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. Six different packaging formulations are available that allow for the production of pseudovirus bearing Wuhan-Hu-1 ("WT"), D614G, or B.1351 spike protein variants 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 (B.1.351 Spike, Full Length) (Cat. No. 631290; blue 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 (B.1.351 Spike, Truncated) (Cat. No. 631291; white 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 Reporter Vector Set: 1 tube each
  - 20 µl pLVXS-ZsGreen1-Puro Vector (500 ng/µl)
  - 20 µl pLVXS-Luciferase-Puro Vector (500 ng/µl)

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. 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 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

The following protocol applies to both packaging mixes included in the kit.

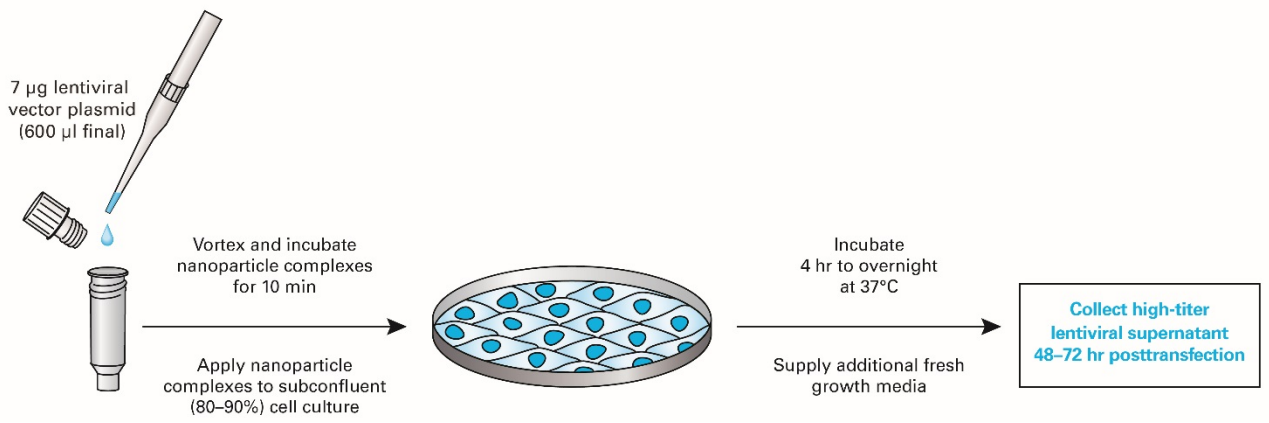

Figure 1. The Lenti-X SARS-CoV-2 Packaging Single Shots protocol.

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.

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 completed.
- 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%.
- 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.

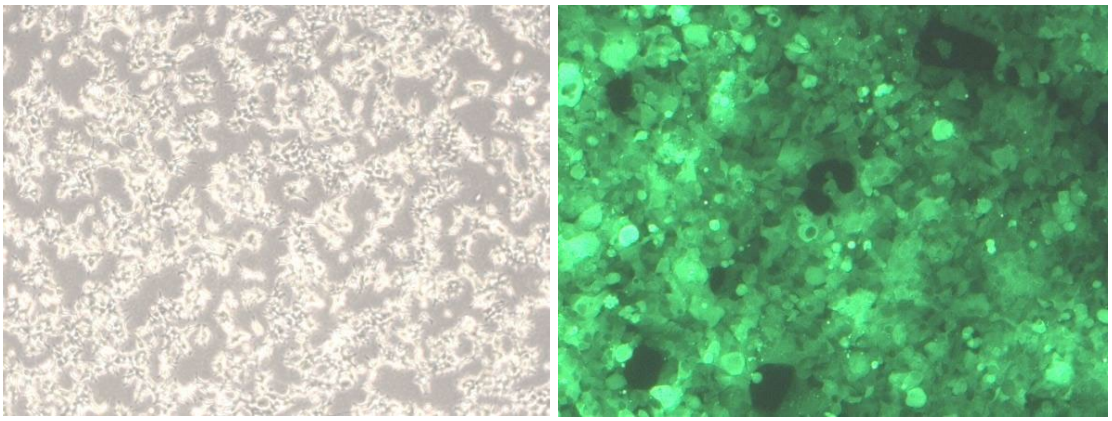

Figure 2. Optimal density of Lenti-X 293T cells at the point of transfection (left panel) and harvest (right panel), shown here using a transfer vector containing ZsGreen1.

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.

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. 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, insert the samples into 1.5-ml microcentrifuge tubes and centrifuge for 2 sec to bring the sample to the bottom of the tube.

5. Transfer the entire 600 µl of nanoparticle complex solution dropwise to the 8 ml of cell culture prepared in Step 1. It is normal for the medium to change color slightly upon addition of the nanoparticle complex solution. Gently rock the plate back and forth to mix.

