Generating Lentiviral Particles in the Corning® HYPER*Flask*® Cell Culture Vessel

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A brief technical report from the Corning Applications Group

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Introduction

Lentiviruses are enveloped retroviruses that are becoming major 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 T-175 flask, Corning offers the HYPER*Flask* vessel. The HYPER*Flask* Cell Culture Vessel features Corning's HYPER (**H**igh **Y**ield **PER**formance) technology which consists of a gas permeable film that serves as the attachment surface and eliminates the headspace within a vessel. This allows for an increase in the number of layers and corresponding cell growth surface area, as compared to traditional rigid single layer culture vessels. The HYPER*Flask* Vessel cell growth surface area is 1,720 cm^2 across 10 layers, and has the same spatial footprint of a T175 vessel.

Lentiviral particles are produced when cells (e.g., HEK-293LTV) are transfected with DNA encoding various genes (e.g., genes encoding viral packaging and envelope proteins) needed for lentivirus propagation. The focus of this study was to determine the efficacy of generating virus using the unique Corning HYPER technology compared to traditional T-flask vessels. Utilizing the HYPER*Flask* vessel, researchers can generate higher yields in a smaller spatial footprint that saves time and space. The results described here demonstrate that the experimental approach to generate lentivirus in the HYPER*Flask* vessel led to equivalent titers and higher yields compared to that generated using a standard T-175 flask.

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-GFP Lentiviral Control Vector (Cell BioLabs, Cat. No. LTV-400), and 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 h 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).

Preparation of the DNA:$CaPO_4$ Complex

On the day of transfection, a master mix of DNA containing $CaPO_4$ was prepared based on a total growth area of 1945 cm^2 (1720 cm^2 [HYPER*Flask*] + 175 cm^2 [comparison T-175] + 50 cm^2 [extra]). To prepare the mix, 19 mL of freshly made 2X HBS buffer (50 mM HEPES, 250 mM NaCl, 1.5 mM $Na2HPO_4$, pH 7.07) was added to a 50 mL conical tube (Corning, Cat. No. 430921) labeled Tube A. To a separate 50 mL conical tube (Tube B), the following DNA components were added at a combined ratio of 0.55 µg/cm^2:

- pLenti Green Fluorescent Protein (GFP) Control Vector (465 µg)
- pREV-RSV (packaging vector) (202 µg)
- pCgpV (packaging vector) (202 µg)
- pCMV-VSVG (envelope vector) (202 µg)

Cell culture grade water (Corning cellgro, Cat. No. 25-055-CV) and 1M $CaCl_2$ (Sigma-Aldrich,® Cat. No. 21115) were added to Tube B to yield a final volume of 19 mL, and incubated at room temperature for 5 minutes. Following the 5 minute incubation, the contents of Tube B were added dropwise 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 $CaCl_2$-DNA solution had been added, the mixture would exhibit a cloudy appearance. 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 these precipitates greatly reduces the transfection efficiency. Each experiment was performed six times in replicate.

Transfection of HEK-293LTV Cells

Cells were seeded onto a T-175 Corning CellBIND® Surface flask (Corning, Cat. No. 431328) or Corning HYPER*Flask*® vessel (Corning, Cat. No. 10034) at 100,000 cells/cm^2 (0.326 mL/cm^2) and incubated overnight at 37°C (5% CO_2, 98% relative humidity). The following day, 5 to 6 hours prior to the transfection, the media was removed and replaced with fresh media (0.326 mL/cm^2) containing 25 µM chloroquine (Sigma, Cat. No. C6628). For optimal

transfection efficiency, the pH of the medium at the time of the transfection was between 7.5 to 7.6. To transfect the 293LTV cells, the medium containing chloroquine was removed from each vessel and combined into a 1L storage bottle (Corning, Cat. No. 430518). Thirty-seven mL of the medium was removed, and 37 mL of the DNA:$CaPO_4$ solution prepared above was added dropwise at a rate of approximately 7 mL/min. The medium containing the DNA:$CaPO_4$ solution was added slowly back to each vessel and incubated for 16 hours at 37°C (3% CO_2, 98% relative humidity). Following the incubation, the transfection medium was removed, fresh medium was added to the vessels and then collected 48 hours later. GFP expression was monitored throughout the course of the experiment using the AMG EVOS® FI microscope.

Lentivirus Harvest

Viruses can undergo a lytic or lysogenic life cycle. Lentiviruses undergo the lysogenic cycle, which results in the release of virus into the medium without cell lysis (Fig. 1). As a result, the medium was collected approximately 65 hours post transfection, and the cells were discarded. To remove any cell debris and multivesicular bodies (MVB), the medium was filtered through a 0.45 µM cellulose acetate filter (Corning, Cat. No. 430514 or 430627) and collected into an autoclaved glass bottle (Corning, Cat. No. 1395-1L or 1395-100). 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.

