Generating Crude Adenoviral Particles in the Corning® HYPERFlask® Cell Culture Vessel

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Application Note

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Introduction

Adenoviruses are non-enveloped DNA viruses that play a vital role in research and industrial applications. Some of these advantages include: (i) the ability to infect a wide range of actively dividing and non-dividing mammalian cells (e.g., primary neurons); (ii) minimum risk of integration into the host genome (the virus is epichromosomal); and (iii) the capacity to be amplified to very high titers in tissue culture.[1] To allow researchers and vaccine manufacturers the opportunity to produce even higher yields in the same spatial footprint as a T-175 flask, Corning offers the HYPERFlask vessel. The Corning HYPERFlask cell culture vessel features Corning's HYPER (High Yield PERformance) technology which utilizes a gas permeable film 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 compared to traditional rigid single layer culture vessels. The HYPERFlask vessel cell growth surface area is 1,720 cm^2 across 10 layers and has the same spatial footprint of a T-175 vessel.

Crude adenoviral particles are produced when cells (e.g., HEK-293AD) are transfected with DNA encoding the various genes needed for adenovirus generation. After the crude adenoviral stocks have been obtained the virus may then be amplified by transducing particular cell lines (e.g. HEK-293AD or A549). The focus of this study was to determine the efficacy of generating crude virus using the unique Corning HYPER technology. Standard methodologies utilize traditional T-flask vessels for virus generation. Utilizing the HYPERFlask vessel, researchers can generate higher titers in a smaller spatial footprint saving both time and space. The results depicted here demonstrate that the experimental approach to generate adenovirus in the HYPERFlask vessel led to higher titers and yields compared to a standard T-175 flask.

Materials and Methods

Cell Culture

HEK-293AD cells (Cell BioLabs AD-100) were maintained in DMEM without sodium pyruvate (Corning 10-017-CM), 10% FBS, and 1X MEM Nonessential Amino Acids (Corning 25-025-CI). HEPES (10 mM) (Corning 25-060-CI) was added to this medium during adenovirus production.

DNA Preparation

GC10 competent cells (Sigma-Aldrich) were heat shocked with the DNA obtained from the RAPAd® CMV Adenoviral Bicistronic Expression System (GFP) (Cell BioLabs VPK-254) and cultured in LB-Broth (MilliporeSigma L3022) in a 1L Erlenmeyer flask (Corning 431403) at 37°C for 16 h at 250 rpm. Plasmid DNA was purified by maxi prep and quantified with the EnVision® multimode plate reader (PerkinElmer). Purified plasmid DNA was digested with Pac-I (New England BioLabs R0547L) at 37°C for at least 4 hours then heat inactivated at 65°C for 30 minutes.

Preparation of the DNA: Lipofectamine Complex

On the day of transfection, a master mix of DNA: Lipofectamine solution was prepared based on a total growth area of 1,945 cm^2 (1,720 cm^2 (HYPERFlask) + 175 cm^2 (comparison T-175) + 50 cm^2 [extra]). To prepare the mix, Opti-MEM (Thermo Fisher 31985070) was added to two 150 mL storage bottles (Corning 431175) at a ratio of 0.032 mL/cm^2 (approx. 63 mL) per bottle. Lipofectamine 2000 (Thermo Fisher 11668019) was added to one storage bottle at a ratio of 3 µL of reagent per 1 µg of DNA (approx. 1.5 mL Lipofectamine 2000) and incubated at room temperature for 5 minutes. To the second storage bottle containing Opti-MEM the following heat inactivated Pac-I digested DNA components (see above for preparation) were added at a combined ratio of 0.245 µg/cm^2:

- pacAd5 CMV-Green Fluorescent Protein (GFP) Control Vector (335 µg)
- pacAd5 9.2-100 Vector (145 µg)

Following the 5 minute incubation, the Opti-MEM containing DNA was added to the Opti-MEM containing Lipofectamine, briefly swirled, incubated for 20 minutes at room temperature, and transferred to a 1L storage bottle (Corning 430518) containing 508 mL of media for adenovirus production. Each experiment was performed 3 individual times.

