell line.

Suggestion: A pilot experiment should be performed to determine the minimum concentration of antibiotics required to kill the untransduced cells before this experiment.

Day 5-6: Replace medium with 2 ml fresh medium containing antibiotic every 2 days.

Day 7-8: Allow antibiotic-resistant colonies to form in dilution wells. No live cells should be growing in the mock control well.

Note: The number of days required for the formation of visible colonies may vary among different cell lines.

Wash wells twice with 2 ml PBS

Stain cells with 1 ml 0.5% crystal violet solution in 20% ethanol and incubate for 30 minutes at room temperature.

Wash wells with distilled water by submerging the plate in a tray full of water, and repeat the wash one more time.

Dry the plate and count the number of blue-stained colonies.

The titer should be the average colony number times the dilution factor.

Note: The transduction efficiency varies from cell line to cell line. The lentiviral titer in the cell line of your interest may be lower (sometimes more than 10-fold) or higher than the virus titer in commonly used cell lines such as 293T cells.

B. Determination of lentiviral titers by flow cytometry.

Day 0: Seed the cell of your choice in a 6-well plate so that the cell density that will be ~25-50% confluent at the time of transduction.

Note: The number of cells seeded in the well is required to calculate lentiviral titer later.

Day 1: Thaw lentiviral stock, gently mix virus and then prepare 2 ml 10-fold serial dilutions ranging from 10^{-1} to 10^{-4} in complete medium containing 5-8 μg/ml polybrene.

Remove the medium from previous day and add 2 ml fresh dilutions into each well.

Suggestion: Leave one well uninfected for mock control. Spin transduction in a desktop centrifuge (e.g. Sorvall RT6000) at 1,000 × g for 30-60 min at room temperature may be necessary if titering virus in cell lines other than 293T.

Day 2: Replace the medium containing virus and polybrene with 2 ml of complete medium.

Day 3-4: Follow your lab protocol to collect and resuspend cells for flow cytometry to determine the percentage of green or red fluorescent cells.

Note: At least 48 hours of transduction allows lentivirus to integrate into the host genome.

The titer should be the average number of live fluorescent cells times the dilution factor.

Note: The transduction efficiency varies from cell line to cell line. The lentiviral titer in the cell line of your interest may be lower (sometimes more than 10-fold) or higher than the virus titer in commonly used cell lines such as 293T cells.

Example: 1×10^5 cells were seeded on Day 0 and the cell doubling time is around 24 hours. The FACS data showed 5% and 40% of fluorescent cells in the 10^{-3} and 10^{-2} dilution wells, respectively. The lentiviral titer is calculated by multiplying the fraction of transduced cells by 2×10^5 (the expected number of cells in the well on Day 1, the time of transduction), and by the dilution factor.

$0.05 \times (2 \times 10^5) \times 10^3 = 1 \times 10^7$

$0.4 \times (2 \times 10^5) \times 10^2 = 8 \times 10^6$

The average lentiviral titer is 9×10^6 IU/ml

Optional: Concentration of Lentivirus by Ultracentrifugation

1. Filter lentivirus through a 0.45 μm filter.

2. Centrifuge at 25,000 rpm for 90 minutes in a SW-28 or SW-41 rotor.

3. Discard the supernatant and use a Pasteur pipette with an attached P100 tip to carefully remove the remaining medium.

4. Gently resuspend viral pellet in 1/100 volume of DMEM. Let the virus suspension sit for overnight at 4° C.

5. On the following day, mix gently, aliquot and freeze virus.

Safety Guidelines for Working with Lentiviruses.

The recombinant lentiviruses have been designated as Level 2 organisms by NIH and CDC. A Biosafety Level 2 (BSL-2) facility is required in order to work with lentiviruses. The information of Biosafety in Microbiological and Biomedical Laboratories (BMBL) can be downloaded from the following link:
http://www.cdc.gov/od/ohs/biosfty/bmbl5/BMBL_5th_Edition.pdf

Please be aware that you are working with media containing lentiviral particles which could transduce human cells.

User Notification

Biosettia products are sold to be used for research purposes only and may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without written approval of Biosettia, Inc. They may not be used for any other purpose, including, but not limited to, use in drugs, in vitro diagnosis, or therapeutics, or in humans. Products may be covered by pending or issued patents or may have certain limitations. Please contact Biosettia for more information. All prices and specifications are subject to change without prior notice. Product claims are subject to change. Please access the Biosettia online catalog for the most up-to-date information.

Biosettia warrants that all of its products will perform according to commercially reasonable standards. The company will replace, free of charge, any product that does not meet those specifications. Customer must notify Biosettia within thirty days of delivery. This warranty limits Biosettia's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Biosettia reserves the right to select the methods used to analyze a product unless Biosettia agrees to a specified method in writing prior to acceptance of the order.