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 Cq values determined by the software.

Transduction of Vero and MDBK Cells

To verify that the virus was functional, Vero (ATCC,™ Cat. No. CCL-81) and Mardin-Darby Bovine Kidney (MDBK) (ATCC, Cat. No. CCL-22) cells 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/cm^2 and incubated overnight at 37°C (5% CO, 98% relative humidity). The following day, the concentrated lentiviruses encoding GFP were added to the cells at a multiplicity of infection (MOI) of 20. 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:

[(Cells/cm^2) x (cm^2 of well) x (MOI 20 (TU/cells)] / (TU/mL)

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

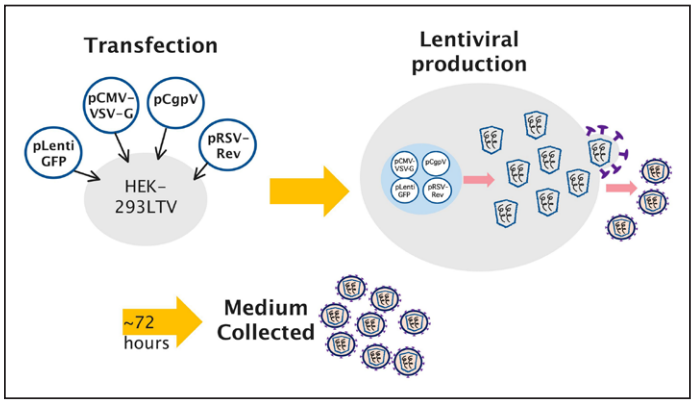

Figure 1. Schematic of Lentivirus production.

Flow Cytometry

To assess GFP expression, Vero and MDBK cells transduced with lentivirus encoding GFP were harvested, centrifuged to remove trypsin/media, and then resuspended in 500 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 using a T-175 flask compared to a HYPER*Flask* 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. 2) throughout the course of the experiment.

Lentiviral Production

Once collected and filtered, the lentivirus' encoding GFP from each vessel were then titered using the Lenti-X™ qRT-PCR Titration Kit to determine copies/mL. Lentivirus obtained from the HYPER*Flask* vessel yielded similar copies/mL (Fig. 3A), similar copies/cm^2 (Fig. 3B), and approximately 10 times more copies (Fig. 3C) compared to lentivirus obtained from the T-175 flask. These results indicate that lentivirus particles may be generated in the HYPER*Flask*® vessel with similar titers, but larger yields are obtained when compared to a standard T-175 vessel with a similar spatial footprint.

GFP Expression in Vero and MDBK Cells

To verify that the virus obtained from the HYPER*Flask*® vessel was as functional as virus obtained from the T-175 flask, Vero and MDBK cells were transduced with lentivirus encoding GFP. Each cell type was transduced with virus obtained from either the HYPER*Flask* vessel or a T-175 flask at a MOI of 20. After 72 hours, the cells were collected and analyzed via flow cytometry. The average GFP fluorescence in each cell line infected with lentivirus was greater than 90.0% (Fig. 4, two independent experiments). Taken together, these results indicate that the HYPER*Flask* vessel produces similar infectious lentiviral particles per cm^2 compared to a traditional T-175 flask.

Summary

In summary, this study demonstrates the utility of the HYPER Technology as an alternative to traditional cell culture flasks for scale up production of Lentivius.

- Lentiviral particles can be amplified in the Corning HYPER*Flask* 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.
- Furthermore, the HYPER technology platform of products includes larger vessels with larger surface areas that provide researchers with the ability to further increase Lentivirus production.

