

Generating Lentiviral Particles in the Corning® HYPERStack® Cell Culture Vessel

Application Note

CORNING

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Introduction

Lentiviruses are enveloped retroviruses that are increasingly utilized biological tools in cell therapy applications. These types of retroviruses are unique for their ability to: (a) deliver a stable gene(s) into most non-dividing cells, (b) integrate their genetic cargo into a target chromosome, and (c) increase their versatility by modifying various molecular properties (e.g., modification of glycoproteins around the envelope).¹ To allow researchers and manufacturers the opportunity to produce even higher yields in the same spatial footprint as a stacked vessel, Corning offers the HYPERStack Cell Culture Vessel. The HYPERStack vessel features Corning's HYPER (High Yield PERFORMANCE) technology, which consists of a gas permeable film that serves as the cell attachment surface and eliminates the headspace within a vessel (Fig. 1). This design allows an increase in the number of layers and corresponding cell growth surface area, as compared to traditional, rigid, single-layer culture vessels.

Standard methodologies utilize rigid cell culture vessels to culture cells (e.g., HEK-293LTV) that are transfected with DNA encoding genes (e.g., envelope and viral packaging) needed for lentivirus propagation. The focus of this study was to determine the efficacy of generating lentivirus using the unique Corning HYPER technology. The results described here demonstrate that the experimental approach to generate lentivirus in the HYPERStack Vessel led to equivalent titers and higher yields compared to those generated using standard, rigid, stacked vessels.

Methods and Materials

Cell Culture

HEK-293LTV cells (Cell BioLabs, Cat. No. LTV-100) were maintained in DMEM with sodium pyruvate (Corning cellgro,® Cat. No. 10-013-CM), 10 % FBS (Corning cellgro, Cat. No. 35-010-CV), and 1X MEM Nonessential Amino Acids (Corning cellgro, Cat. No. 25-025-Cl).

DNA Preparation

GC10 competent cells (Sigma-Aldrich® Cat. No. G2544) were heat shocked with the DNA obtained from the ViraSafe™ Lentiviral Bicistronic Expression System (GFP), Pantropic (Cell BioLabs, Cat. No. VPK-218-PAN), and pLenti-Green Fluorescent Protein (GFP) Lentiviral Control Vector (Cell BioLabs, Cat. No. LTV-400). The cells were cultured in LB-Broth (Corning cellgro, Cat. No.46-050-CM) in a 1L Erlenmeyer flask (Corning, Cat. No. 431403) at 37°C for 16 hours at 250 rpm. Plasmid DNA was purified using the AxyPrep™ Plasmid Maxiprep kit (Axygen,® Cat. No. AP-MX-P-25) and quantified with the EnVision® multimode plate reader (Perkin Elmer).