Transfection of HEK-293AD Cells

Cells were seeded onto a T-175 Corning® CellBIND® surface flask (Corning 431328) or Corning HYPERFlask® vessel (Corning 10034) at 40,000 cells/cm^2 (0.326 mL/cm^2) and incubated overnight at 5% CO_2, 98% relative humidity, 37°C. The following day the media was removed and fresh media (0.326 mL/cm^2) containing the DNA: Lipofectamine complex (see above) was added. Post-transfection (16 hours) the media was changed and the cells were incubated at 37°C for 11 to 14 days. Maintaining proper pH conditions (approx. pH 7.2) is critical for optimal virus production[2] and therefore half media changes were performed when phenol red in the media indicated a pH shift (approx. every 2 to 3 days). Additionally, to help control the pH, HEPES (10 mM) was added to the medium. GFP expression was monitored throughout the course of the experiment using the EVOS® FL microscope (Thermo Fisher).

Adenovirus Harvest

When approximately half of the cells appeared attached to the vessels and half were floating in the medium, both medium and cells were collected (Figure 1). To collect the cells still attached to the vessel, PBS (without Ca^{2+} and Mg^{2+}) (Corning 21-040-CM) was added to each vessel (0.029 mL/cm^2) and incubated at 37°C for 3 to 5 minutes. One or more PBS washes were performed depending on the overall density of the cells that remained attached to the surface and added to the media containing cells. To minimize volume, and concentrate the crude adenovirus preparation, the cells were centrifuged at 500 x g for 10 minutes at room temperature and the cell pellet was resuspended in 10 mM Tris, pH 8.0, 100 mM NaCl (0.023 mL/cm^2). The cell suspension was then subjected to 3 freeze/thaw cycles (-80°C/37°C), and centrifuged at 3000 x g for 10 minutes at 4°C to pellet the cell debris. The supernatant containing the adenoviruses released from the cells was collected. The adenovirus encoding GFP was then aliquoted and stored at -80°C.

Adenovirus Titer

The QuickTiter™ Adenovirus Titer ELISA kit was purchased from Cell BioLabs (VPK-110). 293AD cells were seeded on a Poly-D-Lysine coated 96-well microplate (Corning 3841) at 156,250 cells/cm^2 (0.3125 mL/cm^2) and incubated for 2 hours to allow for cell attachment. Adenovirus isolated from each vessel was thawed, added to multiple wells at various dilutions (10^{-3} to 10^{-5}), and incubated at 37°C for 48 hours. The ELISA assay was performed according to manufacturer's instructions and the signal in the wells was measured utilizing a Perkin-Elmer EnVision® Multilabel Reader.

Transduction of Vero and MDBK Cells

To verify that the crude virus was functional, Vero (ATCC CCL-81) and MDBK (ATCC CCL-22) cells were transduced. Cells were seeded onto a 6-well plate (Corning 3506) at 10,000 cells/cm^2 and incubated overnight at 5% CO_2, 98% relative humidity, 37°C. The following day the adenovirus encoding GFP was added to the cells at a multiplicity of infection (MOI) of 200 (based on previous results). The amount of each virus (mL) added to each well was calculated using the following formula:

$$\frac{(\text{Cells/cm}^2\ [\text{cm}^2\ \text{of well}]) \times (\text{MOI 200 [IFU/cells]})}{\text{IFU/mL}}$$

