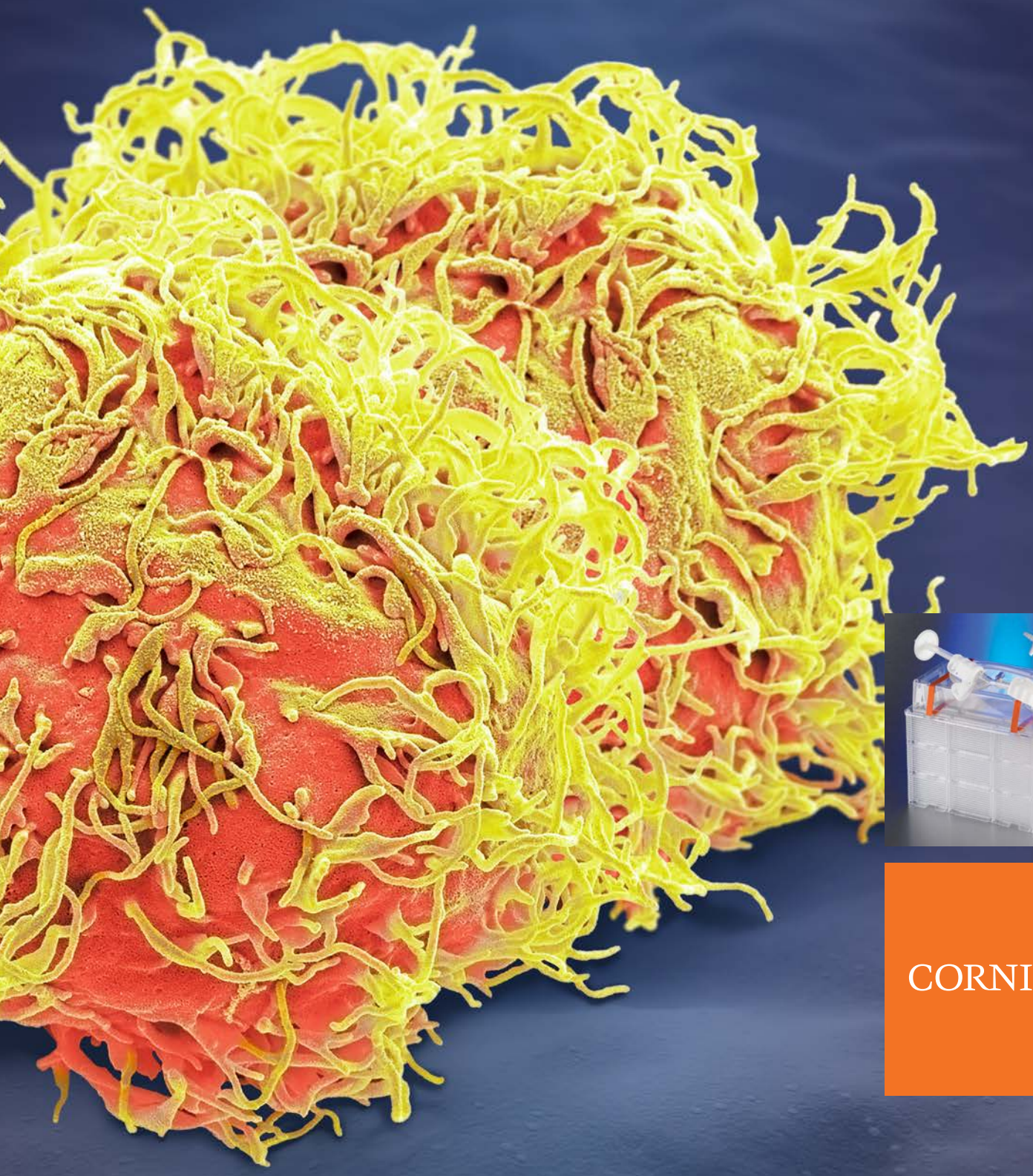
Improving Scale-up Efficiency with Corning® HYPER Technology

A compilation of research experiences in stem cell, viral vector and vaccine production.



CORNING

Introduction

This e-book contains some of our most popular application notes related to the Corning® HYPER*Stack*® cell culture vessel. Introduced in 2010, Corning HYPER*Stack* vessels have become an industry-standard for a variety of adherent cell expansion applications, including viral vector manufacturing and stem cell expansion.

We hope this e-book provides a compelling introduction to Corning HYPER technology. Our Field Application Scientists are eager to help you address your cell culture scale-up needs. Contact your regional scientific support team via the one of these email addresses:

Americas
ScientificSupport@corning.com

Asia/Pacific (Chinese Mainland, India, Korea, Singapore, and Taiwan)
ScientificSupportAP@corning.com

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ScientificSupportEMEA@corning.com

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About HYPER*Stack* Cell Culture Vessels

The Corning HYPER*Stack* vessel is one of two Corning products utilizing our proprietary High Yield PERformance (HYPER) technology, which lets you grow large quantities of adherent cells in a compact space. (The other product is the Corning HYPER*Flask*® vessel, a smaller vessel used for research applications.)

HYPER technology uses a unique gas-permeable film which maximizes the surface growth area relative to cubic footprint of the vessel. This results in a substantial increase in cell growth surface area compared to traditional vessels of comparable footprint.

Features and benefits of the HYPER*Stack* vessel include:

- Higher total cell yields than vessels with similar spatial footprints
- Similar cell morphology, phenotype, and growth compared to traditional vessels
- Closed system design ideal for protein therapeutics, as well as vaccine and virus production including those targeting SARS-CoV-2.
- Scalable product – multiple size offerings, can be manifolded to scale to hundreds of thousands of square centimeters of cell growth surface area
- Ergonomic design – easier manipulation and fits the Corning Automated Manipulator
- Fixed media volume – 0.2 mL/cm² fills vessel for optimal handling
- Innovative assembly – no adhesives, low particulates
- Less volumetric waste – less waste per growth area lowers overall costs

For more information visit **www.corning.com/hyper.**

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Large Scale Expansion of Human Mesenchymal Stem Cells using Corning® stemgro® hMSC Medium and Corning CellBIND® Surface HYPER*Stack*® Cell Culture Vessels

Application Note

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Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that exhibit the capacity to differentiate into multiple cell lineages such as osteoblasts, chondrocytes and adipocytes. The ability to self-renew and differentiate under appropriate culture conditions not only makes MSCs valuable for T-cell therapy and tissue engineering applications, but also drives the demand for large scale expansion. Traditional MSC culturing methods involve the use of serum-containing media, but the undefined nature of serum is a concern in clinical applications. While chemically defined, serum-free media formulations are available[1], many of these media require pre-coating of the growth surface with biological substrates. Biological coating of growth surfaces may be both challenging and expensive during large scale production.