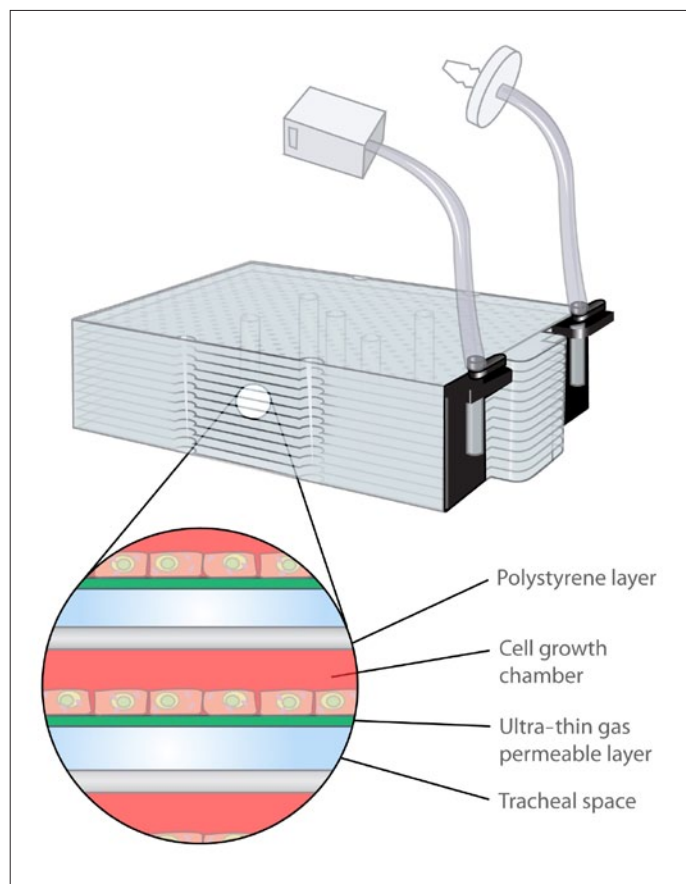


Figure 1. Corning HYPER technology eliminates the headspace found in traditional cell culture technology.

Preparation of the DNA:CaPO₄ Complex

On the day of transfection, a master mix of DNA containing CaPO₄ was prepared based on a total growth area of 7,400 cm² (6,000 cm² [Corning® HYPERStack®-12 Vessel] + 1,272 cm² [comparison 2-layer Corning CellSTACK® Chamber] + 128 cm² [extra]). To prepare the mix, 48 mL of freshly made 2X HBS buffer (50 mM HEPES, 250 mM NaCl, 1.5 mM Na₂HPO₄, pH 7.07) were added to a 250 mL storage bottle (Corning, Cat. No. 430281) labeled Tube A. To a 50 mL conical tube (Tube B), the following DNA components were added at a combined ratio of 0.55 µg/cm²:

- ▶ pLenti Green Fluorescent Protein (GFP) Control Vector (1.8 mg)
- ▶ pREV-RSV (packaging vector) (768 µg)
- ▶ pCgpV (packaging vector) (768 µg)
- ▶ pCMV-VSVG (envelope vector) (768 µg)

Cell culture grade water (Corning cellgro®, Cat. No. 25-055-CV) and 1 M CaCl₂ (Sigma-Aldrich, Cat. No. 21115) were added to Tube B to yield a final volume of 48 mL and incubated at room temperature for 5 minutes. Following the 5 minute incubation, the contents of Tube B were added drop by drop to Tube A at a rate of approximately 5 mL/min and incubated for 20 minutes at room temperature. After approximately one-third of the CaCl₂-DNA solution was added, the mixture became cloudy. Each experiment was performed three independent times. *Please note:* if white precipitates form while mixing, then the solution in Tube B was added to Tube A either too quickly or with too much agitation. The formation of precipitates greatly reduces the efficiency of the transfection.

Transfection of HEK-293LTV Cells

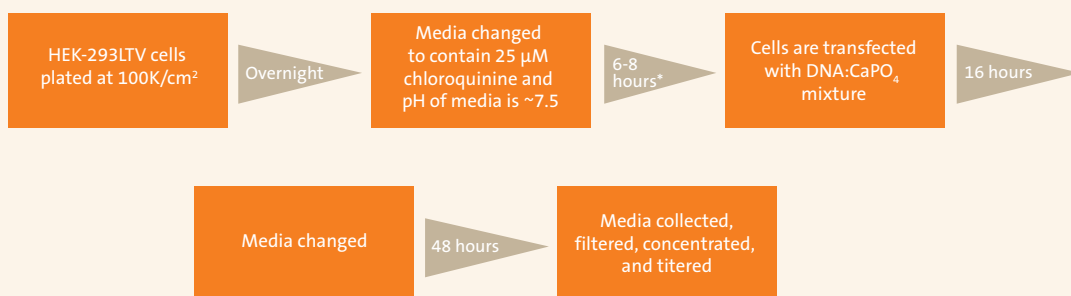
Cells were seeded onto a 2-layer Corning CellSTACK® with CellBIND® Surface (Corning, Cat. No. 3310) and a Corning HYPERStack-12 Cell Culture Vessel (Corning, Cat. No. 10012) at 100,000 cells/cm² (0.217 mL/cm²) and incubated overnight at 37°C (5% CO₂, 98% relative humidity). The following day, the media was removed 8 hours before the transfection and replaced with fresh media

(0.217 mL/cm²) containing 25 µM chloroquine (Sigma, Cat. No. C6628). *Please note:* For optimal transfection efficiency, the pH of the medium at the time of the transfection was between 7.5 and 7.6. For larger vessels (e.g., HYPERStack-36 vessel), the medium containing chloroquine may need to incubate longer (e.g., >8h) for the medium to equilibrate with the gases and temperature. Shorter incubation times (less than 6 hours) typically lead to a decrease in transfection efficiency.

