I. Introduction

This protocol is provided for transfection and lentivirus production with **Lenti-X SARS-CoV-2 Packaging Single Shots**, 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 spike protein from SARS-CoV-2. Four different packaging formulations are available that allow for the production of pseudovirus bearing wild-type or D614G variant spike protein in full-length or truncated forms (truncations involve deletion of the final 19 amino acids of the C-terminus).

- Lenti-X SARS-CoV-2 Packaging Single Shots (WT Spike, Full length) (Cat. No. 632672; green caps)
- Lenti-X SARS-CoV-2 Packaging Single Shots (D614G Spike, Full length) (Cat. No. 632673; purple caps)
- Lenti-X SARS-CoV-2 Packaging Single Shots (WT Spike, Truncated) (Cat. No. 632674; blue caps)
- Lenti-X SARS-CoV-2 Packaging Single Shots (D614G Spike, Truncated) (Cat. No. 632675; red caps)

Lenti-X SARS-CoV-2 Packaging Single Shots provide a simple method to transfect 293T cells with lentiviral vector DNA. 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.

Also provided is control packaging mix for production of lentiviral particles lacking an envelope protein, for use as a negative control, and two self-inactivating lentiviral plasmids encoding either ZsGreen1 or firefly luciferase, to be used as reporters (generating fluorescence or luminescence, respectively) for lentiviral transduction.

II. Components

Lenti-X SARS-CoV-2 Packaging Single Shots kits include the following:

- Lenti-X SARS-CoV-2 Packaging Mix (spike variant): 12 tubes
- Lenti-X SARS-CoV-2 Packaging Mix (No-Envelope Control): 6 tubes
- Lenti-X SARS-CoV-2 Vector Set:
    - pLVXS-ZsGreen1-Puro Vector: 1 tube (20 µl, 500 ng/µl)
    - pLVXS-Luciferase-Puro Vector: 1 tube (20 µl, 500 ng/µl)

III. General Considerations

A. Storage and Handling

- Store Lenti-X SARS-CoV-2 Packaging Mixes in the supplied foil pouch with the desiccant sachet at –20°C.
- Return any unused Lenti-X SARS-CoV-2 Packaging Mixes to the supplied foil pouch with the desiccant sachet, and store at –20°C.
- Store plasmids at –20°C.

IV. Transfection and Virus Production Protocol

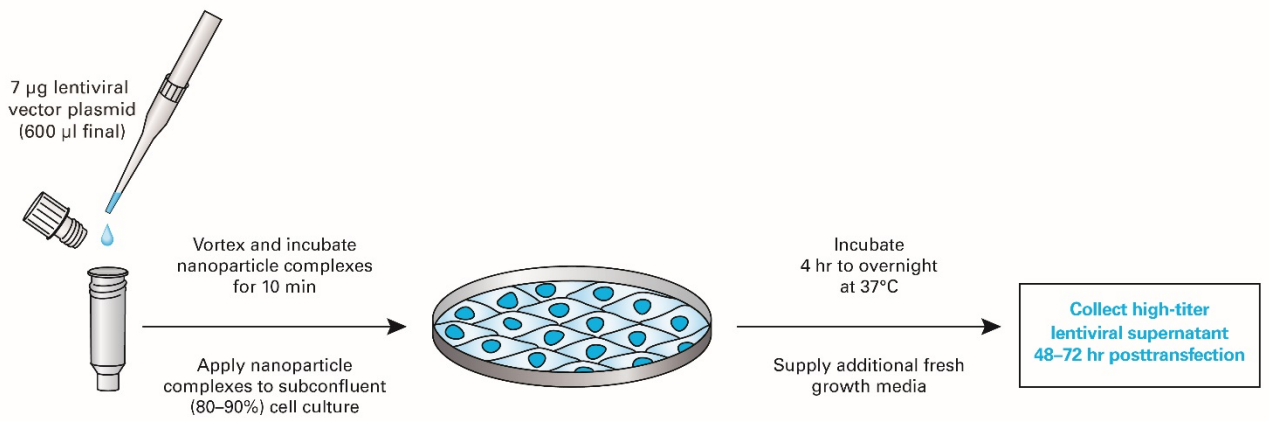


Figure 1. The Lenti-X SARS-CoV-2 Packaging Single Shots protocol.