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

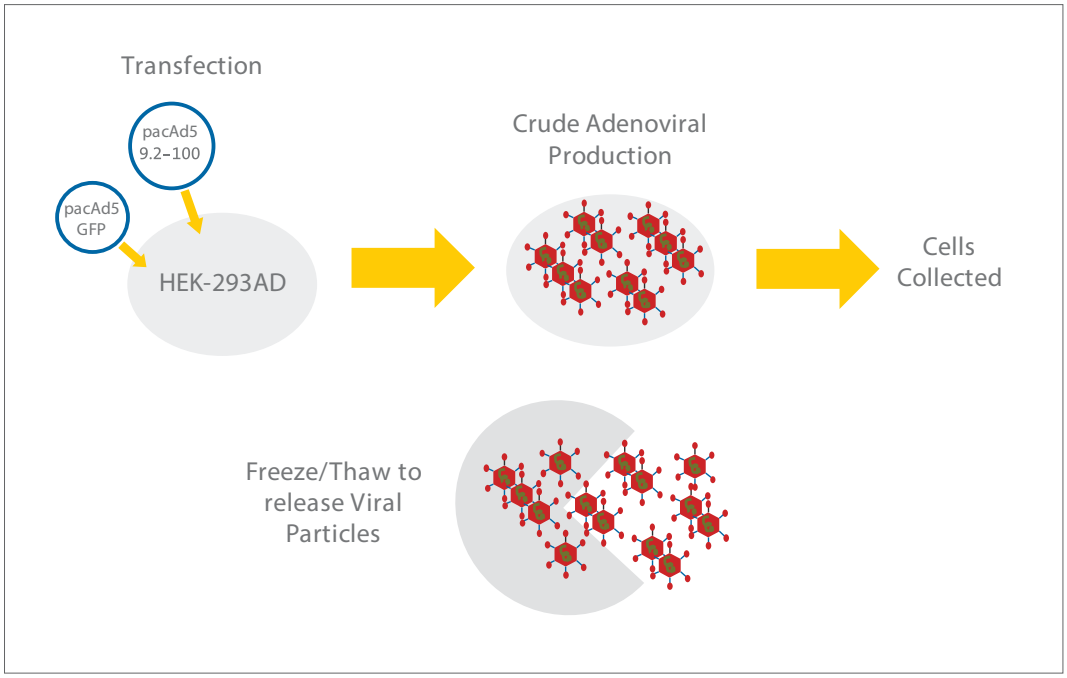

Figure 1. Schematic of crude adenovirus production.

Flow Cytometry

To assess GFP expression, Vero and MDBK cells transduced with adenovirus encoding GFP were harvested, centrifuged to remove trypsin/media, and resuspended in 1 mL of PBS (Corning 21-040-CM). Cell suspensions were analyzed using the MACSQuant® Analyzer instrument (Miltenyi Biotec).

Results and Discussion

GFP Expression Throughout Production

To assess adenoviral production on a T-175 flask compared to a HYPERFlask vessel, 293AD cells were transfected with DNA obtained from the RAPAd CMV Adenoviral Bicistronic Expression System (GFP) and GFP expression was monitored throughout the course of the experiment. After 72 hours post-transfection, similar GFP expression was observed in both vessels (Figure 2). However, approx. 10 days post-transfection, differences in GFP expression became visible between the two vessels. 293AD cells cultured/transfected in the T-175 flask had disproportionate GFP expression throughout the vessel. Some areas had little observed GFP expression (Figure 3, Panel A) whereas other areas had higher expression indicating either the beginning (Figure 3, Panel B), or mid-late stages of plaque formation (Figure 3, Panel C). In the HYPERFlask vessel, the GFP distribution was more uniform. In areas where plaque formation had not yet begun, GFP expression appeared higher than the expression level detected in the T-175 flask (Panel D versus Panel A [Figure 3]). Additionally, there appeared to be a higher amount of plaque formations occurring in the beginning (Panel E) and late (Panel F) stages compared to the T-175 flask. Lastly, after approximately 11 to 14 days post-transfection, the 293AD cells were harvested from the vessels. The cells cultured on the T-175 flask were ready to be collected on approx. day 13, whereas cells cultured on the Corning HYPERFlask vessel were, on average, collected 1 to 2 days earlier compared to the T-175 flask (Figure 4).

Adenoviral Production

Once collected, the adenovirus encoding GFP from each vessel was then titered using the QuickTiter ELISA kit to determine infectious forming units (IFU)/mL. Adenovirus obtained from the HYPERFlask vessel yielded higher IFU/mL (Figure 5A), IFU/cm^2 (Figure 5B), and greater than 20 times more total IFU (Figure 5C) compared to adenovirus obtained from the T-175 flask. The average titers produced on the HYPERFlask vessel were 1.8 x 10^9 IFU/mL and on the T-175 flask were 6.4 x 10^8 IFU/mL. These results demonstrate that higher infectious adenoviral particles/cm^2 occurred in the HYPERFlask vessel compared to a traditional T-175 flask.

