pPACK-ID: Integrase-Defective Lentiviral Packaging Mix

Cat# LV520A-ID (15 reactions)

Cat# LV525A-ID (30 reactions)

User Manual

Store kit at -20°C on receipt

Version 1
7/29/2016

Contents

Product Description

pPACK-ID is an integrase-defective lentiviral packaging plasmid mixture. The pPACK-ID lentiviral packaging plasmids provide all of the proteins essential for transcription and packaging of an RNA copy of the expression construct into recombinant pseudoviral particles, except that the integrase gene has been mutated. This allows any third generation lentivectors to be packaged into viral particles, but the resulting expression construct remains as an episomal vector in the target cell, instead of being integrated into the host cell genome. In dividing cells, the episome is able to produce high levels of effector or reporter molecules in target cells in a transient manner, eventually decreasing over time due to cell division and dilution of the episome. In non-dividing cells (e.g. neurons), the expression of the episome is sustained for at least several weeks, allowing for longer-term gene functional studies.

The pPACK-ID Packaging Plasmid Mix consists of an optimized mixture of three plasmids: pPACK-ID-GAG, pPACK-ID-REV and pVSV-G.[1]

- The pPACK-ID-GAG plasmid contains the structural (Gag), and replication (Pol) genes which code for some of the proteins required to produce the lentivirus. It also encodes the viral Env gene, which encodes the envelope protein that defines the tropism (i.e. the range of infectable cells). The defective integrase is also located on this plasmid.
- The pPACK-ID-REV plasmid contains the regulatory protein Rev that is required for HIV replication.
- The pVSV-G plasmid expresses the envelope glycoprotein of vesicular stomatitis virus (VSV-G) from the CMV promoter. VSV-G pseudotyped viral particles mediate viral entry through lipid binding and plasma membrane fusion and can infect both mammalian and non-mammalian cells.

Available Products

Item	Catalog #	Storage temperature	Shelf-life
pPACK-ID (15 reactions)	LV520A-1	-20°C	12 months
pPACK-ID (30 reactions)	LV525A-1	-20°C	12 months

Storage

The kits are shipped on blue ice or dry ice and should be stored at -20°C upon receipt. Properly stored kits are stable for 1 year from the date received.

General Information

Lentiviral expression vectors are the most effective vehicles for transducing and stably expressing different effector molecules (siRNA, cDNA, DNA fragments, antisense, ribozymes, etc.) or reporter constructs in almost any mammalian cell, including non-dividing cells and whole model organisms. By packaging the lentiviral expression construct into pseudoviral particles, you can obtain highly efficient transduction (up to 100%), even with the most difficult to transfect cells, such as primary, stem, and differentiated cells. Lentivectors also offer advantages such as large cargo capacity and low immunogenicity compared to other delivery vehicles. However, one of the big disadvantages of lentivectors is their ability to randomly integrate into the genome, which can lead to insertional mutations and unwanted side effects. Some applications of integration-defective lentivectors are in the following areas:

1. Stem cell therapies and induced pluripotent stem cell generation
2. Immunizations
3. Delivery to sites like the spinal cord or the central nervous system

[1] SBI does not provide individual plasmids in the pPACK-ID kit for sale nor is able release their sequences as they are proprietary in nature.

4. Cancer therapies

There are several properties that distinguish integrating lentivectors from integration defective lentivectors. First of all, gene expression from the episome is generally less efficient when compared to an integrated construct. Secondly, the expression of integration-deficient episomes is transient in dividing cells, and will eventually become diluted in daughter cells. Expression of integration-deficient lentivectors lasts longer in non-dividing cells, although it is still episomal. pPACK-ID generally has a lower transduction efficiency than wild-type (approximately 50% of wild-type when measured by GFP expression in target cells). Transduction efficiency also depends on the size of the insert being packaged.[2]