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. Know and use appropriate safety precautions.

- The following protocol applies to both packaging mixes included in the kit.
- 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.
- One day prior to transfection, plate cells in 8 ml of complete growth medium so that the cells will be 80–90% confluent at the time of transfection.

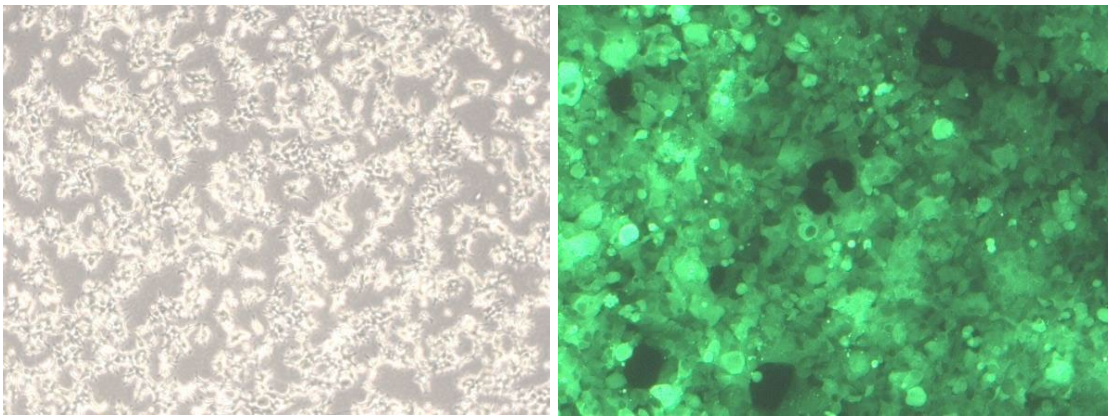


Figure 2. Optimal density of Lenti-X 293T cells at the point of transfection (left panel) and harvest (right panel), shown here using a transfer vector containing ZsGreen1.

NOTE: To achieve the highest titers, it is critical to pay close attention to the transfection. You should be able to achieve transfection efficiencies of greater than 90%.

1. Approximately 24 hr before transfection, seed 4–5 x 10^6 Lenti-X 293T 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. In a sterile microcentrifuge tube, dilute 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. 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 Mix. (Undiluted DNA should not be mixed with the transfection reagent).

3. Add the 600 µl of diluted DNA to a tube of Lenti-X SARS-CoV-2 Packaging Mix, 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 600 µl of nanoparticle complex solution dropwise to the 8 ml of cell culture 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 a 4-hr to overnight 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.

   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), instead of nitrocellulose. Nitrocellulose binds proteins present in the membrane of lentivirus and destroys the virus.

9. 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. Users should avoid multiple freeze/thaw cycles

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

V. Expected Results

Typical results are shown in Figures 3 and 4. High infectious titers (IFU/ml) are observed using Lenti-X SARS-CoV-2 Packaging Single Shots (WT or D614G spike, truncated) according to the protocol described in Section IV.