Corning stemgro hMSC medium is a chemically defined, serum-free medium formulated for maximum expansion of hMSCs. When used in conjunction with the Corning CellBIND surface, stemgro hMSC medium enables attachment and growth comparable to that of serum-containing cultures, including maintenance of MSC multipotency[2], but eliminates the need for biological coatings.

Corning CellBIND surface HYPER*Stack* cell culture vessels (Figure 1) use the patented HYPER technology platform, consisting of a gas permeable film to eliminate the requirement for the air gap normally found in standard cell culture vessels. This allows for a compact vessel with increased growth area. The HYPER*Stack*-12 layer (6,000 cm^2, A) and 36 layer (18,000 cm^2, B) vessels are designed for closed system applications and are manufactured using methods acceptable for large-scale cell propagation.

The following study demonstrates the large scale expansion of hMSCs in a serum-free environment using stemgro hMSC medium in combination with HYPER*Stack*-12 Layer cell culture vessels. To monitor the health and functionality of the hMSC cultures, cell yields, viability and surface antigen profiles (positive antigens: CD105, CD166, and CD29; negative antigens: CD14 and CD34) were assessed throughout the scale up process. To confirm the functionality of expanded cultures of hMSCs, the cells were harvested from HYPER*Stack*-12 vessels and induced to differentiate into adipogenic and chondrogenic lineages.

Methods and Materials

Cell Cultures

Bone marrow-derived human mesenchymal stem cells; hMSC (STEMCELL™ Technologies Cat. No. MSC-001F, Lot No. BM2758) were thawed and maintained in complete stemgro hMSC medium (Corning Cat. No. 40-410-KIT) or stemgro basal medium, without phenol red – cGMP grade (Corning Cat. No. 40-415-KIT) supplemented with 2 mM L-glutamine, (Corning Cat. No. 61-030-RM). Freeze banks of 1.0 x 10^6 cells/vial (passage #2) were prepared using 95% complete growth medium and 5% DMSO.

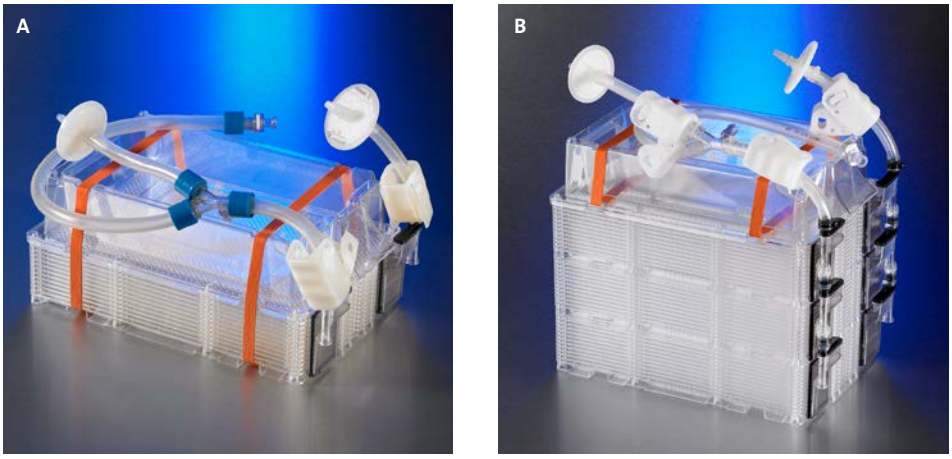


Figure 1. Corning CellBIND surface HYPER*Stack* cell culture vessels product line. (A) 12-layer and (B) 36-layer.

Thaw and Maintenance

Frozen hMSC stocks were thawed using standard cell culture protocols. Freeze media was removed by centrifugation at 500 x g for 4 minutes and replaced with fresh growth medium. Cells were plated at a concentration of 5,000 cells/cm^2 on Corning® CellBIND® surface flasks and designated passage #0. Cells were maintained in cultured until they reached 75% to 80% confluence (4 to 5 days) in a humidified incubator set to 37°C and 5% CO_2. A full medium change was performed a minimum of 24 hours prior to harvest by aspirating spent medium from the vessel and replacing with freshly prepared room temperature growth medium.

Subculture and Scale-up

On the day of harvest (75% to 80% confluent cultures) medium was aspirated from the vessel followed by a gentle rinse using 1x HBSS (Corning Cat. No. 21-023-CM). To maintain serum-free conditions, cultures were harvested with TrypLE™ Express (Invitrogen™ Cat. No. 12605-028) cell dissociation reagent. The TrypLE solution was added to culture vessels using approximately 0.03 mL of reagent per cm^2 and incubated at room temperature for 3 to 5 minutes. After most of the cells were released from the growth surface, the cell suspension was collected into an equal volume of complete growth medium. The empty vessel was further incubated at room temperature for approximately 3 more minutes to allow the remaining cells to detach. Cells were collected from the vessel with an HBSS rinse and pooled with the initial collection. When needed, a second rinse was done using complete growth medium. The cell suspension was concentrated by centrifugation at 500 x g for 5 to 10 minutes (centrifugation time based upon volume of cell collection, see table 1) and resuspended in fresh medium. Cell concentration and viability were assessed using a BioProfile® FLEX instrument (Nova Biomedical). Cells were plated at a concentration of 5,000 cells/cm^2 into larger surface area vessels such as the Corning CellBIND surface HYPER*Flask*® M cell culture vessel, 1,720 cm^2 (Corning Cat. No. 10034) or Falcon® cell culture Multi-Flask, 525 cm^2 (Corning Cat. No. 353143). Scale up continued until enough cells were collected to seed a HYPER*Stack*®-12 layer cell culture vessel (Corning Cat. No. 10012) and a T-175 Corning CellBIND surface control flask (Corning Cat. No. 3292) at a concentration of 5,000 cells/cm^2 in 0.217 mL/cm^2 complete Corning stemgro® hMSC medium. At the end of each harvest a small sample of cell suspension (1 to 5 mL) was saved for phenotypic analysis of cells. The study was independently repeated four times.

Morphologic and Proliferation Assessment

To monitor the general health of the hMSC cultures throughout the scale up process, population doublings and morphologic changes were assessed via microscopic examination using an inverted Olympus microscope. Population doublings were assessed using the equation n = 3.32 (logN-logX); where n = population doubling, N = total number of cells harvested and X = initial number of cells plated.