Reagents Required for the Protocol, but Not Included in the Kit

- Lentiviral expression vector, purified with an endotoxin-free plasmid preparation kit
- 293TN producer cells (Cat # LV900A-1)
- Purefection transfection reagent (Cat # LV750A-1)
- Dulbecco's Modified Eagle's Medium (D-MEM) (high glucose with sodium pyruvate and L-glutamine; Invitrogen, Cat. # 11995073)
- Fetal Bovine Serum (Invitrogen, Cat. # 16000036)
- Puromycin (Sigma, Cat. # P8833)
- Penicillin/Streptomycin (Invitrogen, Cat. # 15070063)
- Trypsin-EDTA (Sigma, Cat. # T3924)
- Tissue Culture Plates and Related Tissue Culture Supplies
- Sterile TE Buffer (10 mM Tris pH 8.0, 0.1 mM EDTA pH 8.0)
- PEG-it Viral Concentration Solution (Cat # LV810A-1) (optional)
- Transdux Viral transduction reagent (Cat # LV850A-1) (optional)

Protocol:

Lentiviral expression construct quality

Transfection efficiency significantly depends on the quality of plasmid DNA. We recommend purifying plasmid DNA with a QIAGEN Endotoxin-free Plasmid Kit. You will need 2 µg of lentiviral expression construct in sterile TE buffer with a concentration ranging from 0.2 – 2 µg/µl for each transfection in a 10-cm culture plate (or 75 cm^2 flask).

[2] The following SBI lentivectors are recommend for use with the pPACK-ID system: CD510B-1, CD511B-1, CD710B-1, CD711B-1. The single marker lentivectors perform most reliably with pPACK-ID.

Maintaining 293TN cell line

The 293TN cell line is a highly transfectable derivative of the HEK293 cell line with constitutive expression of SV40 T-antigen and neomycin resistance gene. The 293TN cells should be grown at 37°C in a humidified chamber with 5% CO2 in D-MEM medium supplemented with 4 mM L-glutamine, 4.5 g/l glucose, 100 units/ml penicillin G, 100µg/ml streptomycin, and 10% fetal bovine serum. With a doubling time of less than 24 hours, the 293TN cells should be split every 1 – 2 days when they reach 70 – 80% confluency. For subculturing, detach the cells with 0.25% trypsin, 0.03% EDTA at 37°C, add fresh culture medium, and split at a ratio of 1:3 – 1:5. Alternatively, 293TN cells can be subcultured every 3 to 4 days by splitting cells 1:10 or 1:20, respectively. The cells should never reach more than 90% confluency in order to keep the culture continuously in logarithmic growth phase.

Transfection of 293TN Cells with PureFection™ reagent (100 mm plate protocol)

To make lentivirus, cotransfect your lentivector construct with the pPACK-ID plasmid mix into 293TN cells using PureFection reagent. For some viruses, you may need to seed several plates of cells to obtain a high enough titer for transduction of target cells.

1. 18 - 24 hours prior to transfection, seed 3.0 – 4.0 x10^6 293TN cells per 100 cm^2 cell culture plate[3] in 9 ml of normal culture medium (without antibiotics) so that the cell density reaches ~60 - 80% confluency at the time of transfection.
2. Add 0.8 ml DMEM (serum free) to an autoclaved 2 ml Eppendorf tube.
3. Add 20 µl pPACK-ID and 2 µg of your lentivector construct into the DMEM. Mix by pipetting.
4. Add 24µl PureFection into the same tube. Vortex for 10 seconds.
5. Incubate DMEM-Plasmid-PureFection mixture at room temperature for 15 minutes.
6. Add DMEM-Plasmid-PureFection mixture drop-wise to the dish, and swirl to disperse evenly throughout the plate.
7. Return the dish to the cell culture incubator at 37°C with 5% CO_2.
8. Change the medium 12-24 hours after transfection (optional).
9. At 48 hours and 72 hours after transfection, collect the medium (which now contains pseudoviral particles) into a 50-ml sterile, capped conical centrifuge tube. Centrifuge at 3000 x g for 15 minutes at room temperature to pellet cell debris. Transfer the viral supernatant into a new tube.