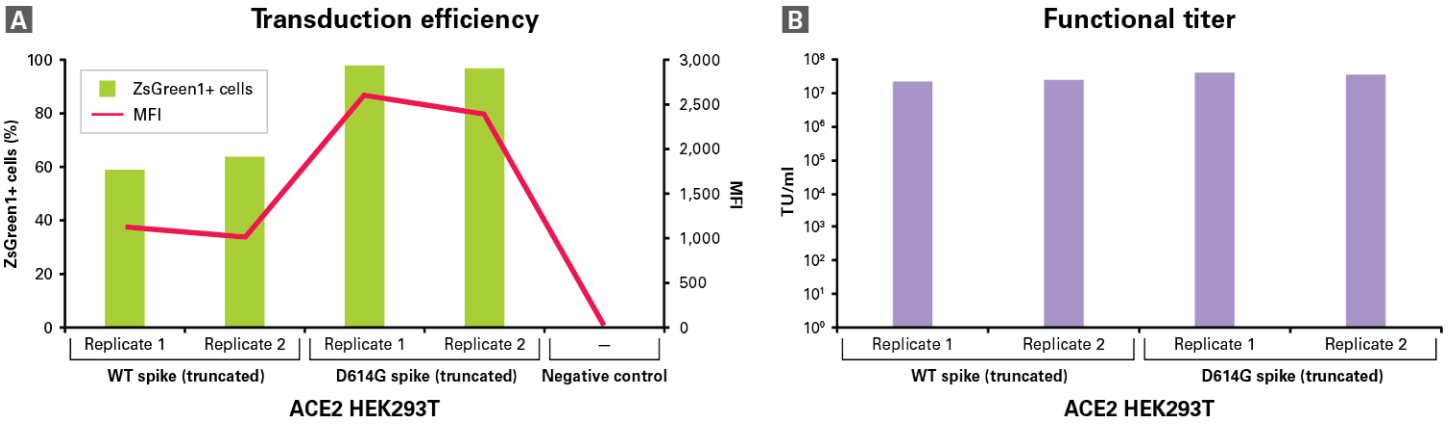


Figure 3. Transduction efficiencies and infectious titers using Lenti-X SARS-CoV-2 Packaging Single Shots. Lenti-X SARS-CoV-2 Packaging Single Shots (WT or D614G spike, truncated) were used to produce pseudovirus encoding the fluorescent protein ZsGreen1 in duplicate experiments. 100 µl of supernatant from each pseudoviral prep was 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 (**Panel A**) and functional titers were also calculated (**Panel B**).