To transfect the 293LTV cells, the medium containing chloroquine was removed from each vessel and combined into a 2L roller bottle (Corning, Cat. No. 431644). Ninety-five mL of the medium was removed and 95 mL of the DNA:CaPO₄ solution prepared above was added drop by drop at a rate of approximately 7 mL/min. The medium containing the DNA:CaPO₄ solution was added back to each vessel and incubated for 16 hours at 37°C (3% CO₂, 98% relative humidity). Following the incubation, the transfection medium was removed. Fresh medium was added to the vessels, then collected 48 hours later. GFP expression was monitored throughout the course of the experiment using the AMG EVOS® FI microscope.

Lentivirus Harvest

Lentiviruses undergo the lysogenic cycle, which results in the release of virus into the medium without cell lysis. Therefore, the medium was collected approximately 65 hours post transfection, and the cells were discarded. To remove any cell debris and multi-vesicular bodies (MVB), the medium was filtered through a 0.45 µM cellulose acetate filter (Corning, Cat. No. 430514 or 430516) and collected into an autoclaved glass bottle (Corning, Cat. No. 1395-1L or 1395-2L). To concentrate the virus, 35 mL of the virus solution obtained from each vessel was transferred to a Spin-X® filter (MWCO 100,000) (Corning, Cat. No. 431491) and centrifuged at 3000 x g (4°C) until a final volume of ~300 µL was collected (two spin steps were performed to concentrate 35 mL). The lentiviruses encoding GFP (concentrated and non-concentrated) were then aliquoted and stored at -80°C. Figure 2 shows an overview of the protocol outlined above.



Media: DMEM with sodium pyruvate, 10% FBS with 1X NEAA.

*Time of incubation may need to be increased for larger vessels (e.g., HYPERStack-36 Vessel) to equilibrate the larger volumes of media.

Figure 2. Protocol for CaPO₄ transfections using the Corning HYPERStack-12 Cell Culture Vessel.

Lentivirus Titer

The Lenti-X™ qRT-PCR Titration Kit was purchased from Clontech (Cat. No. 631235), and the assay was performed according to manufacturer's instructions using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The copies/mL were calculated based on the C_q, quantification cycle, values determined by the software.

Transduction of MDBK Cells

To verify that the virus was functional, Mardin-Darby Bovine Kidney cells (MDBK (NBL1), ATCC,® Cat. No.CCL22) were transduced. Previous titration results from FACS suggested that approximately 1,000 copies/mL is equivalent to 1 TU/mL (transduction unit/mL). Cells were seeded onto a 24 well plate (Corning, Cat. No. 3527) at 5,000 cells/cm² and incubated overnight at 37°C (5% CO₂, 98% relative humidity). The following day, the concentrated lentiviruses encoding GFP were added to the cells at a multiplicity of infection (MOI) of 10. Polybrene® (Sigma-Aldrich,® Cat. No. H9268), at a final concentration of 10 µg/mL, was also added to the medium to improve lentivirus transduction efficiency. The amount of each virus (mL) added to each well was calculated using the following formula:

$$([\text{Cells}/\text{cm}^2] * [\text{cm}^2 \text{ of well}] * (\text{MOI } 10 [\text{TU}/\text{cells}]) / (\text{TU}/\text{mL})$$

The cells were harvested 72 hours later, and the GFP expression was analyzed by flow cytometry.

Flow Cytometry

To assess GFP expression, MDBK cells were transduced with lentivirus encoding GFP and harvested 72 hours later, centrifuged to remove trypsin/media, and then resuspended in 250 µL of PBS (Corning® cellgro®, Cat. No. 21-040-CM). Cell suspensions were analyzed using the MACSQuant® Analyzer instrument (Miltenyi Biotec).