6. Incubate the cells at 37°C, 5% CO_2 for a minimum of 4 hrs.

   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 cellulose acetate or polysulfone (low protein binding) filter to remove cellular debris.

   NOTE: The filter should NOT be 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.

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.

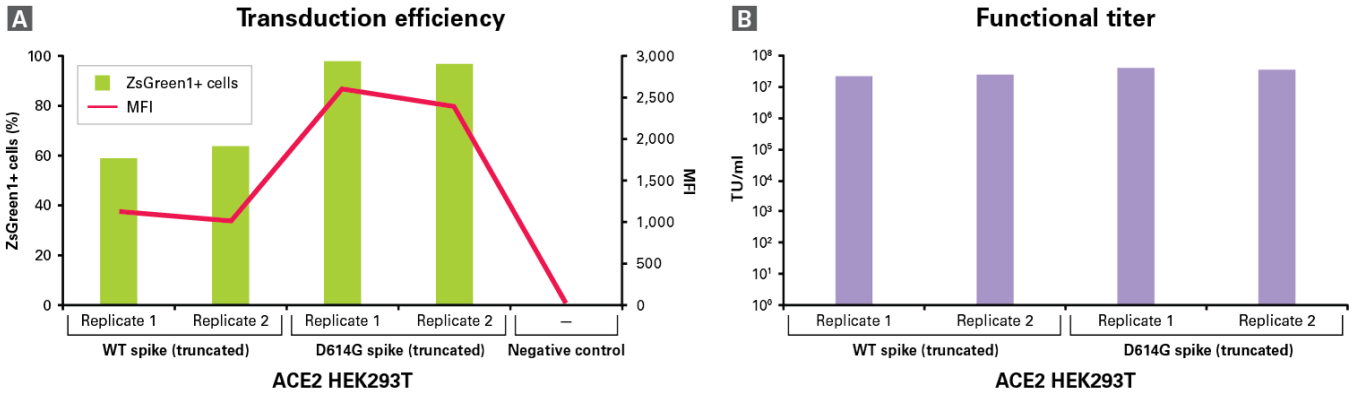

Figure 3. Transduction efficiencies and infectious titers using Lenti-X SARS-CoV-2 Packaging Single Shots. Lenti-X SARS-CoV-2 Packaging Single Shots (WT or D614G spike, truncated) were used to produce pseudovirus encoding the fluorescent protein ZsGreen1 in duplicate experiments. 100 µl of supernatant 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).

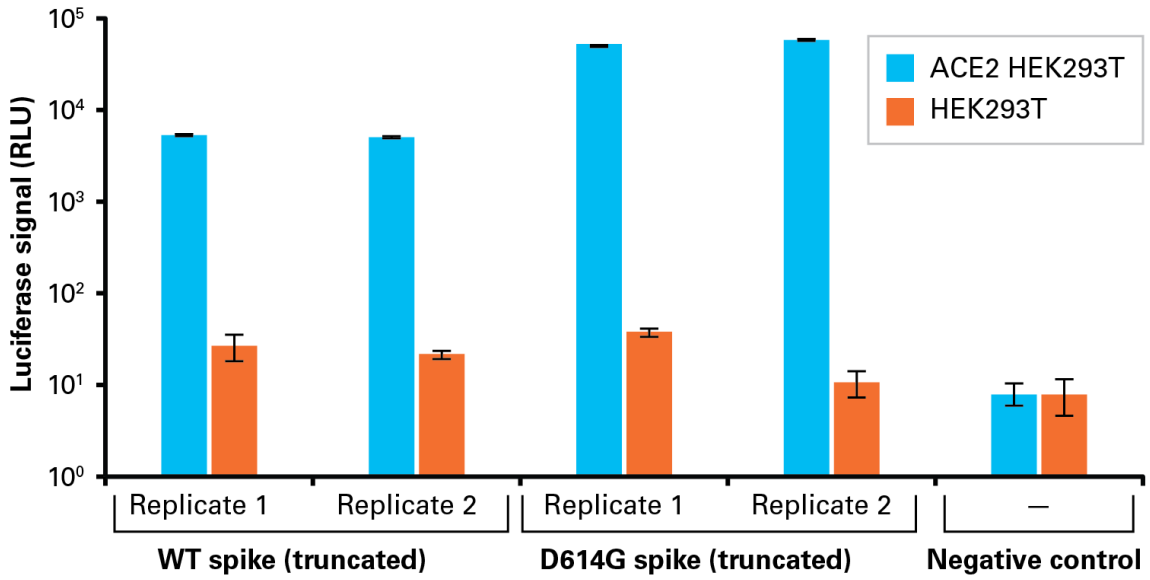

Figure 4. Transduction of ACE2 HEK293T cells using SARS-CoV-2 pseudovirus encoding luciferase. Lenti SARS-CoV-2 Packaging Single Shots (WT or D614G spike, truncated) were used to produce pseudovirus encoding firefly luciferase. 100 µl of supernatant from each prep was used to transduce an HEK293T cell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

For specific applications (e.g., neutralization assays) the pseudovirus needs to be concentrated; consult Table 1 for guidance regarding the fold concentration(s) required for your experiments, as they will vary depending on which pseudovirus variants are being used.