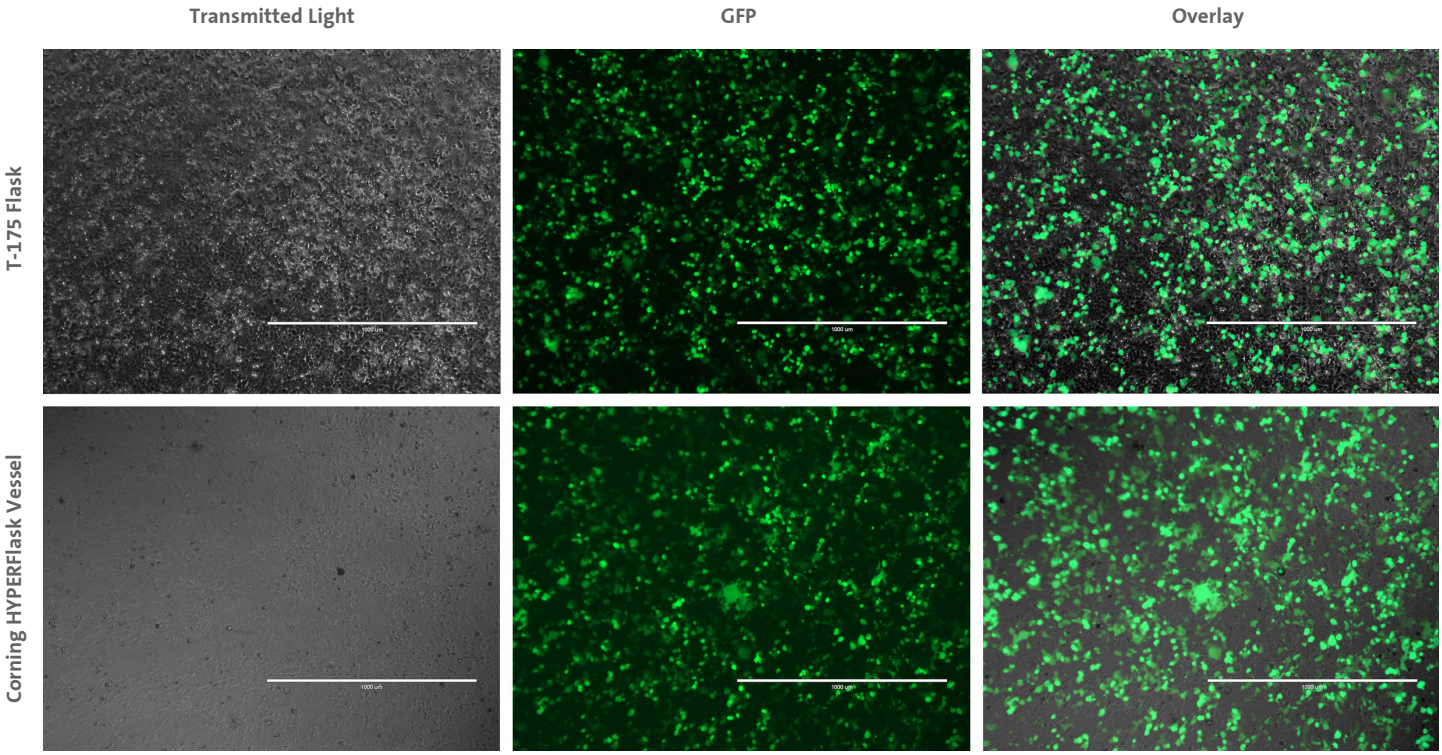

Figure 2. Similar GFP expression between vessels was observed 72 hours post- transfection. Representative images from the same experiment demonstrating similar GFP expression in both vessels. Scale bar represents 1,000 µm. These trends were observed with all experiments. Images were captured using the EVOS® FL microscope.