Caution: You are working with infectious pseudoviral particles at this stage. Please follow the recommended guidelines for working with BSL-2 safety class.

[3] If you use 15 cm plates, seed 7-8x10^6 cells/ dish in 20 ml of normal culture medium without antibiotics.

- In step 2, 1-1.6ml serum free medium should be used for each 15 cm plate.
- In step 3, 45 µl pPACK-ID and 4.5 µg of plasmid should be used for each 15 cm plate.
- In step 4, 55 µl PureFection should be used for each 15 cm plate.

10. The pseudoviral particles can now be concentrated using SBI's PEG-it viral concentration reagent, or used directly for transduction into target cells.

Validation Data

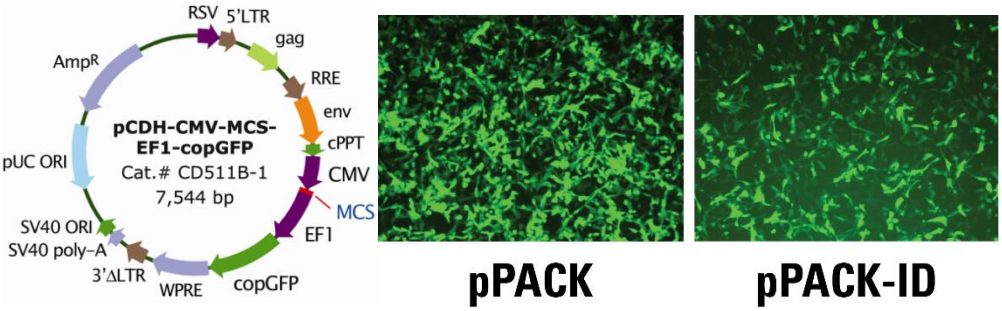

Figure 1. pPACK-ID provides good transduction efficiency. (Left panel) We packaged SBI's third-generation lentiviral vector pCDH-CMV-MCS-EF1-copGFP (Cat. # CD511B-1) into lentiviral particles using either standard pPACK which supports lentiviral integration, or pPACK-ID which keeps the transduced lentivector episomal. Transduction efficiency as assessed by the fraction of GFP-positive cells is within a factor of two between pPACK (middle panel) and pPACK-ID (right panel).

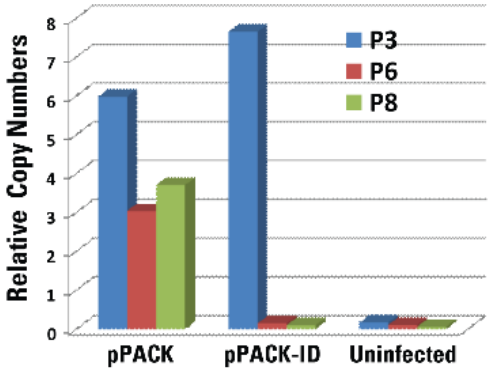

Figure 2. Lentivectors delivered by pPACK-ID are rapidly lost in dividing cells. HT1080 cells infected with pPACK or pPACK-ID lentivirus containing a GFP marker (Cat. # CD511B-1) were cultured and carried through 8 passages. The relative copy number of GFP was measured by qPCR after passages 3, 6, and 8 in cells infected with pPACK lentivirus, pPACK-ID lentivirus, and uninfected HT1080 cells. After 3 passages, the transgene copy number is similar in both pPACKand pPACK-ID-infected cells, but drops to background levels in pPACK-ID cells by passage 6.