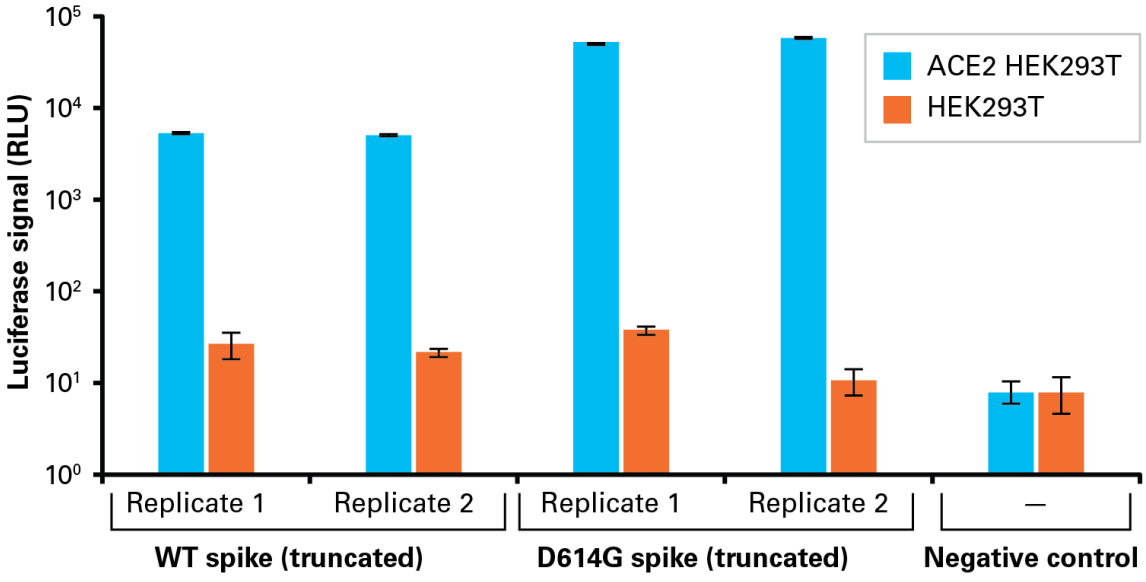
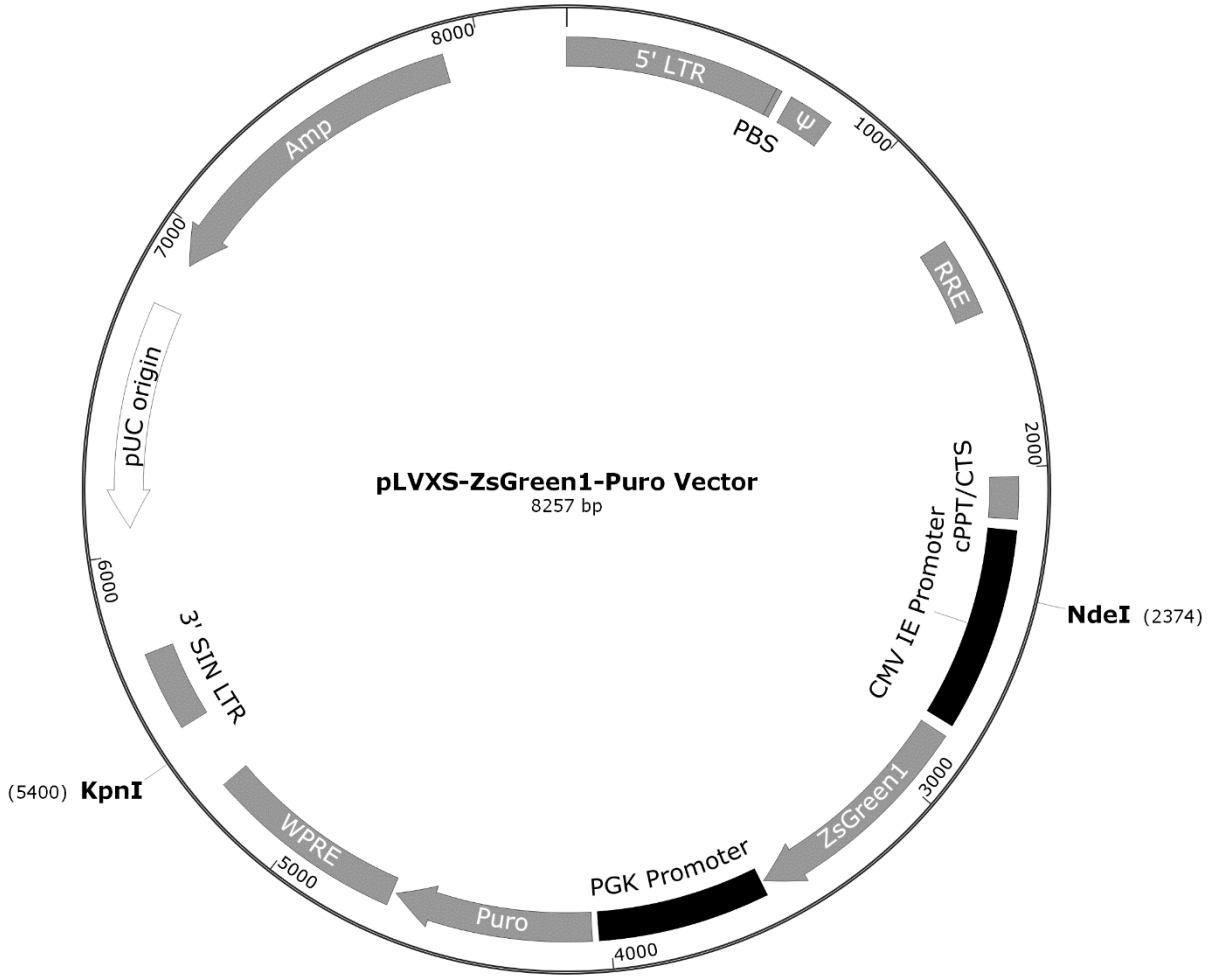


Figure 4. Transduction of ACE2 HEK293T cells using SARS-CoV-2 pseudovirus encoding luciferase. Lenti SARS-CoV-2 Packaging Single Shots (WT or D614G spike, truncated) were used to produce pseudovirus encoding firefly luciferase. 100 µl of supernatant from each prep was used to transduce an HEK293T cell 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. Luminescence values for each sample were measured 6 days post-transduction.

