Lenti-X™ Packaging Single Shots (Envelope-Free) Protocol-At-A-Glance

I. Introduction

This protocol is provided for transfection and lentivirus production with **Lenti-X Packaging Single Shots (Envelope-Free)**, a kit consisting of single tubes pre-aliquoted with lyophilized Xfect™ Transfection Reagent premixed with an optimized formulation of Lenti-X lentiviral packaging plasmids to enable streamlined production of lentiviral particles pseudotyped with the envelope protein of choice.

Lenti-X Packaging Single Shots (Envelope-Free) provide a simple method to transfect 293T cells with lentiviral vector DNA producing lentiviral particles, expressing your envelope of interest. The amount of reagent and packaging vectors in each tube is optimized for pseudotyped lentivirus production in a 10-cm dish. Transfections can be carried out entirely in the presence of serum. Use of tetracycline-free FBS is critical for achieving high titers with this technology.

A vector for cloning the envelope protein of choice (pEmpty) is included with the kit. The pEmpty vector contains a DNA fragment encoding a partial *lacZ* gene flanked by restriction sites on both the 5' and the 3' end. The excision of this DNA fragment is a clear indication of successful plasmid digestion.

Also provided are two self-inactivating lentiviral plasmids encoding either ZsGreen1 or firefly luciferase, to be used as reporters (generating fluorescence or luminescence, respectively) for monitoring lentiviral transduction.

II. Components

Lenti-X Packaging Single Shots (Envelope-Free) kits include the following:

- Lenti-X Packaging Single Shots (Envelope-Free): 16 tubes
- pEmpty Vector: 1 tube (20 µl, 500 ng/µl)
- Lenti-X Reporter Vector Set:
    - pLVXS-ZsGreen1-Puro Vector: 1 tube (20 µl, 500 ng/µl)
    - pLVXS-Luciferase-Puro Vector: 1 tube (20 µl, 500 ng/µl)

NOTE: The plasmids provided in the Lenti-X Reporter Vector Set and the pEmpty Vector must be amplified in bacteria to obtain sufficient quantities for cloning or pseudovirus production using the kit. We recommend NucleoBond Xtra Midi EF or NucleoBond Xtra Maxi EF kits (Cat. Nos. 740420.10 and 740424.10) for efficient production of endotoxin-free, transfection-grade plasmid DNA.

III. General Considerations

Storage and Handling

- Store Lenti-X Packaging Single Shots (Envelope-Free) in the supplied foil pouch with the desiccant sachet at –20°C.
- Return any unused Lenti-X Packaging Single Shots (Envelope-Free) to the supplied foil pouch with the desiccant sachet, and store at –20°C.
- Store plasmids at –20°C.

IV. pEmpty Vector Manipulations

The pEmpty Vector allows for expression of the desired envelop protein under the control of a CMV promoter. Cloning of the desired envelope protein gene sequence into the pEmpty Vector can be accomplished using In-Fusion® Snap Assembly (Cat. No. 638949) or traditional cloning techniques. The pEmpty Vector has two different regions of restriction sites flanking a small DNA stuffer fragment (partially encoding the *lacZ* gene) to simplify cloning. Please see the GenBank file for a detailed list of these restriction sites.

Plasmid Vector Propagation & Construction of Your Customized Envelope Protein Vector

1. To ensure that you have a renewable source of plasmid DNA, transform each of the plasmid vectors provided in this kit into an *E. coli* host strain, such as Stellar™ Competent Cells (Cat. No. 636763).

2. To purify plasmid DNA for cloning purposes, use a suitable NucleoBond or NucleoSpin kit. See takarabio.com for available kits and options.

3. Using standard cloning techniques, insert your desired envelop protein coding sequence into the pEmpty Vector's multiple cloning sites (MCS). We recommend choosing two different restriction sites, one from the 5' MCS and one from the 3' MCS that flank the *lacZ* fragment, for linearization of and cloning into the pEmpty Vector. We recommend using In-Fusion Snap Assembly for plasmid construction, which allows for directional, seamless cloning of the desired envelop protein PCR product into the linearized pEmpty Vector. In addition, the online In-Fusion Cloning Primer Design Tool (www.takarabio.com/learning-centers/cloning/primer-design-and-other-tools) allows for easy and convenient primer design.

   NOTE: the desired envelope protein sequence (cDNA or gene fragment) may require an ATG initiation codon. In such cases, addition of a Kozak consensus ribosome binding site (Kozak, 1987) may improve expression levels, but this is generally not required.