Immunophenotyping Assessment

Surface marker analysis of the hMSCs were performed throughout each cell passage in order to monitor potential changes to the cells. Cell samples were washed twice (600 x g for 5 minutes) with 5 mL of flow cytometry staining buffer (SB) (R&D Systems® Cat. No. FC001) to remove residual culture medium. Cells were fixed by resuspending cell pellets at a concentration of 5.0 x 10^6 cells/mL in a 1:1 mixture of SB and 4% paraformaldehyde (PFA) (Boston BioProducts Cat. No. BM-155-250 mL) and stored at 4°C for up to 48 hours. (Note: a marked decrease in CD29+ expression was observed if the sample was not analyzed within 48 hours after collection. All other markers remained stable for up to 5 days in storage.)

On the day of analysis, the fixed samples were pelleted by centrifugation at 600 x g for 5 minutes to remove fixing solution, resuspended in blocking buffer (0.5% BSA in staining buffer) to a density of approximately 10 x 10^6 cells/mL and incubated for 15 minutes at room temperature. After blocking, 100 µL of samples (~1.0 x 10^6 cells/mL) were aliquoted into 2 mL tubes and marked as follows: no stain control, IgG1 isotype control, CD105+, CD166+, CD29+, CD14- and CD34- (R&D Systems Cat. Nos. IC002P, FAB10971P, FAB6561P, FAB17781P, FAB3832P, and FAB7227P, respectively). Ten microliters of the corresponding conjugated antibody or isotype control were then added to each sam

Table 1. Reagent volumes and centrifugation times. Reagent and media volumes used during expansion procedure as well as centrifugation times and speeds.

	Falcon Multi-Flask (525 cm^2)		Corning CellBIND Surface HYPER*Flask* Vessel (1,720 cm^2)		Corning CellBIND Surface HYPER*Stack*-12 Vessel (6,000 cm^2)	
	mL/cm^2	mL	mL/cm^2	mL	mL/cm^2	mL
HBSS Rinse	0.03	15	0.03	50	0.03	200
TrypLE	0.03	15	0.03	50	0.03	200
Quench Media	0.03	15	0.03	50	0.03	200
HBSS Rinse	0.03	15	0.03	50	0.03	200
Media Rinse	0.03	15	0.03	50	0.03	200
Total Volume		60		200		800
Centrifuge Tube		2 x 50 mL		250 mL		2 x 500 mL
Centrifuge Time (@ 500 x g)		5 min		10 min		15 min

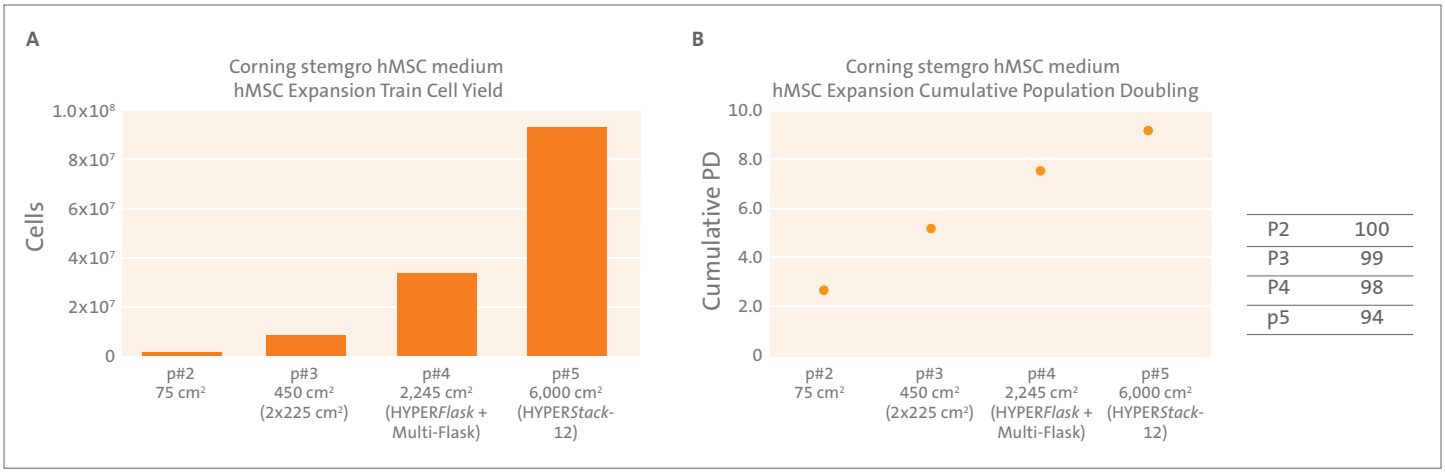


Figure 2. hMSC Expansion. Corning® stemgro® hMSC medium supported expansion of hMSC cultures (a) while maintaining consistent population doublings (b) and cell viability (c). A representative example of typical seed train (data from first expansion study).

ple tube, mixed well and incubated for 30 to 45 minutes at room temperature while being protected from light. After incubation, cells were washed and pelleted by centrifugation 2 times using SB to remove unbound antibody (4 minutes at 300 x g). Cell pellets were resuspended in 200 to 500 µL of SB (volume based on size of cell pellet) for flow cytometry analysis using a MACSQuant® Analyzer (Miltenyi Biotec).

Multipotency Assessment

To evaluate whether multipotency was maintained throughout the course of the expansion, hMSCs harvested from Corning® CellBIND® surface HYPER*Stack*®-12 vessels and Corning CellBIND surface T-175 control flask were differentiated into adipocytes and chondrocytes. Adipogenic Differentiation Bullet Kit® (Lonza Cat. No. PT-3004) was utilized for adipogenic differentiation of hMSCs. Following the kit's protocol, hMSCs were plated at a concentration of 21,000 cells per cm^2 in Corning CellBIND surface 12 well multiple well plates (Corning Cat. No. 3336) and maintained in complete stemgro hMSC medium until they were ready to differentiate using the kit's induction medium. Adipocyte differentiation was confirmed through Oil Red O Staining and quantitated using AdipoRed™ Assay Reagent (Lonza Cat. No. PT-7009) and analyzed on an EnVision® Microplate Reader (PerkinElmer). StemPro® Chondrogenesis Differentiation Kit (Life Technologies™ Cat. No. 10071-01) was used to induce chondrogenic differentiation. Following the manufacturer's protocol, hMSCs were suspended in complete stemgro hMSC medium at a concentration of 1.6 x 10^7 cells/mL and plated on Corning CellBIND surface 12 well multiple well plates by placing a 5 µL droplet in the middle of each well. Differentiation followed using the kit's induction media as follows: after a 2-hour incubation under high humidity, 1 mL of chondrogenic or control media (stemgro hMSC medium) was added to the wells. The cells were then cultured at 37°C in 5% CO_2 for up to 3 weeks with complete media changes every 3 days. Following 22 days in culture, the Alcian Blue Stain (Newcomer Supply® Cat. No. 1003) was added to confirm chondrocyte formation.