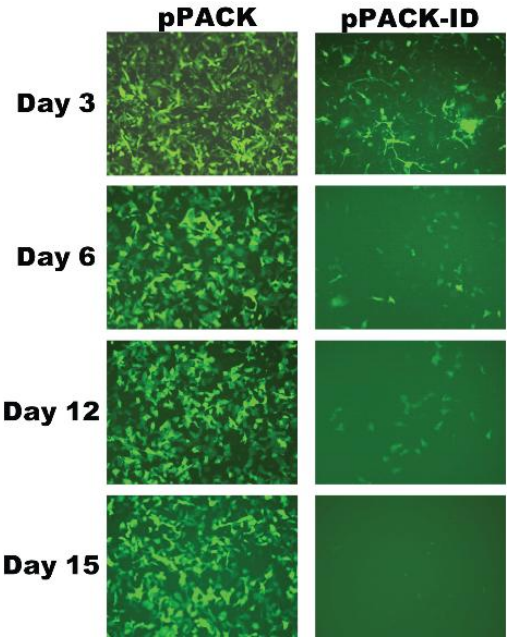

Figure 3. Transgene expression is rapidly lost in pPACK-ID-infected cells. HT1080 cells were transduced with pPACK and pPACK-ID lentivirus carrying a GFP marker Cat. # CD511B-1). Images were taken on days 3, 6, 12 and 15 days post-transduction, and show progressive loss of GFP signal in pPACK-ID-infected cells, but not in pPACK-ID-infected cells.

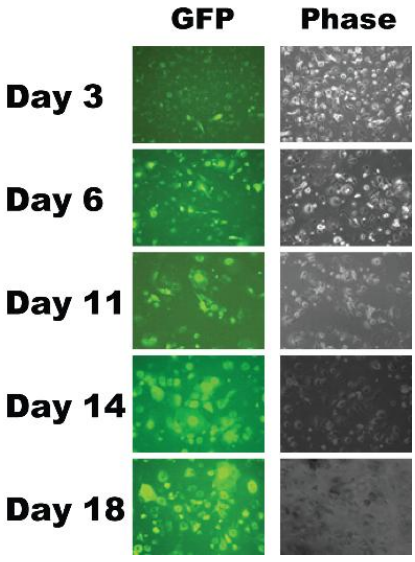

Figure 4. Transgene expression is stable in non-dividing cells infected with pPACK-ID. Mouse embryonic fibroblast (STO) cells were treated with Actinomycin D to prevent cell division, transduced with GFP lentivirus (Cat. # CD511B-1) packaged using pPACK-ID, and cultured for 18 days. GFP expression remained consistent across the timepoints, showing stability of transgene expression in non-dividing cells.

Troubleshooting

Low Viral Titer (<10^5 ifu/ml)

Problem		Solution
Poor transfection efficiency	293TN Cells have too high or too low density	Plate fewer or more cells in order to have about 50 - 80% confluency at transfection stage.
	Lentivector expression construct DNA preparation is of poor quality	Purify plasmid DNA using a QIAGEN Endotoxin-free Plasmid Purification Kit or by phenol/chloroform extraction followed by a CsCl gradient centrifugation.
	Plasmid DNA/PureFection™	Optimize the ratios using the guidelines provided in the PureFection™ protocol.

Inefficient Production of the Pseudovirus

Problem	Solution
293TN cells are of poor quality	Check growth medium
	293TN cells should not be grown for more than 20 passages.
	Check for mycoplasma contamination
	Make sure the cells have not been overgrown (do not allow the cells to reach more than 90% confluency in order to keep the culture continuously in logarithmic growth phase).
Pseudoviral supernatant harvested too early or too late	Harvest supernatant every 12 hours starting 24 hours after transfection for 2 – 3 days (24, 36, 48, 60, 72 hours), then titer each batch.
Lentiviral expression construct is too large	The packaging limit for the lentiviral system is 8.5 kb from 5' LTR to 3' dLTR. However, the efficiency of packaging drops significantly at greater than 2 kb of cDNA insert length. For a 3 kb insert, the titers could be 10-fold lower than for a 1 kb insert.
Truncated viral RNA transcript	Re-check the lentivector construct sequence to confirm the absence of a polyadenylation (ATAAA) site between the LTR elements.