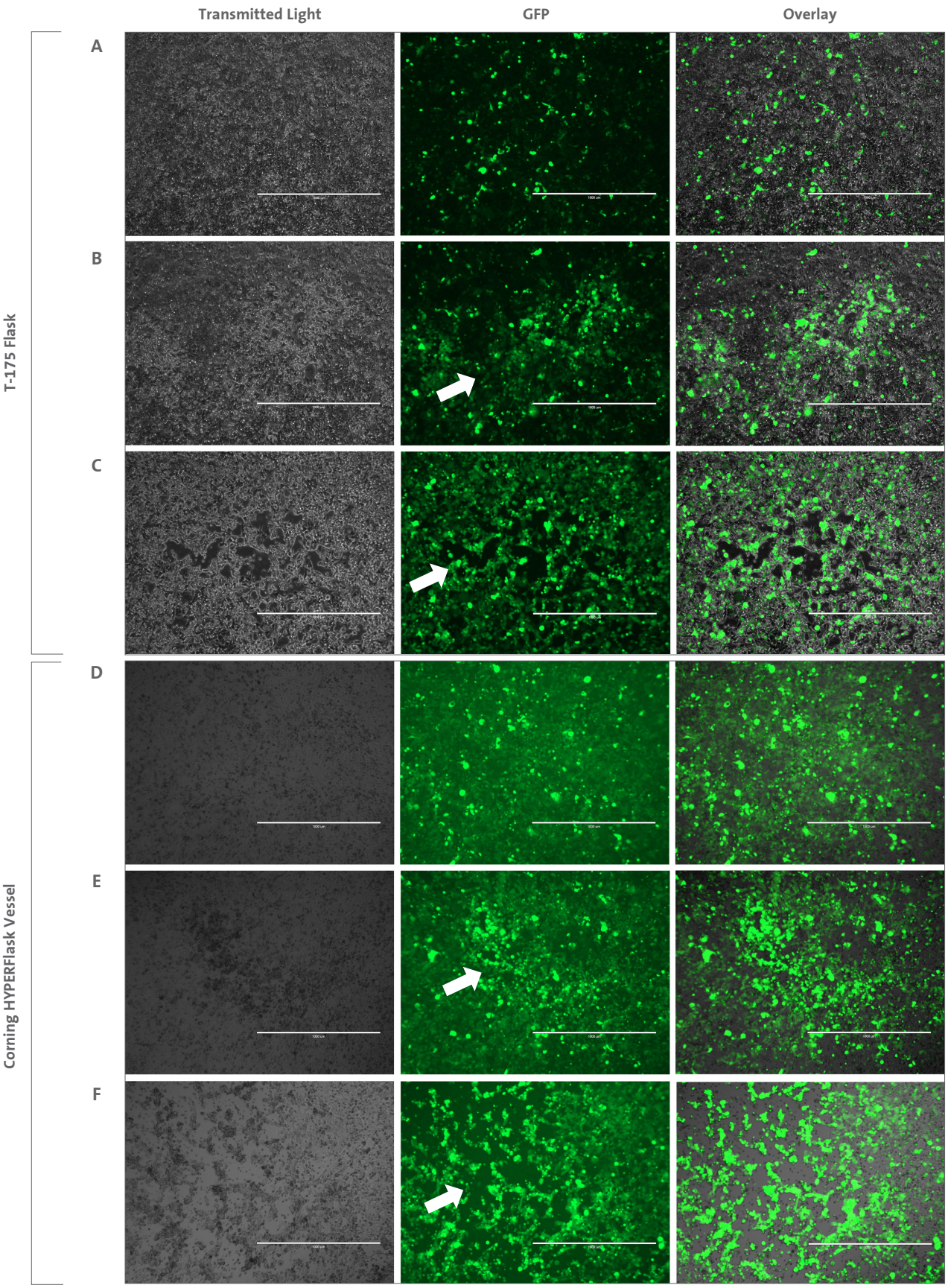

Figure 3. GFP expression in 293AD cells approx. 10 days post-transfection. Representative images from the same experiment demonstrating various GFP expression in each vessel type. (A-C) T-175 vessel: (A) Areas where GFP expression was low; (B) Beginning stages of plaque formation; (C) Mid-late plaque formation. (D-F) HYPERFlask vessel: (D) High GFP expression was detected throughout the vessel; (E) Beginning stages of plaque formation; (F) Late plaque formation. White arrow points to plaque formations. Scale bar represents 1,000 µm. Images were captured using the EVOS® FL microscope. These trends were observed in all experiments.