Results

The International Society for Cellular Therapy lists the following criteria for defining functional MSCs:

- Attach and proliferate *in vitro* under standard culture conditions
- Maintain phenotypical characterization through the expression of surface antigens, and
- Differentiate along the adipogenic, chondrogenic and osteogenic lineages[3]. These criteria were applied to evaluate the quality of the hMSCs that were cultured and expanded in stemgro hMSC medium and on Corning HYPER technology vessels.

Proliferation

The effectiveness of the stemgro hMSC medium as a serum-free medium for the expansion of hMSCs without the need for biological coatings was assessed by the healthy scale up of the hMSCs into the HYPER*Stack*-12 vessel. A primary metric of this study was to maintain the multipotency of the hMSCs. For this study, the hMSC cultures were maintained at a low passage and at approximately 75% to 80% confluence throughout the scale-up to the HYPER*Stack*-12 vessel to conserve the multipotency of the cells. The stemgro hMSC medium supported the expansion of hMSC cultures from thaw to HYPER*Stack*-12 vessels in 3 to 4 passages without effect on rate of cell growth. Figure 2 shows a representative example of a typical expansion process. hMSCs were thawed and seeded at a concentration of 5,000 cells per cm^2. Cultures were then sequentially scaled into larger surface area vessels using the same seeding concentration until reaching the HYPER*Stack*-12 vessel (6,000 cm^2) seeding density. Population doublings were calculated after each harvest using the equation n = 3.32 (logN−logX). The results demonstrate consistent population doublings throughout the length of the expansion process. Cell viability, determined by Trypan blue exclusion (BioProfile® Flex analyzer) also indicated a healthy viable cell population.

Expansion Yields

Expansion of hMSCs into the Corning® CellBIND® surface HYPER*Stack*®-12 and Corning CellBIND surface T-175 control vessels was the final scale up step in this study. Vessels were seeded at a concentration of 5,000 cells/cm² in complete Corning stemgro® hMSC medium (0.217 mL/cm²) and cultured for 4 days (75 to 80% confluence). Cells were harvested with TrypLE reagent and enumerated using the Trypan blue exclusion method.

Viable cell counts from four independent studies show average yields of 1.17 x 10^8 cells from the HYPER*Stack*-12 vessels with viabilities of 92% or better (Figure 3), demonstrating a suitable expansion process. Cells harvested from the T-175 control flasks were compared to those harvested from HYPER*Stack*-12 vessels to evaluate proliferation on the Corning CellBIND surface treated gas-permeable film. The results demonstrated no statistical difference between cell yields per growth area (cm²) (paired t-test value 0.1148) among the two vessel types (Figure 4). The data demonstrate not only comparable cell growth between vessels, but also the ability to expand hMSCs. Each expansion study was independently repeated four times.

Phenotypical Characterization

Cell morphology and immunophenotyping of the hMSCs were indicators of normal cell function throughout the expansion process. Microscopic examination revealed no morphologic changes during the scale up process or the final expansion step. Figure 5 demonstrates the similarities in morphology and confluence of hMSC cultures during different stages of the scale up process.

In addition to cell expansion and morphology, immunophenotyping was performed to further evaluate the hMSC cultures. Samples from each scale-up step were collected and analyzed for the positive and negative expression of surface antigens by flow cytometry analysis. CD105, CD166, and CD29 markers were the positive expression markers while CD14 and CD34 were the negative expression markers. These markers were chosen based on the analysis report generated by STEMCELL™ Technologies. Flow cytometric analysis demonstrated consistent expression of the various surface markers throughout the length of each expansion, with >99% expression for each of the positive markers and <5% expression for the negative markers (Figure 6 and Table 2).

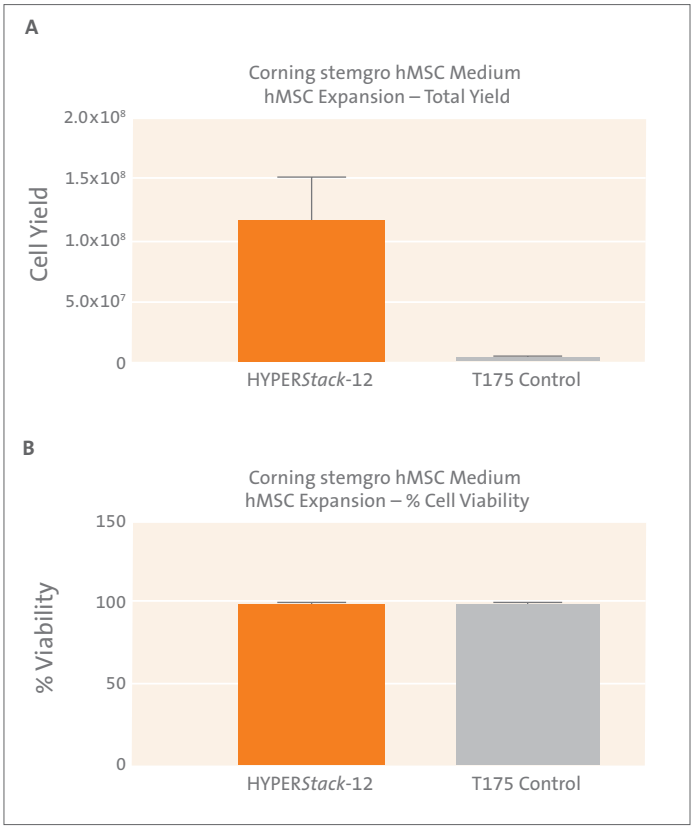


Figure 3. hMSC expansion yields. Final expansion of hMSC in Corning CellBIND surface HYPER*Stack*-12 Layer cell culture vessels using Corning stemgro hMSC medium. (A) Average cell yields and (B) average percent cell viability from the four independent studies.

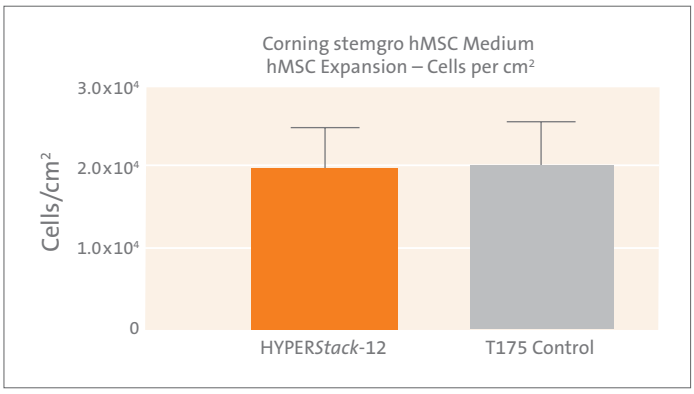


Figure 4. hMSC expansion yields in cells/cm². No statistical significant difference was observed between the Corning CellBIND surface HYPER*Stack*-12 vessels and T-175 Corning CellBIND surface control flask yields (n=4; paired *t*-test value: p<0.05).

