I. Introduction

This protocol is provided for transfection and lentivirus production with **Lenti-X Packaging Single Shots**, pre-aliquoted, lyophilized, single tubes of Xfect™ Transfection Reagent premixed with an optimized formulation of Lenti-X lentiviral packaging plasmids. Three formulations are available: Lenti-X Packaging Single Shots (VSV-G) (Cat. Nos. 631275 & 631276) provide the highest titers and widest tropism; Lenti-X Packaging Single Shots (Ecotropic) (Cat. No. 631278) restrict tropism to cells of mouse and rat origin; and Lenti-X Packaging Single Shots (Integrase Deficient) (Cat. No. 631277) produce non-integrating lentivirus stocks.

Lenti-X 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 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.

II. General Considerations

A. Storage and Handling

- Store Lenti-X Packaging Single Shots at –20°C in the supplied foil pouch containing the desiccant sachet.
- Make sure to return any unused Lenti-X Packaging Single Shots to the supplied foil pouch containing the desiccant sachet, and store at –20°C.

B. Mock Transfections

Use a plasmid that does not contain your gene of interest. You should include a source of nucleic acids to assemble with the Xfect polymer.

III. Transfection and Virus Production Protocol

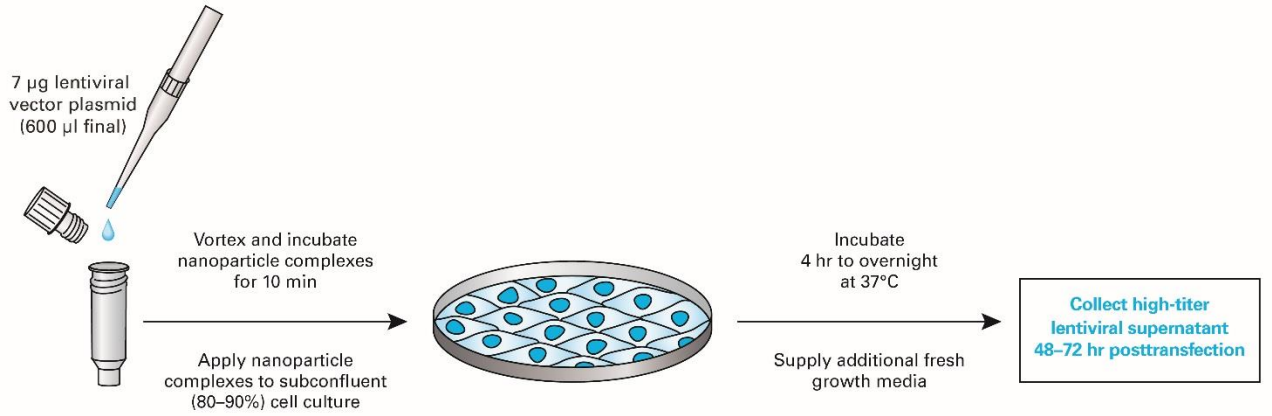

Figure 1. The Lenti-X Packaging Single Shots protocol.

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.

- Transfections should be performed using **10-cm tissue culture dishes**. Tetracycline-free FBS should be used in the transfection medium and in the medium used to collect the virus.
- One day prior to the transfection, plate cells in 8 ml of complete growth medium so that the cells will be 80–90% confluent at the time of transfection.