Inefficient Transduction of Packaged Lentivector Expression Constructs

Problem		Solution
Poor infection efficiency	Your stock contains low titer of virus	Optimize infection protocol by using standard pre-packaged pseudoviral stocks of copGFP positive control which can be purchased from SBI.
	Volume of infecting supernatant is too high	Keep volume as low as possible to achieve maximal adsorption of viral particles to the cells.
	The assay is performed too early	Normally, the maximal expression of integrated provirus is expected to develop by 72 hours after infection. However, some cells display delayed expression. Try the assay at a later time, such as 96 hours.
	Promoter is not functional in target cells	Replace the CMV promoter with the elongation factor 1 (EF1) promoter in the expression construct.
	Target cell line may be difficult to transduce	Check titer with 293TN or H1299 cells. Optimize the transduction protocol. Use a higher MOI.
	Loss of pseudoviral titer during storage	Aliquot and store pseudoviral stock at –80°C. Each freeze-thaw cycle drops the titer about 30%. Use a fresh aliquot for transduction.
Infection affects target cell viability	Pseudoviral stock medium affects target cell growth	Dilute the stock medium or concentrate the pseudovirus by centrifugation to minimize the amount of medium added to the target cells. We recommend using SBI's PEG-it™ Virus Precipitation Solution (Cat. # LV810A-1).
No Expression of Expression Construct	The CMV or H1 promoter is not functional in target cells	We have observed this in primary cells, but the only way to solve this problem is to change the type of target cells or replace the CMV promoter with the EF1 promoter and H1 promoter with the U6 promoter.

Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site: **http://www.systembio.com**

For additional information or technical assistance, please call or email us at:

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Biosafety for pPACK-ID and Lentivectors

SBI's HIV-based lentivector systems are designed to maximize their biosafety features, which include:

- Lentivectors contain a deletion in the enhancer of the U3 region of 3'ΔLTR that ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter in HIV-based vectors upstream of 5'LTR in the lentivector allow efficient Tat-independent production of viral RNA, reducing the number of genes from HIV-1 that are used in this system.
- The 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 (for HIV-based packaging plasmids) that lack packaging signals. The packaging plasmids share no significant homology to any of the expression lentivectors, the pVSV-G expression vector, or any other vector, to prevent generation of recombinant replication-competent virus. The pPACK-ID packaging plasmids have a mutated integrase gene, thus preventing integration in the host cell genome.
- None of the HIV-1 genes (gag, pol, rev) are present in the packaged viral genome, as they are expressed from separate plasmids lacking packaging signal. Therefore, the lentiviral particles generated are replication-incompetent.
- Produced pseudoviral particles will carry only a copy of your expression construct.

The choice of SBI's lentiviral system for experimental studies is driven by functional considerations, including increased productivity and transduction efficiency. The design of SBI's biosafe vectors has benefited researchers allowing them to conduct experimental studies with lower risk. Currently, SBI's vectors combine improved safety features (that decrease the risk of recombination and vector mobilization) with increased transduction efficiency.

Despite the above safety features, use of HIV-based vectors falls within NIH Biosafety Level 2 criteria due to the potential biohazard risk of possible recombination with endogenous viral sequences to form self-replicating virus, or the possibility of insertional mutagenesis. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm. It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and to always follow standard microbiological practices, which include:

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

Licensing and Warranty Statement

Limited Use License

Use of the pPACK-ID (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

The purchaser of the Product is granted a limited license to use the Product under the following terms and conditions:

- The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use.
- The Product may not be resold, modified for resale, or used to manufacture commercial products without prior written consent of SBI.
- This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research.

SBI has pending patent applications related to the Product. For information concerning licenses for commercial use, contact SBI.

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Limited Warranty

SBI warrants that the Product meets the specifications described in this manual. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This

limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

SBI's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

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