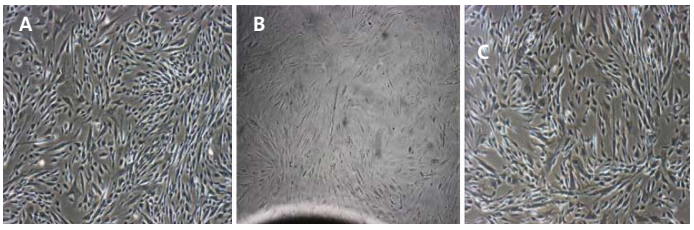


Figure 5. Morphological evaluation of hMSC cultures. Representative micrographs of hMSCs cultured in Corning stemgro hMSC medium. (A) Vendor hMSC stocks (passage #1) 96 hour cultures on a T-225 Corning CellBIND surface flask. (B) hMSC cultures at passage #5 on a Corning CellBIND surface HYPER*Stack*-12 vessel and (C) T-175 Corning CellBIND surface control flask (magnification = 4X using inverted Olympus microscope).

Differentiation

The ability to differentiate into multiple cell lineages is an important property of hMSCs. The differentiation potential of hMSCs cultured in Corning® stemgro® hMSC medium was assessed after expansion in the Corning CellBIND® surface HYPER*Stack*®-12 vessel. The hMSCs were differentiated into adipocytes and chondrocytes using commercially available kits. Intracellular triglyceride droplets were used as markers to help identify MSC differentiation into adipocytes. The lipid droplets (red) were stained with Oil Red O using the AdipoRed™ assay for visual confirmation of adipocyte differentiation (Figure 7). The AdipoRed assay was performed to quantitate adipocyte differentiation of cells harvested from HYPER*Stack*-12 vessels and T-175 control flasks. Results indicated that cells derived from HYPER*Stack* vessels had a significant increase (paired *t*-test value of 0.003) in adipocyte differentiation over cells derived from T-175 control vessels (Figure 8). Further studies of the statistical difference in adipocyte differentiation between both vessel types are ongoing.

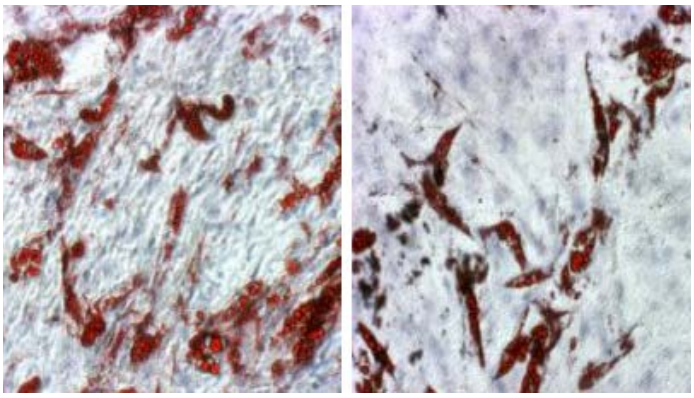


Figure 7. Oil Red O stain of adipocytes. Representative example of adipocyte differentiation of hMSCs. Intracellular lipid droplets stained red with Oil Red O stain. Micrographs of differentiated hMSCs from third expansion study; Corning CellBIND surface HYPER*Stack*-12 vessel (left) and T-175 Corning CellBIND surface control flask (right) derived, (magnification = 10X using inverted Olympus microscope).

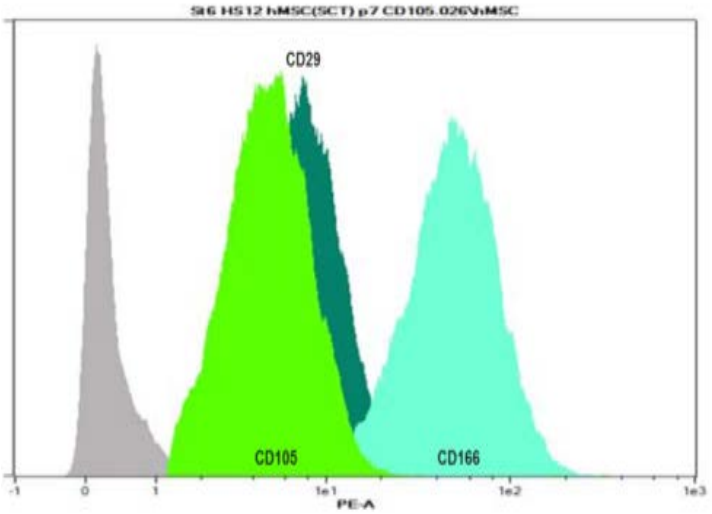


Figure 6. Representative flow cytometry analysis. Flow cytometry histograms of hMSCs harvested from Corning CellBIND surface HYPER*Stack*-12 vessel (second expansion study, passage #7). Positive expression response to CD105, CD166, and CD29 surface markers, gray peak is indicative of the isotype control.

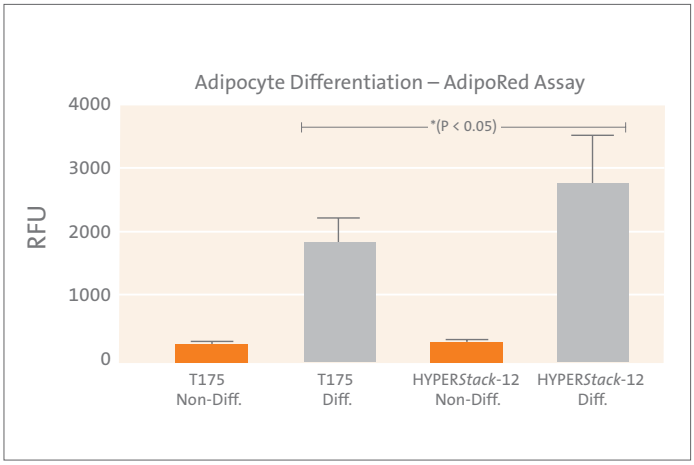


Figure 8. Adipogenic assessment. The AdipoRed™ assay was performed to quantitate adipocyte differentiation. Results indicated statistically higher (paired *t*-test value $p<0.05$) differentiation of cells derived from Corning CellBIND surface HYPER*Stack*-12 vessel compared to cells derived from T-175 Corning CellBIND surface control flask. Results are the average from four independent studies, 3 wells per differentiation condition (n = 12 wells/4 studies).

Table 2. Immunophenotyping assessment of hMSC cultures. Surface marker expression analysis indicated no changes to hMSC line throughout the expansion process. Purity of cells from each expansion was comparable, or better than, the original vendor stock.