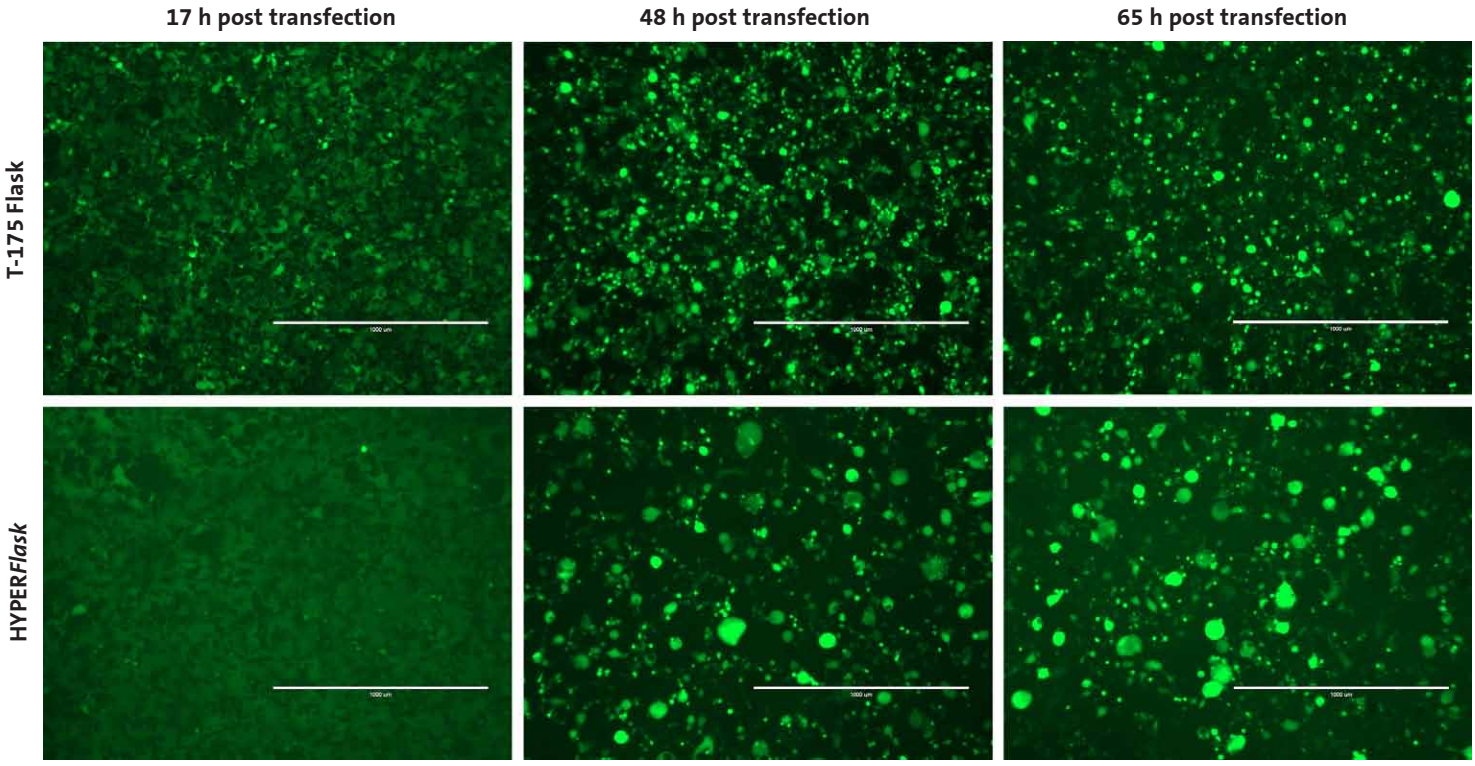

Figure 2. Analysis of GFP expression in the Corning HYPER*Flask*. Representative images from the same experiment demonstrating similar GFP expression in the Corning HYPER*Flask* and T-175 Flask. Scale bar represents 1000 µm. These trends were observed with all experiments. Images were captured using the AMG EVOS® Fl microscope.