GFP Expression in Vero and MDBK Cells

To verify that the virus obtained from the HYPERFlask vessel was as functional as virus obtained from the T-175 flask, Vero and MDBK cells were transduced with adenovirus encoding GFP. Each cell type was transduced with virus obtained from either the HYPERFlask vessel or T-175 flask at an MOI of 200. After 72 hours, the cells were collected and analyzed via flow cytometry. The average GFP fluorescence in each cell line with each adenovirus was greater than 99% (Figure 6, 3 independent experiments). Cells were transduced at a MOI 200 to ensure maximum expression. Previous results also depicted equal GFP expression amongst the vessels when transduced at lower MOIs (10, 50, and 100) for shorter time periods (24 and 48 hours). GFP expression from each experiment varied between 40% and 95% depending on a) MOI and b) time (data not shown). Taken together, these results indicate that the HYPERFlask vessel produces more functional adenoviral particles per cm^2 compared to the traditional T-175 flask.

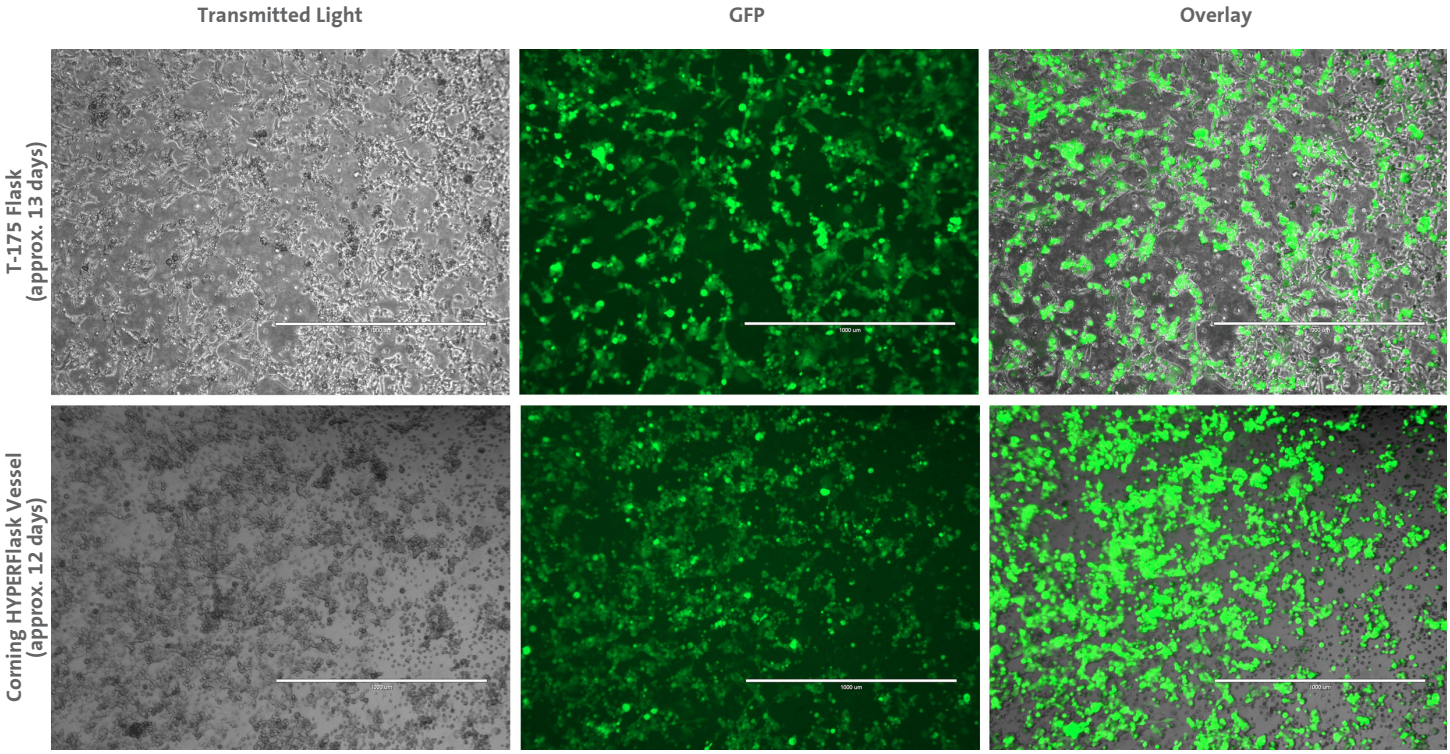

Figure 4. Cell morphology and GFP expression on day of harvest. Representative images from the same experiment demonstrating morphology/GFP expression on the day of harvest. On average, cells were harvested from the T-175 flask on day 13, whereas cells were harvested on day 12 from the HYPERFlask vessel. Scale bar represents 1000 µm. Images were captured using the EVOS® FL microscope.

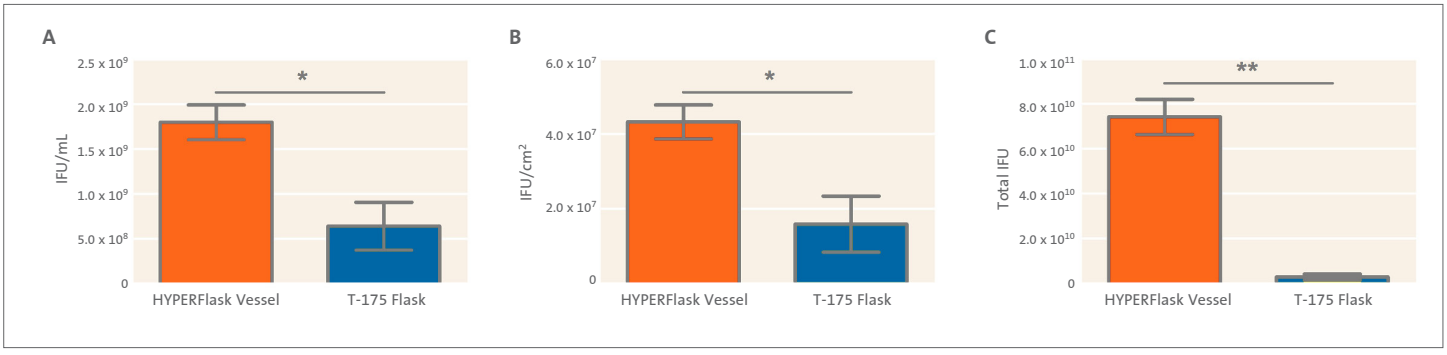

Figure 5. The Corning HYPERFlask vessel leads to higher viral production per cm^2 compared to a T-175 flask. (A) Titers obtained from the QuickTiter ELISA Adenovirus kit. (B) When normalized on a per cm^2 basis the HYPERFlask vessel yields greater than 2.5 times more infectious adenoviral particles. (C) The HYPERFlask vessel generates a significantly higher amount of total infectious adenoviral particles. Paired t-test, *$p<0.05$, **$p<0.01$, N=3.