Results

Cell Morphology and GFP Expression

To assess lentiviral production in a 2-layer stacked vessel compared to a Corning HYPERStack®-12 Vessel, HEK-293LTV cells were transfected with DNA obtained from the ViraSafe™ Lentiviral Bicistronic Expression System (GFP). GFP expression and cell morphology were monitored throughout the course of the experiment. The medium was collected approximately 65 hours post transfection. Similar cell morphology and GFP expression were observed in samples from both vessels (Fig. 3) throughout the course of the experiment.

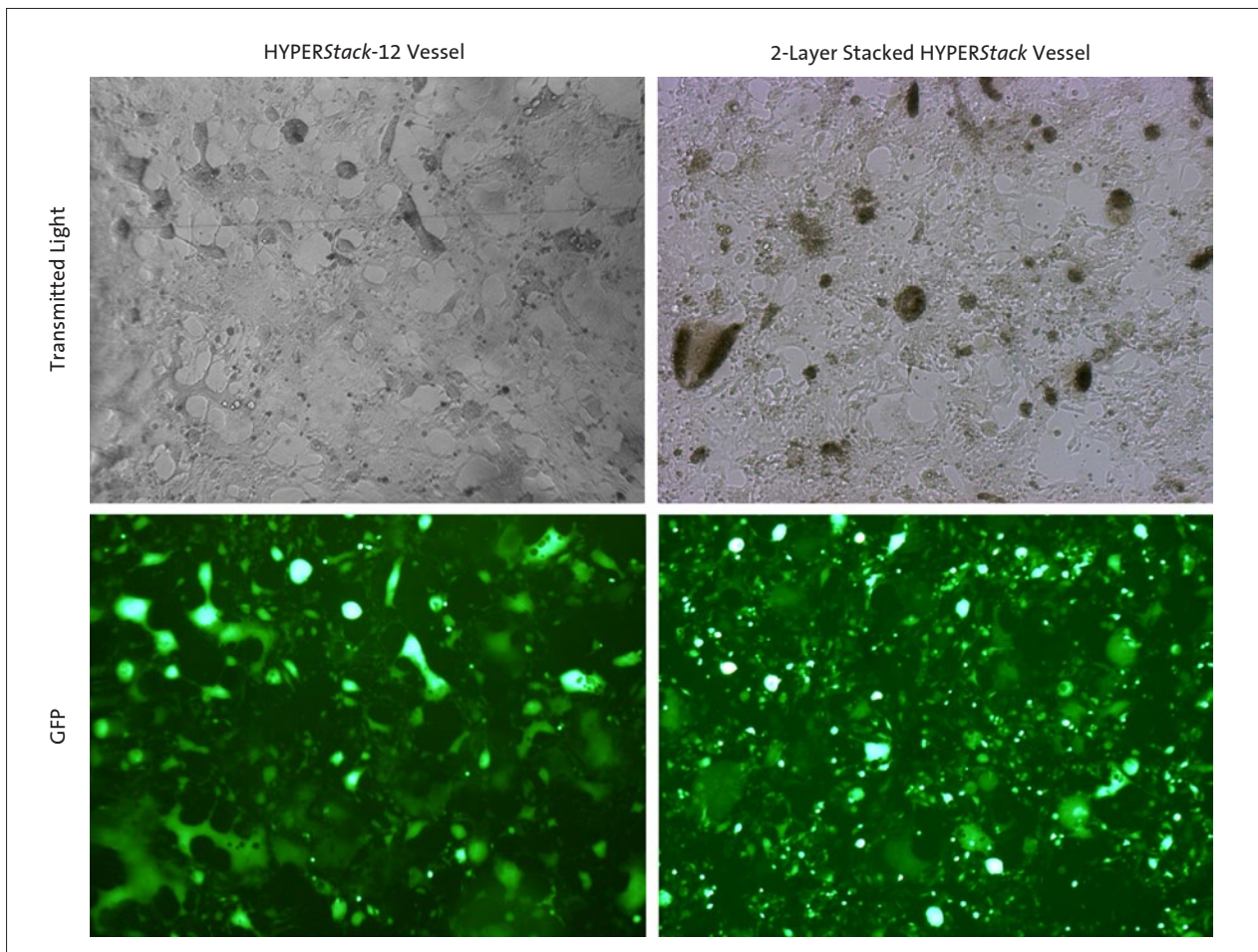


Figure 3. Analysis of GFP expression in the Corning HYPERStack Vessel. Representative images from the same experiment demonstrating similar GFP expression in the Corning HYPERStack Vessel and 2-layer stacked cell culture vessel. These trends were observed with all experiments. Images obtained using an Olympus® IMT-2 inverted fluorescence microscope. Magnification, 10X.

Lentiviral Production

Once collected and filtered, the lentivirus encoding GFP from each vessel was then titered using the Lenti-X™ qRT-PCR Titration Kit to determine copies/mL. Lentivirus obtained from the Corning® HYPERStack® Vessel yielded similar copies/mL (Fig. 4A), similar copies/cm² (Fig. 4B), and approximately 5 times more copies (Fig. 4C) compared to lentivirus obtained from the 2-layer stacked vessel. These