Table 1. *N*-fold concentrations of pseudovirus needed to transduce at least 50% of target cells (using ZsGreen1 as reporter)

Variant		Fold concentration	Volume*
WT	Full length	20x	100 µl
	Truncated	No concentration	100 µl
D614G	Full length	20x	100 µl
	Truncated	No concentration	100 µl
B.1.351	Full length	50x	100 µl
	Truncated	20x	20 µl

*Volume of pseudovirus used in each well of a 48-well plate (total volume = 200 µl)

VI. Appendix

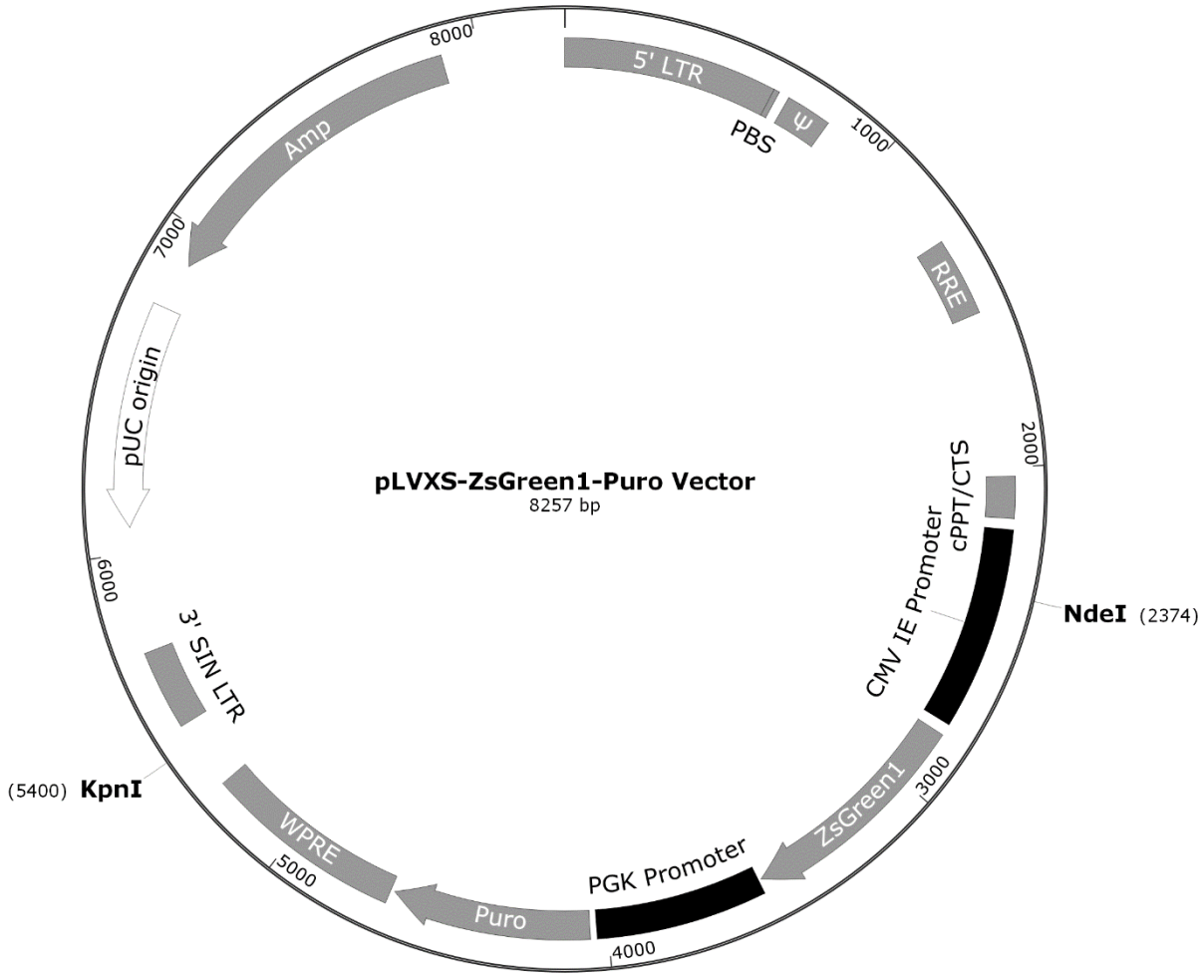

Figure 5. pLVXS-ZsGreen1-Puro vector map.