VI. Appendix


pLVXS-ZsGreen1-Puro Vector
8257 bp
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CMV IE Promoter
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PGK Promoter
Puro
WPRE
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pUC origin
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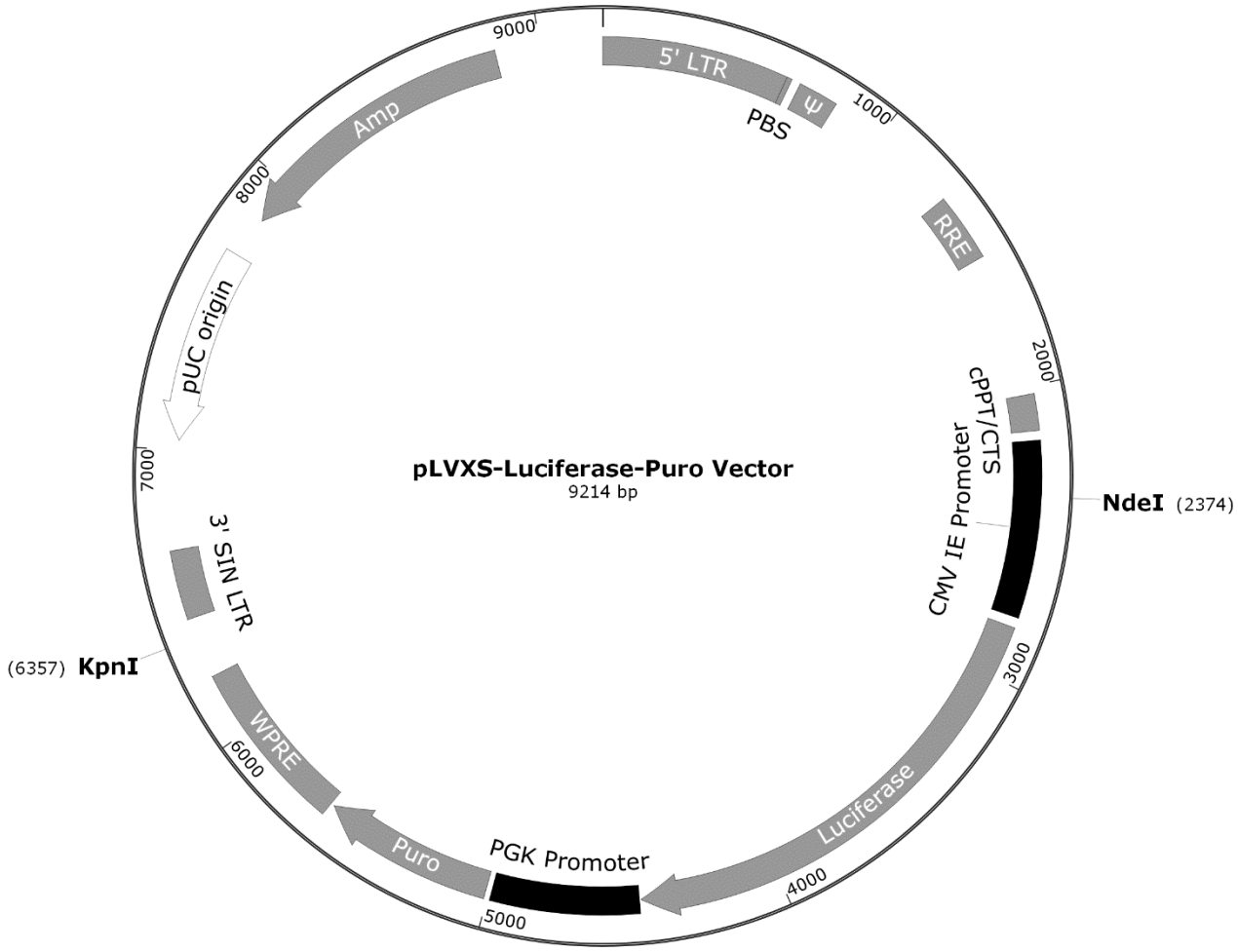

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