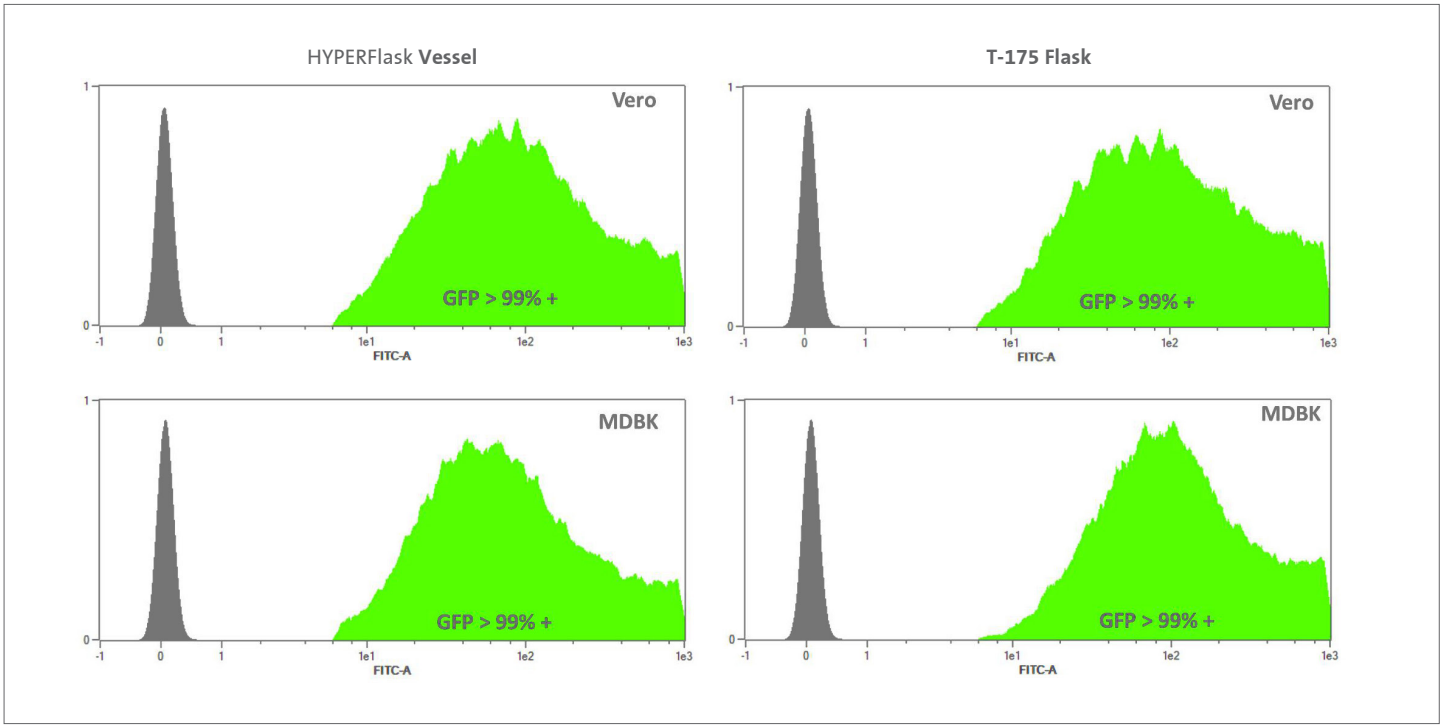

Figure 6. Vero and MDBK cells transduced with adenovirus exhibit similar GFP expression levels. Representative flow cytometry data. Expression of GFP (green) compared to a negative control of non-transduced cells (black). After 3 independent experiments, the GFP expression in both the Vero or MDBK cells was greater than 99%, regardless in which vessel the virus was generated.

Conclusions

In summary, this study demonstrates the utility of the HYPER technology in adenovirus production as a good alternative to traditional cell culture flasks for obtaining higher virus yields. These higher yields could be explained by the improved gas exchange in the Corning® HYPERFlask® vessel compared to standard cell culture flasks. Even though the transfection efficiency appears similar within the first 72 hours (Figure 2) the cells are then continuing to propagate over the next 1 to 2 weeks. Over the course of this time a large oxygen gradient may be forming throughout the media leading to a deprivation of oxygen on standard T-flasks. Cells cultured with HYPER technology are continuously exposed to the same amount of oxygen eliminating the oxygen gradient. As a result, the health of the cells over the course of this study (11 to 14 days) may be significantly improved leading to higher viral yields.

- Adenoviral particles can be produced in the Corning HYPERFlask vessel at higher titers than traditional tissue culture vessels, resulting in greater virus production in a similar spatial footprint.
- Furthermore, the HYPER technology platform of products includes larger vessels with higher surface areas providing a researcher with the ability to further amplify adenoviral particles.[3]

References

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2. Silva AC, et al. Adenovirus vector production and purification. Curr Gene Ther 2010;10(6):437-455. doi: 10.2174/156652310793797694
3. Amplifying Adenoviral Particles in the Corning HYPERStack Cell Culture Vessel. Corning Application Note (CLS-AN-214).

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