		Expansion #1		Expansion #2		Expansion #3		Expansion #4	
		Corning CellBIND Surface		Corning CellBIND Surface		Corning CellBIND Surface		Corning CellBIND Surface	
Culture	Stock Lot Analysis	T-175	HYPER*Stack*-12	T-175	HYPER*Stack*-12	T-175	HYPER*Stack*-12	T-175	HYPER*Stack*-12
IgG2 Iso		0.7%		0.4%		14.0%		0.3%	
CD105	83.25%	97.9%	98.1%	97.6%	97.2%	98.6%	99.1%	99.5%	99.5%
CD166	86.17%	98.7%	99.9%	99.7%	99.7%	99.8%	99.9%	99.9%	99.9%
CD29	92.98%	N/A	N/A	98.8%	99.5%	99.8%	99.8%	99.9%	100.0%
CD14	3.36%	2.2%	0.4%	1.2%	0.4%	0.8%	0.3%	1.5%	0.4%
CD34	0.00%	1.2%	0.5%	2.3%	1.1%	0.2%	0.2%	1.3%	1.0%

For additional verification, hMSCs cultured in the Corning® CellBIND® surface HYPER*Stack*®-12 vessels were also induced to differentiate into chondrocytes using the StemPro® Chondrocygenesis Differentiation kit (Life Technologies™). When MSCs are differentiated into chondrocytes, the cells form a micro-mass of chondrogenic (cartilage) cells. This mass will begin to synthesize extracellular matrix (ECM) proteins such as proteoglycans. To visualize differentiation, Alcian Blue stain was applied to the micro-mass to stain proteoglycans (blue stain) produced by the chondrogenic cells (Figure 9). Based on a visual comparison, there was no difference detected in differentiation between hMSCs cultured on T-175 Corning CellBIND surface control flasks and HYPER*Stack* vessels; however, no histological studies were performed.

The ability of the hMSCs cultured in Corning stemgro® hMSC medium to differentiate into adipogenic and chondrogenic lineages, further demonstrates the capability of this system to support large scale expansion without the loss of cellular function.

Summary/Conclusion

Regenerative medicine, tissue engineering and bio-banking have created a demand for large scale production of hMSCs. Corning stemgro hMSC medium in combination with Corning CellBIND surface and HYPER technology have been successfully utilized as a method to scale-up hMSCs in serum-free conditions while still maintaining all characterizations/criteria of hMSCs.

- stemgro hMSC medium enables serum-free proliferation of hMSC cultures on the Corning CellBIND surface without the need for biological coatings.
- Successful expansion of hMSCs in stemgro hMSC medium can be achieved while retaining the three main criteria used to define MSCs: proliferation, phenotypical characterization and multipotency.
- stemgro hMSC medium enables easy expansion into the HYPER*Stack* cell culture vessel for large-scale expansions. We observed significantly higher rates of adipocyte differentiation of hMSCs harvested from the HYPER*Stack*-12 cell culture vessel.

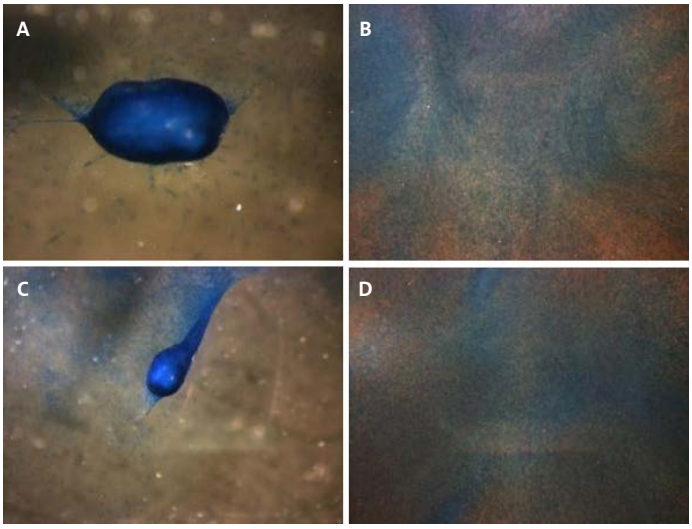


Figure 9. Chondrogenic assessment using Alcian blue stain. Representative example of chondrocyte differentiation from fourth expansion study, Alcian blue staining of proteoglycans (blue) synthesized by chondrocytes. Top: hMSCs harvested from Corning CellBIND surface HYPER*Stack*-12 vessel; (A) differentiated and (B) non-differentiated. Bottom: hMSCs harvested from T-175 Corning CellBIND surface control flask; (C) differentiated and (D) non-differentiated.

Since the original publication of this application note, Corning has discontinued selling stemgro hMSC medium. It is important to note that hMSCs can be expanded using Corning HYPER technology and other stem cell media.

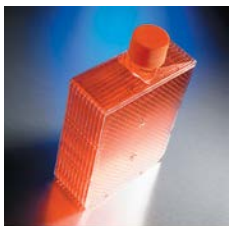
Solutions Applied

A CORNING CUSTOMER CASE STUDY

Researchers from the Ottawa Hospital Research Institute successfully take their innovative research to the next level—scaling up to prepare for large-scale clinical research that may reframe the approach to treating patients with septic shock.

Introduction

Research using the production of mesenchymal stem cells (MSCs) is expanding—leading to clinically relevant discoveries across therapeutic areas. Led by Dr. David Courtman, Director Biotherapeutics, the Ottawa Hospital Research Institute's Cell Manufacturing Facility successfully conducted a Phase I clinical trial examining the safety and efficacy of allogeneic bone marrow-derived MSCs as a treatment for patients experiencing septic shock.



Corning HYPERFlask Cell Culture Vessels were used to manufacture single dose treatments for patients with septic shock.

In their Phase I trial, freshly cultured allogeneic bone marrow-derived MSCs were dosed into patients with septic shock.[1] A single dose was manufactured using the Corning® HYPERFlask® on an as-needed basis. Since dosing was developed on an emergent per-patient basis, researchers were notified of a patient enrollment and had to deliver the dose within 6 hours.

The Challenge? Large-Scale Production of MSCs in a Space-restricted Facility

Larger-phase clinical trials were needed to determine true clinical impact and this required a significant scale-up of MSCs. That is what Courtman and his colleagues tackled next.

Before initiating clinical Phase II studies, the protocol for generating large quantities of MSCs had to be established to reduce end-product variability, minimize production costs, and to ensure sufficient yield to treat multiple patients. Maximizing the yield of viable cells proved challenging in a limited footprint.