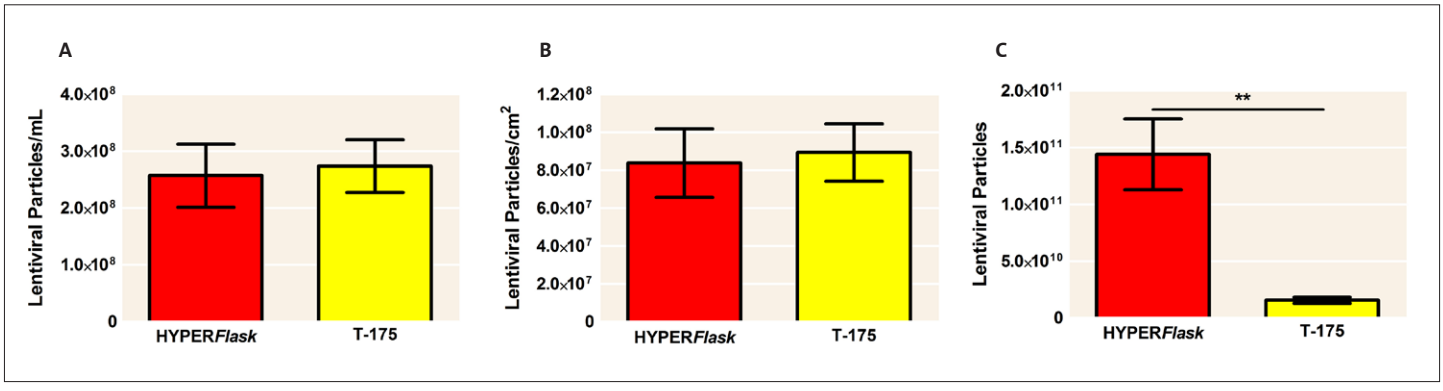

Figure 3. The Corning HYPER*Flask* vessel supports comparable viral production, with a higher yield of total virus compared to a T-175 flask. (A) Titers obtained from the Lenti-X™ qRT-PCR Titration Kit. (B) When normalized on a per cm^2 basis the HYPER*Flask* vessel yields similar amount of lentiviral particles. (C) The HYPER*Flask* vessel generates a significantly higher amount of total infectious adenoviral particles. Paired t-test, **p <0.01, N=6.

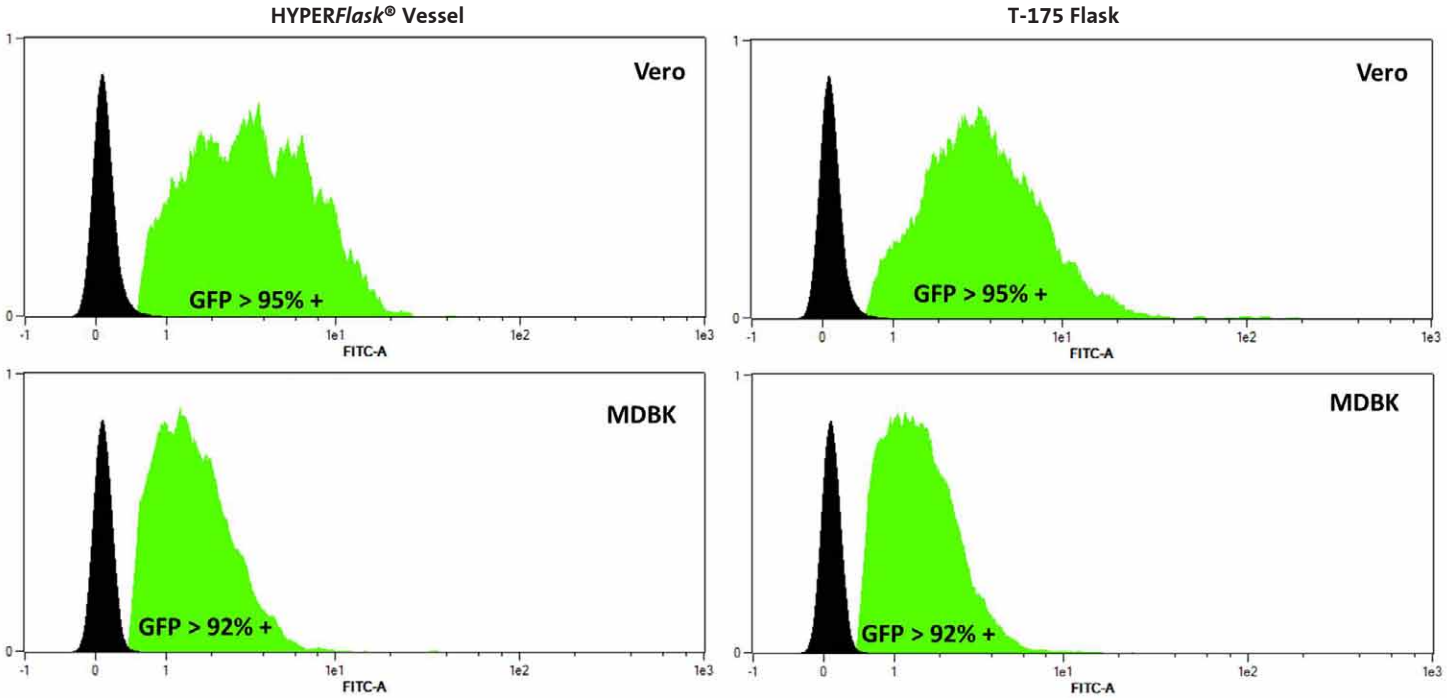

Figure 4. Vero and MDBK cells transduced with Lentivirus exhibit comparable levels of GFP expression. Representative flow cytometry data. Expression of GFP (green) compared to a negative control of non-transduced cells (black). After two independent experiments, the GFP expression in both the Vero or MDBK cells was greater than 90%, regardless of which vessel in which the virus was generated.

Reference

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

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