4. Perform a midi- or maxi-scale plasmid DNA preparation for each plasmid that will be transfected into the packaging cells. For guaranteed transfection-grade plasmid DNA, we recommend using NucleoBond Xtra Midi Plus or Maxi Plus kits (Cat. Nos. 740412.10 and 740416.10).

V. Transfection and Virus Production Protocol

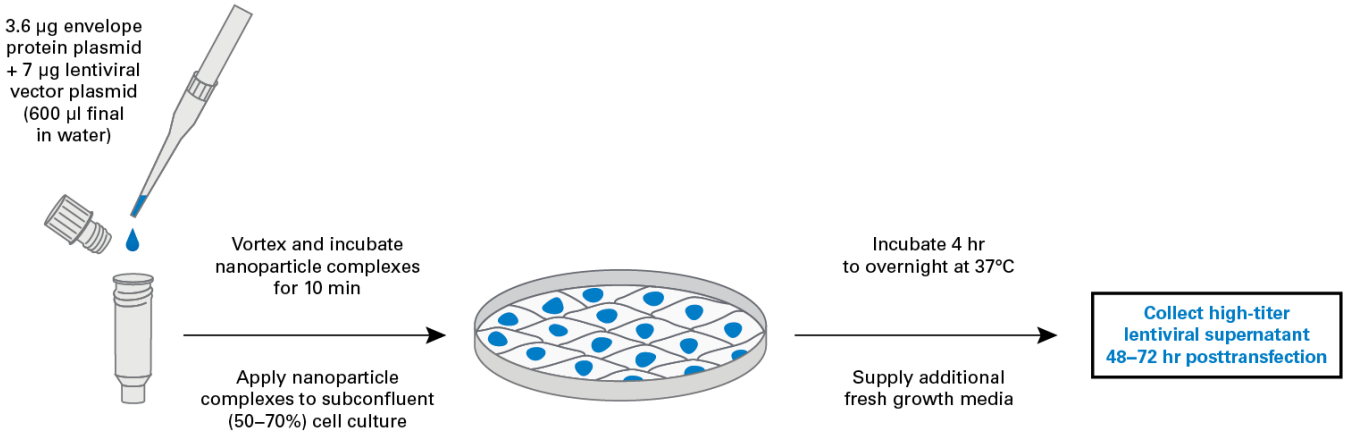

Figure 1. The Lenti-X Packaging Single Shots (Envelope-Free) protocol.

IMPORTANT: All of the following steps should be performed in a sterile tissue culture hood. Lentivirus requires the use of a Biosafety Level 2 facility. Pseudotyped lentiviruses packaged from HIV-1-based vectors are capable of infecting human cells. Use appropriate safety precautions.

A. Before you begin

- The plasmids provided in the Lenti-X Reporter Vector Set must be amplified in bacteria to obtain sufficient quantities for pseudovirus production using the kit. Do not start the cells until after this amplification is complete.

- Transfections should be performed using **10-cm tissue culture dishes**. Tetracycline-free FBS should be used at a final concentration of 10% in both the transfection medium and the medium used to collect the virus.

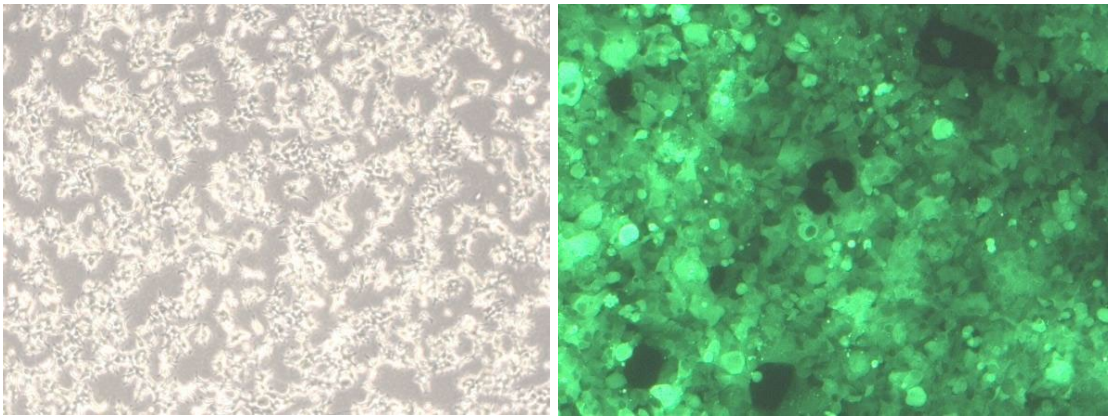

Figure 2. Optimal density of Lenti-X 293T cells (Cat. No. 632180) at the point of transfection (left panel) and harvest (right panel), shown here using a transfer vector containing ZsGreen1.