"Hospital-based cell manufacturing facilities often have significant space and staffing constraints making the more standard scale up process difficult to accomplish. The Corning HYPER*Stack* system provides a much more manageable platform to reach batch production levels appropriate for the typical scale up required in academic trials with MSCs."

—Dr. David Courtman
Director Biotherapeutics

Perfecting the Protocol with Customized Support from Corning

For the Phase I trial, the cell manufacturing lab utilized the Corning HYPERFlask to grow and reproduce allogeneic bone marrow-derived MSCs. To take the research to the next level, researchers needed to be able to work within the same isolator units, following the same protocol, with successful, reproducible cell culture on a much larger scale. "We were aware of the Corning® HYPER*Stack*® and needed to make the most of our limited footprint while scaling up and maintaining successful cell harvest. The Corning HYPER*Stack* seemed like the logical next step," says Saad Khan, Senior Research Associate in the Cell Manufacturing Facility and lead researcher in the scale-up study.

"We were aware of Corning HYPER*Stack* and needed to utilize the same footprint while scaling up and maintaining successful cell harvest. The Corning HYPER*Stack* seemed like the logical next step."

- Saad Khan
Senior Research Associate

Using the Corning HYPERStack cell culture vessels, Khan and colleagues successfully scaled up the production of bone marrow-derived MSCs while maintaining cellular characteristics such as identity and potency.[2]

Corning HYPERStack cell culture vessel provides increased surface area which enables more cell growth per volumetric footprint compared to a traditional stacked vessel.

Says Courtman, "We were concerned about the challenge of getting the cells out." Michael Bagguley, Bioprocess Specialist, stepped in to quickly assist the lab in creating a customized strategy for scale-up. "Michael was extra helpful in providing consultation on manipulating the cells in the vessel, cell seeding, media changes, and harvesting—all within the isolator units. He was there to offer suggestions and expertise along the way," adds Courtman.

Potential Significant Impact on Patients' Lives

For the cell manufacturing team, the end goal is to develop a safe, potent, and effective cell therapy product. "If successful, this could have a significant impact on the course of disease for patients," says Courtman.

Learn more about this research and other innovative discoveries taking place at the Ottawa Hospital Research Institute by visiting http://www.ohri.ca/home.asp

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Efficient Expansion of Human Mesenchymal Stem Cells (hMSCs) on Corning® Enhanced Attachment Microcarriers Using a Continuous Agitation Protocol

Application Note

CORNING

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Introduction

Microcarriers are small spheres used to culture adherent cells at scales that are impractical using traditional two-dimensional (2D) cultureware. Microcarriers maximize the surface area for adherent cell growth while minimizing the total culture volume and/or footprint. Microcarrier-based cultures, in which cells are grown on suspended microcarrier beads, can be maintained at small scale in spinner flasks (100 mL to 3L) or at large scale in stirred tank bioreactors (5L to 10,000L). Although 2D culture methods are well established for the majority of cell types, protocol development and optimization are required for microcarrier-based cell culture. Usually, optimization is accomplished at small scale in spinner flasks and utilizes offline measurements to monitor cell growth, gas levels, and nutrient exchange. Culture conditions are optimized to enable a uniform cell distribution on microcarriers with minimal shear while allowing sufficient exchange of nutrients and gases.

Corning has commercialized sterile, ready-to-use microcarriers with different surface chemistries for different cell types. Here we describe a protocol for human mesenchymal stem cell (hMSC) culture on Corning microcarriers in 1L glass spinner flasks. Two main challenges for microcarrier culture with hMSCs are: (1) these primary cells are sensitive to agitation, especially during cell attachment[1,2], and (2) they tend to form large cell-microcarrier aggregates during expansion[3,4]. Our goal was to develop a continuous agitation protocol for efficient (>8-fold) expansion of hMSCs with minimal microcarrier clumping, while maintaining cell multipotency. The following parameters were evaluated: cell seeding density, microcarrier surface type, microcarrier concentration, agitation rate, working culture volume, and serum concentration during cell attachment. We achieved consistent hMSC yield of >40,000 cells per cm^2 (160,000 cells/mL) across multiple cell donors with minimal microcarrier clumping (2 to 3 beads/clump). Higher cell densities could be attained but with larger clump sizes (>5 beads/clump). After culture on microcarriers, cells retained typical spindle-like morphology, multipotent cell surface marker expression profile, and normal karyotype.

Materials and Methods

Cells

- Bone marrow-derived human mesenchymal stem cells (AllCells Cat. No. MSC-001F)

Microcarriers

- Corning® enhanced attachment microcarriers (Corning Cat. No. 3779)
- Corning Collagen-coated microcarriers (Corning Cat. No. 3786)
- 1L glass spinner flask (Corning Cat. No. 4500-1L), siliconized with Sigmacote® siliconizing reagent (Sigma Cat. No. SL2-100ML)

Vessels

- Corning CellBIND® surface 175 cm^2 angled neck cell culture flask with vent cap (Corning Cat. No. 3292)
- Corning CellBIND HYPER*Flask*® M cell culture vessel (Corning Cat. No. 10020)
- Corning CellBIND surface 6-well clear multiwell plates (Corning Cat. No. 3335)

Media and Reagents

- DMEM medium (Corning Cat. No. 10-013)
- MSC-qualified fetal bovine serum (Life Technologies Cat. No. 12662-011)
- Sterile, cell culture grade water (Corning Cat. No. 25-055-CM)
- DPBS (Dulbecco's phosphate buffered saline) (Corning Cat. No. 21-031-CV)
- TrypLE™ Select enzyme (1X) (Life Technologies Cat. No. 12563-011)

Consumables

- Corning 70 µm cell strainer (Corning Cat. No. 431751)
- Corning Easy Grip polystyrene storage bottles (Corning Cat. Nos. 431175, 430282)
- Stripette™ serological pipets (Corning Cat. Nos. 4487, 4488, 4489, 4490, 4491)
- Falcon® 50 mL high clarity PP centrifuge tube (Corning Cat. No. 352070)

Lab Equipment

- Wheaton Micro-Stir® magnetic stirrer (VWR Cat. No. 12000-908)
- Vi-CELL® Analyzer (Beckman Coulter Cat. No. 731050)

hMSC Scale-up from Thaw

hMSCs (designated as passage 1) were thawed from a cryogenic storage vial and seeded at 5,000 cells/cm² on Corning® CellBIND® surface T-175 flasks in 10% hMSC-qualified FBS/DMEM.

NOTE: the cell seeding density of 5,000 cells/cm² was selected for optimal passage [e.g., every 5 days]. Seeding density should be optimized for different cell donors and/or expansion rate.