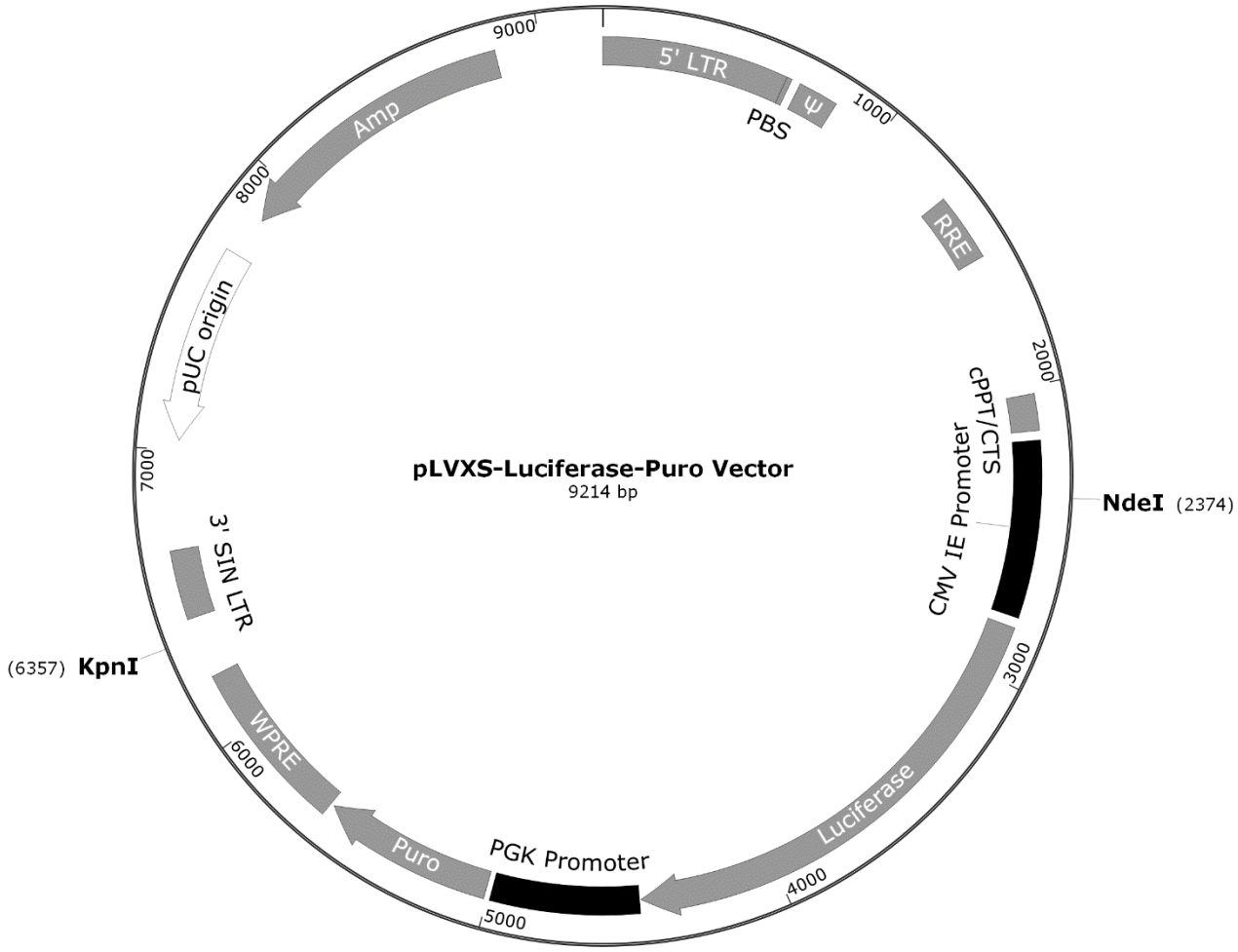

Figure 6. pLVXS-Luciferase-Puro vector map.

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: 800.662.2566 (toll-free)	tel: 800.662.2566 (toll-free)
fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
web: takarabio.com/service	web: takarabio.com/support
e-mail: ordersUS@takarabio.com	e-mail: technical_support@takarabio.com

Notice to Purchaser

Our products are to be used for **Research Use Only**. They may not be used for any other purpose, including, but not limited to, use in humans, therapeutic or diagnostic use, or commercial use of any kind. Our products 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 our prior written approval.

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product's web page at takarabio.com. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements

This document has been reviewed and approved by the Quality Department.