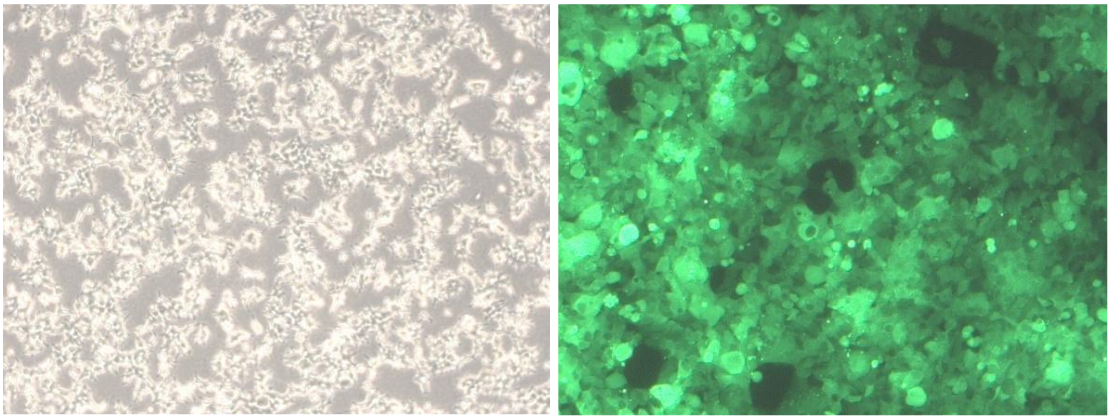

Figure 2. Optimal density of Lenti-X 293T cells at the point of transfection (left panel) and harvest (right panel), shown here using a transfer vector containing ZsGreen1.

NOTE: To achieve the highest titers, it is critical to pay close attention to the transfection. You may want to perform a cotransfection with a lentiviral vector that contains a fluorescent protein. 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 microfuge tube, dilute 7.0 µg of your lentiviral vector plasmid DNA 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 Lenti-X Packaging Single Shot. Do not add water and DNA separately (since undiluted DNA should not be mixed with Xfect Transfection Reagent).
3. Add the 600 µl of diluted DNA to a tube of Lenti-X Packaging Single Shots, replace the cap, and vortex well at a high speed for 20 sec. The pellet should dissolve completely.

   NOTE: In some cases, some 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 microfuge tubes for a brief centrifugation.
5. Add the entire 600 µl of nanoparticle complex solution dropwise to the 8 ml of cell culture prepared in Step 1. Rock the plate gently back and forth to mix.

   NOTE: It is normal for the medium to change color slightly upon addition of nanoparticle complex solution.
6. Incubate the cells at 37°C supplied with 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 4 hr to overnight, 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 lentiviral 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 lentivirus.

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.

NOTE: Titers can drop as much as 2–4 fold with each freeze-thaw cycle

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

IV. Expected Results

Typical results are shown in Figures 3, 4, and 5. High infectious titer (IFU/ml) are observed using Lenti-X Packaging Single Shots according to the protocol described in Section III (Figures 3 & 4). Integrase-deficient lentivirus can be used to obtain transient expression in dividing cells (Figure 5).