results indicate that lentivirus particles may be generated in the HYPERStack Vessel with similar titers, but larger yields are obtained when compared to a standard stacked vessel with a similar spatial footprint.

GFP Expression in MDBK Cells

To verify that virus obtained from the HYPERStack Vessel was as functional as virus obtained from the 2-layer rigid stacked vessel, MDBK cells were transduced with lentivirus encoding GFP. Each cell type was transduced with virus obtained from either vessel at a MOI of 10. After 72 hours, the cells were collected and analyzed via flow cytometry. The average GFP fluorescence in each cell line transduced with lentivirus was greater than 80.0% (Fig. 5, two independent experiments). Taken together, these results indicate that the HYPERStack Vessel produces similar infectious lentiviral particles per cm² compared to a traditional stacked vessel.

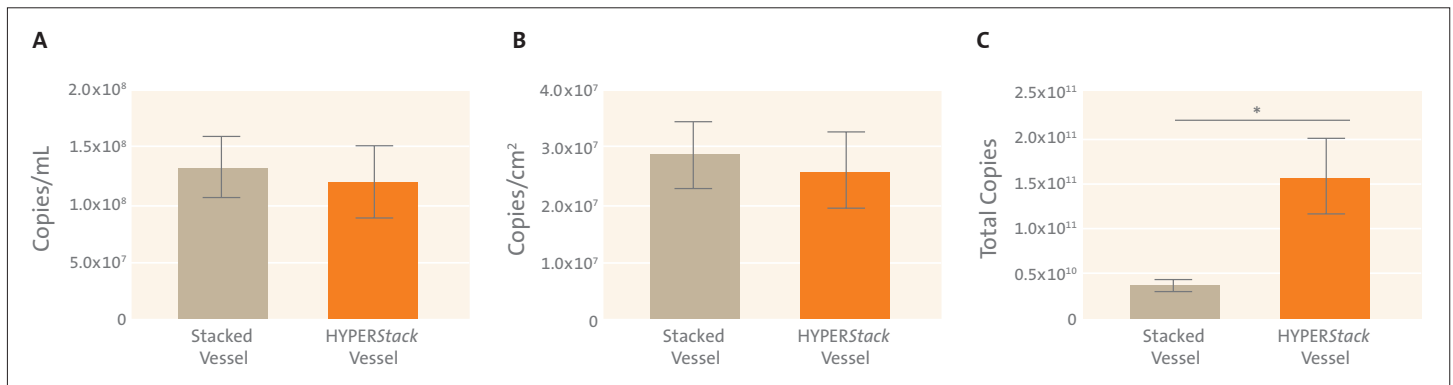


Figure 4. The Corning HYPERStack Vessel supports comparable viral production with a higher yield of total virus compared to a 2-layer stacked vessel. (A) Titers obtained from the Lenti-X™ qRT-PCR Titration Kit. (B) When normalized on a per cm² basis, the HYPERStack Vessel yields similar amount of lentiviral particles. (C) The HYPERStack Vessel generates a significantly higher amount of total infectious lentiviral particles. Paired t-test, *p < 0.05, N=3.