B. Protocol

NOTE: The plasmids provided in the Lenti-X Reporter Vector Set must be amplified in bacteria to obtain sufficient quantities for pseudovirus production using the kit.

Lenti-X™ Packaging Single Shots (Envelope-Free) Protocol-At-A-Glance

1. Approximately 24 hr before transfection, seed 4–5 x 10^6 Lenti-X 293T packaging cells/10-cm plate in 8 ml of growth medium. Make sure that the cells are plated evenly. Incubate at 37°C, 5% CO_2 overnight.

   Continue to incubate the cells until you are ready to add the transfection mixture in Step 5. The cells should be 80–90% confluent at the time of transfection.

2. Dilute 3.6 µg of your plasmid encoding the desired envelope protein and 7.0 µg of your lentiviral vector plasmid DNA (pLVXS-ZsGreen1-Puro or pLVXS-Luciferase-Puro) with sterile water to a final volume of 600 µl in the same sterile, microcentrifuge tube. Mix thoroughly by vortexing.

   NOTE: Always dilute your DNA in water prior to adding it to a tube containing Lenti-X SARS-CoV-2 Packaging Single Shots (Envelope-Free). Undiluted DNA should not be mixed with the transfection reagent.

3. Add the 600 µl of diluted DNA to a tube of Lenti-X Packaging Single Shots (Envelope-free), replace the cap, and vortex at high speed for 20 sec. The pellet should dissolve completely.

   NOTE: In some cases, insoluble material may be visible after vortexing. This material does not have a negative effect on transfection efficiency or virus yields.

4. Incubate the samples for 10 min at room temperature to allow nanoparticle complexes to form. After the 10 min incubation, centrifuge the tube for 2 sec to bring the sample to the bottom of the tube.

   NOTE: Sample tubes can be inserted into 1.5-ml microcentrifuge tubes for a brief centrifugation.

5. Transfer the entire nanoparticle complex solution dropwise to the Lenti-X 293T packaging cells prepared in Step 1. Gently rock the plate back and forth to mix.

   NOTE: It is normal for the medium to change color slightly upon addition of the nanoparticle complex solution.

6. Incubate the cells at 37°C, 5% CO_2.

   NOTE: A 4-hr incubation with Xfect-DNA nanoparticles is sufficient for optimal transfection. Incubation may be continued overnight for convenience but does not generally increase transfection efficiency or titer.

7. After incubation, add an additional 6 ml of fresh complete growth medium and incubate at 37°C, 5% CO_2 for an additional 24–48 hr. Virus titers will generally be highest 48 hr after the start of transfection.

8. Harvest the supernatants and pool similar stocks, if desired (a 48-hr sample may be stored at 4°C until a 72-hr sample is harvested).

   CAUTION: Supernatants contain infectious pseudovirus. Use appropriate safety precautions.

9. Centrifuge briefly (500*g* for 10 min) or filter through a 0.45-µm filter to remove cellular debris.

   NOTE: The filter used should be made of cellulose acetate, or polysulfone (low protein binding). Do NOT use filters made of nitrocellulose. Nitrocellulose binds proteins present in the membrane of lentivirus and destroys the virus.

10. Verify virus production using Lenti-X GoStix™ Plus (for details, see the Lenti-X GoStix Plus Protocol-At-A-Glance). Alternatively, titrate the virus stock, then use the virus to transduce target cells, or store at –80°C. Avoid multiple freeze/thaw cycles.

11. For protocols describing how to transduce your target cells or create frozen stocks, see the Lenti-X Lentiviral Expression Systems User Manual.

VI. Expected Results

Typical results are shown in Figures 3 and 4. High transduction efficiencies are observed using pseudotyped lentivirus encoding ZsGreen or firefly luciferase produced with Lenti-X Packaging Single Shots (Envelope-Free) according to the protocol described in Sections IV and V. In addition, the results obtained with Lenti-X Packaging Single Shots (Envelope-Free) are comparable to those obtained with Lenti-X SARS-CoV-2 Packaging Single Shots.