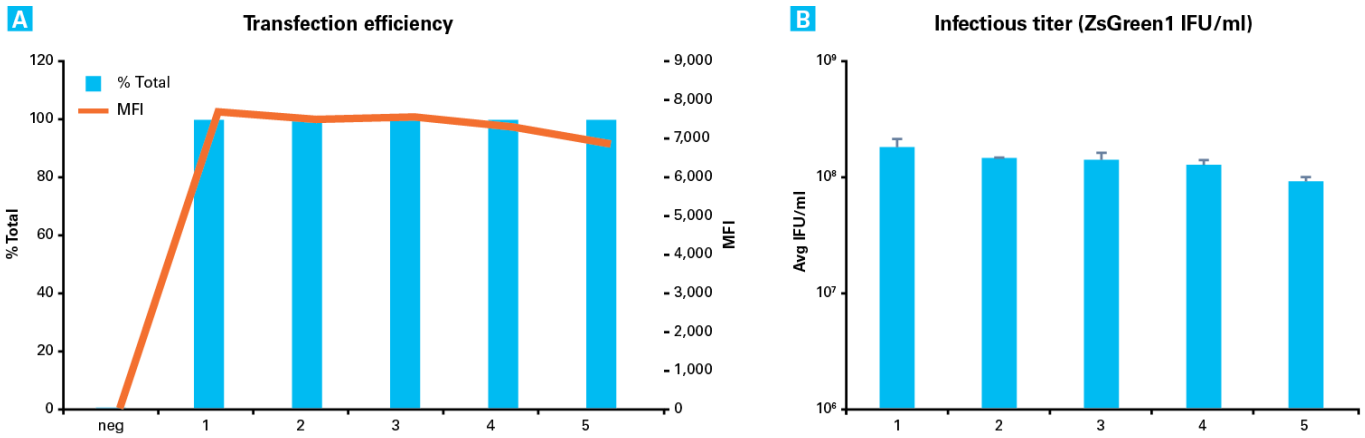

Figure 3. Transfection efficiency and infectious titer using Lenti-X Packaging Single Shots (VSV-G). Lenti-X Packaging Single Shots (VSV-G) were used to produce lentivirus containing the fluorescent protein ZsGreen1 in five experiments. The transfection efficiencies for each sample **(Panel A)** were measured by flow cytometry (left panel), and were shown to be ~100% for all five experiments, as determined by measuring the mean fluorescence intensity (MFI). Clarified supernatant was then serially diluted and titrated using HT1080 cells in the presence of 6 µg/ml polybrene. Transduced cells were analyzed by flow cytometry 72 hr post-transduction (right panel). Titers **(Panel B)** were calculated using culture volumes that resulted in cell populations transduced at <10% efficiency to ensure one copy per cell. The resulting titers for all five experiments were ~1 x 10^8 IFU/ml.

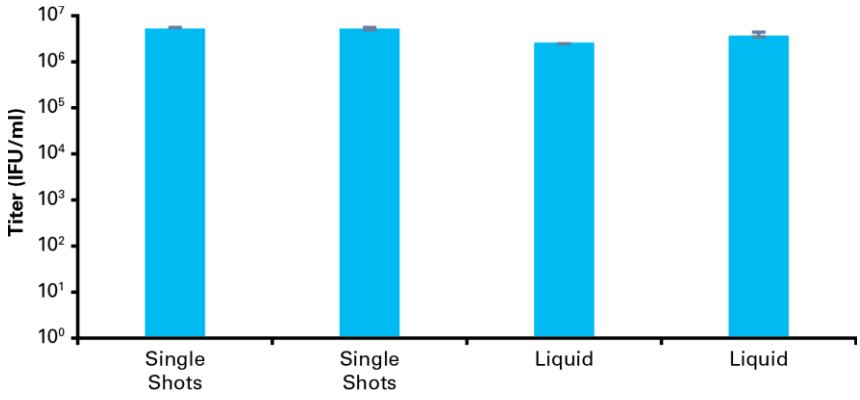

Figure 4. High ecotropic titers obtained using Lenti-X Packaging Single Shots (Ecotropic). Lenti-X Packaging Single Shots (Ecotropic) were used to produce ecotropic lentivirus containing ZsGreen1 (Single Shots). A traditional liquid format of the packaging mix (Liquid) was used in parallel as a control. Clarified supernatant was then serially diluted and titrated using NIH3T3 cells in the presence of 6 µg/ml polybrene. Transduced cells were analyzed by flow cytometry 72 hr post-transduction. Titers were calculated using culture volumes which resulted in cell populations transduced at <10% efficiency to ensure one copy per cell. The resulting titers were similar for both formats (~1 x 10^6 IFU/ml).

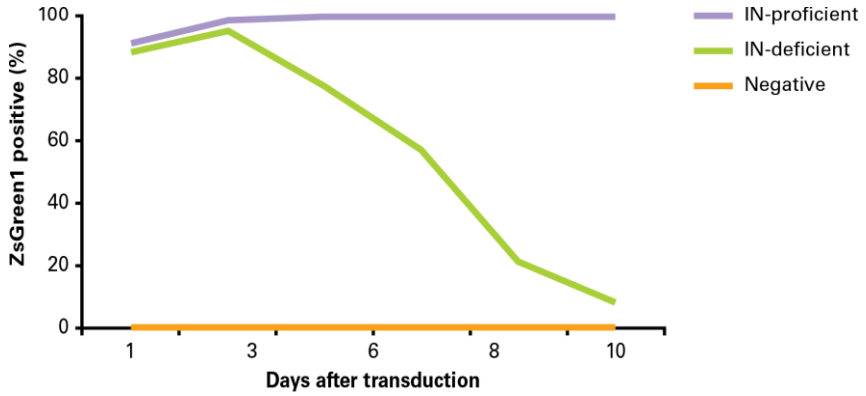

Figure 5. Transient expression in dividing cells using integrase-deficient lentivirus. Both integrase-deficient (IN-deficient) and -proficient (IN-proficient) lentivirus containing ZsGreen1 were produced. Viral dosage was normalized to a MOI of 20 and used to infect HT1080 cells. The percentage of ZsGreen1 positive cells was determined by flow cytometry on the days indicated. Compared to the integration-proficient lentivirus, which maintained expression over the course of the experiment in these dividing cells, the integrase-deficient lentivirus exhibited an expression pattern typical of transient episomes, with expression decreasing to near background levels after 10 days.

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