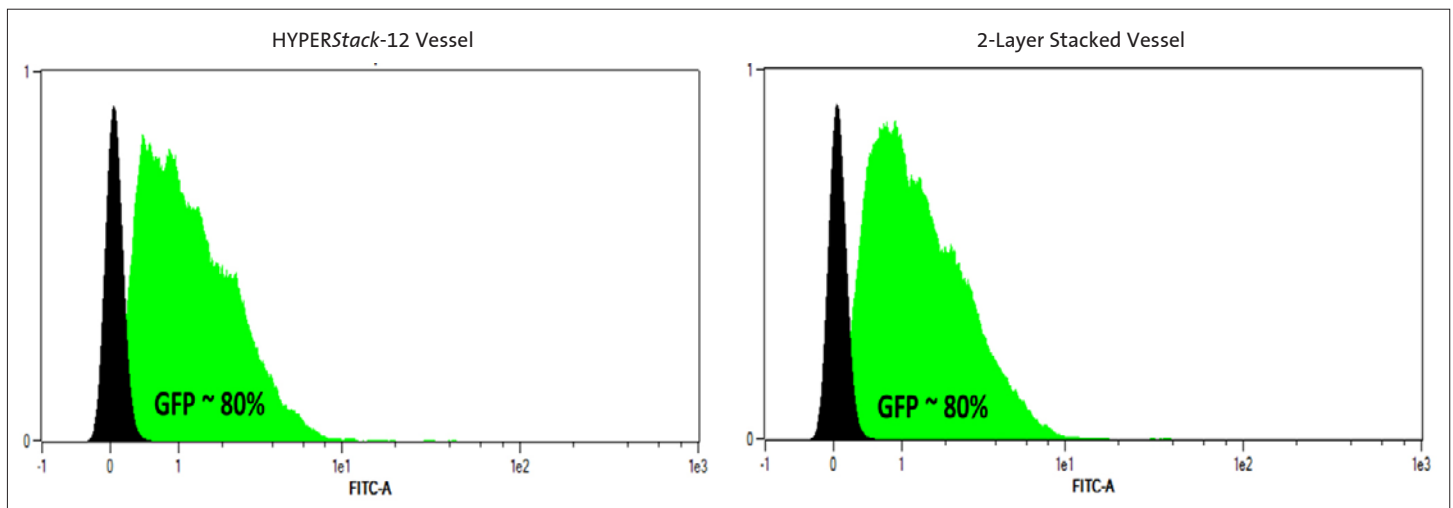


Figure 5. MDBK cells transduced with lentivirus exhibit comparable levels of GFP expression. Representative flow cytometry data show the expression of GFP (green) compared to a negative control of non-transduced cells (black). After two independent experiments, the GFP expression in MDBK cells was greater than 80%, regardless of which vessel the virus was generated in.

Summary

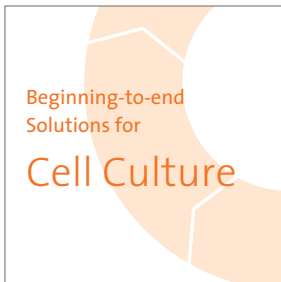
This study demonstrates the utility of the HYPER technology as an alternative to traditional cell culture flasks for scale up production of lentivirus.

- ▶ Lentiviral particles can be amplified in the Corning® HYPERStack® Cell Culture Vessel at similar titers compared to traditional tissue culture vessels, while allowing for greater virus production in a smaller footprint.
- ▶ Lentiviral particles generated on the HYPER technology platforms also exhibit similar levels of infection compared to traditional culture vessels.

- ▶ The HYPER technology platform of products includes larger vessels with larger surface areas that provide researchers with the ability to further increase lentivirus production.

References

1. Giry-Laterrière, M., Verhoeyen, E., and Salmon, P. Viral Vectors for Gene Therapy: Methods and Protocols. *Meth. Molec. Biol.* 2011. 737:183-209.



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