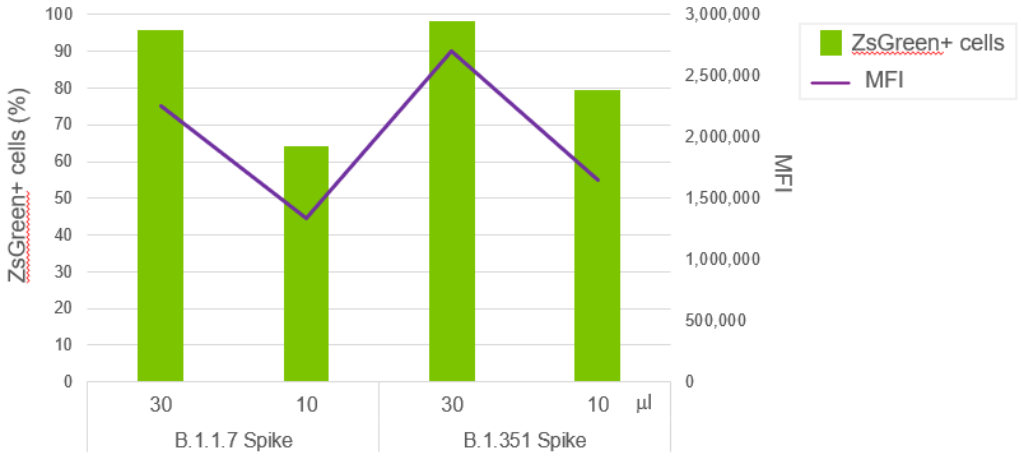

Figure 3. Transduction of ACE2 HEK293T cell line (Cat. No. 631289) using SARS-CoV-2 pseudovirus encoding ZsGreen. Lenti-X Packaging Single Shots (Envelope-Free) were used to produce truncated SAR-CoV-2 spike B.1.1.7 pseudovirus encoding the fluorescent protein ZsGreen. Simultaneously, truncated SARS-CoV-2 spike B.1.351 pseudovirus encoding ZsGreen was produced using the Lenti-X SARS-CoV-2 Packaging Single Shots (B.1.351 Spike, Truncated) (Cat. No. 631293). Different volumes of the concentrated supernatant (20X) from each prep (30 or 10 µl) were used to transduce a HEK293T cell line stably expressing the human ACE2 receptor in the presence of 6 µg/ml polybrene in 48-well plates. The transduction efficiencies for each sample were measured by flow cytometry 6 days post-transduction. The percentage of ZsGreen-positive (ZsGreen+) cells and mean fluorescence intensity (MFI) are shown.

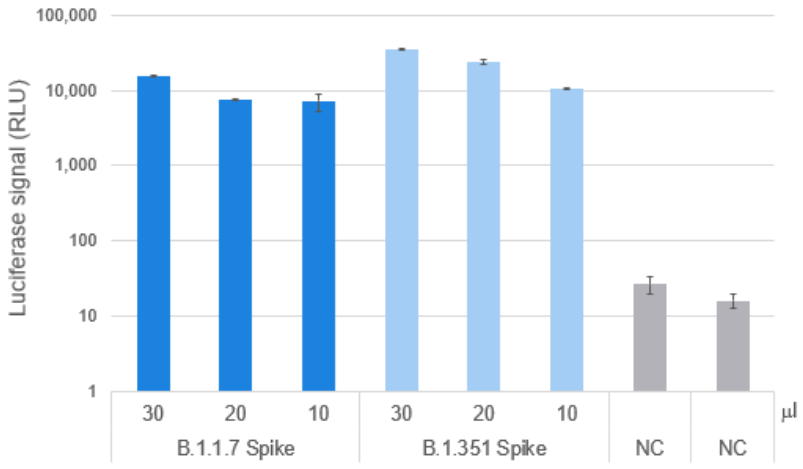

Figure 4. Transduction of ACE2 HEK293T cell line (Cat. No. 631289) using SARS-CoV-2 pseudovirus encoding firefly luciferase. Lenti-X Packaging Single Shots (Envelope-Free) were used to produce truncated SARS-CoV-2 spike B.1.1.7 pseudovirus encoding firefly luciferase (dark-blue bars). Simultaneously, truncated SARS-CoV-2 spike B.1.351 pseudovirus encoding firefly lucifersase was produced using the Lenti-X SARS-CoV-2 Packaging Single Shots (B.1.351 Spike, Truncated) (Cat. No. 631293; light-blue bars). Different volumes of the concentrated supernatant (20X) from each prep (30, 20, or 10 µl) were used to transduce an HEK293T cell line stably expressing the human ACE2 receptor in the presence of 6 µg/ml polybrene in 48-well plates. HEK293T cells lacking the *ACE2* transgene were transduced to determine background luminescence levels (NC; negative control). Luminescence values for each sample were measured 6 days post-transduction.

VII. References

Kozak, M. (1987) At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J Mol Biol*. **196**:947–50

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Customer Service/Ordering	**Technical Support**
tel: 800.662.2566 (toll-free)	tel: 800.662.2566 (toll-free)
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web: www.takarabio.com/service	web: www.takarabio.com/support
e-mail: ordersUS@takarabio.com	e-mail: technical_support@takarabio.com

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