Cells were sub-cultured after 5 days when 75% confluent (~20,000 cells/cm²) and seeded at 5,000 cells/cm² on Corning CellBIND surface HYPER*Flask*® vessels (passage 2). At 75% confluence, cells were harvested (passage 3) and seeded onto microcarriers (1,600 cm² per 1L siliconized glass spinner flask, 11 g/L) at 6,000 cells/cm² in a 400 mL final volume.

NOTE: a slightly higher seeding density may improve cell attachment when transitioning from 2D to microcarrier cultures.

Cell Attachment and Expansion on Microcarriers

Cell attachment to microcarriers occurred under constant agitation at 15 rpm for 20 hours. After the attachment phase, the agitation was increased to 30 rpm. Microcarrier clumping was monitored daily, and the agitation was increased by 10 rpm every 2 days starting on day 3 to prevent microcarrier clumping. The maximum agitation rate was 80 rpm. Half-volume medium changes were performed on day 3 and then every 2 days thereafter.

Cell Counts

For daily assessments of cell growth, 5 mL samples were collected, and cells were harvested from microcarriers via a 5-minute TrypLE™ treatment followed by filtration through a 70 µm cell strainer. Cell number and viability were assessed using an automated Vi-CELL analyzer.

Cell Characterization

Morphology

To assess cell morphology after microcarrier culture, cells were removed from microcarriers using TrypLE and re-seeded onto Corning CellBIND surface 6-well plates.

hMSC Surface Markers

Cells were removed from microcarriers using TrypLE, washed with PBS, and fixed in 4% paraformaldehyde. Quantitative phenotypic marker expression was evaluated for CD73, CD90, CD105, CD14, and CD34 via standard immunofluorescent staining and flow cytometry.

Karyotype

Cells were removed from microcarriers using TrypLE and re-seeded into Corning CellBIND surface T-75 flasks for karyotype analysis at WiCell Research Institute.

A

	Passage 1	Passage 2	Passage 3
Surface	Corning CellBIND	Corning CellBIND	Corning Enhanced Attachment Microcarriers
Vessel	T-Flask	Corning HYPER*Flask* vessel	1L glass spinner flask
Surface area (cm²)	175	1,720	1,600
Cell yield (millions)	3.5	35	64

Figure 1A. hMSC scale-up for glass spinner flask inoculum.

B

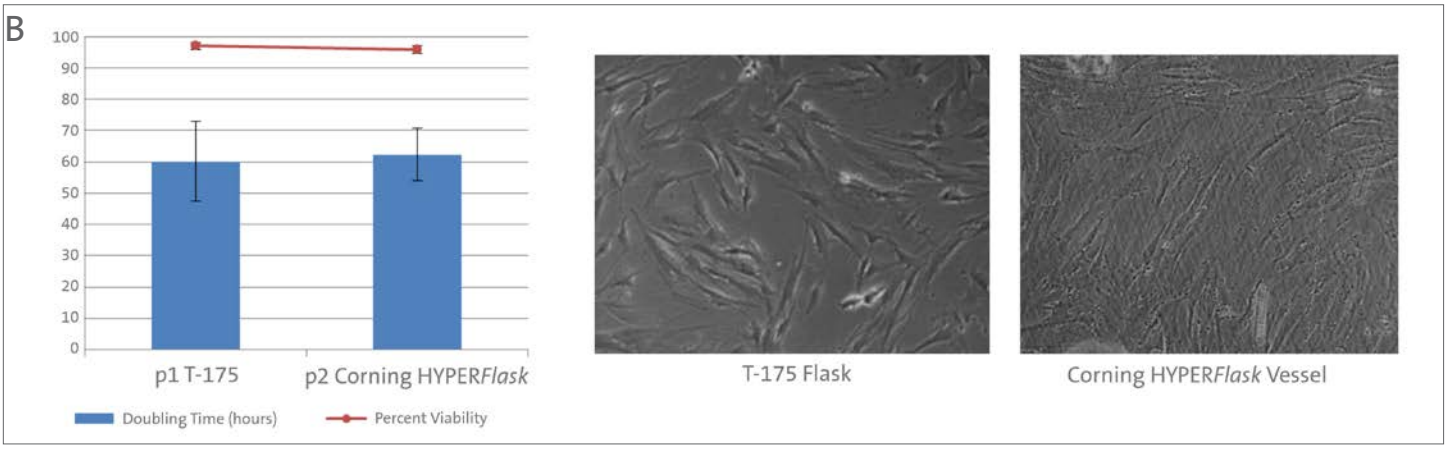

Figure 1B. No significant change in cell morphology, doubling time, or viability was observed for hMSC scale-up.

Results and Discussion

hMSC Expansion Protocol to Generate Sufficient Number of Cells for Microcarrier Inoculum

For microcarrier culture at liter(s) scale, 20 to 100 million cells are required for cell seeding. Due to limited hMSC yields at isolation, an efficient and consistent method is required to generate a large number of cells for a microcarrier inoculum. Here we describe a cell scale-up protocol using Corning HYPER*Flask* vessels. As shown in Figure 1A, passage 1 cells were thawed into T-175 flasks and then passaged to HYPER*Flask* vessels before being seeded in glass spinner flasks. This scale-up method was evaluated twice for 3 bone marrow donors, and no significant change in cell morphology, doubling time, or viability was observed (Figure 1B, error bars represent n = 6). This method was used to generate the cell inoculum required for the optimization of microcarrier culture conditions in 1L glass spinner flasks.

Continuous Agitation Protocol for Attachment and Expansion of hMSC on Corning Microcarriers in 1L Glass Spinner Flasks

To determine optimal culture conditions, cells were seeded on microcarriers in 1L glass spinner flasks (Figure 2A). Cultures were expanded for 9 to 11 days, after which cells were evaluated for percent viability, total yield, and retention of typical morphology, surface marker expression, and normal karyotype. The following culture parameters were evaluated during protocol optimization: cell seeding density, microcarrier surface type, microcarrier concentration, agitation rate, working culture volume, and serum concentration during attachment. Using the optimized parameters shown in Figure 2B, cells were expanded on microcarriers
under continuous agitation in glass spinner flasks. Cell yields greater than 50,000 cells/cm^2 (200,000 cells/mL) were achieved while maintaining high cell viability (Figure 3A). Duplicate spinner flask cultures were evaluated for each microcarrier type. Small microcarrier clump size (2 to 3 microcarriers per clump) was maintained with Corning® enhanced attachment microcarriers by increasing the agitation rate after medium re-feeds (Figure 3B, phase images). For Corning Collagen-coated microcarriers, it was more difficult to maintain small clump size with agitation, resulting in sampling error. Therefore, there are larger error bars starting at day 6 (Figures 3A graph and 3B phase images). Day 7 cells were stained with a viability stain, Calcein AM, to better visualize cell morphology, microcarrier coverage, and microcarrier clump size (Figure 3B, FITC images).

A

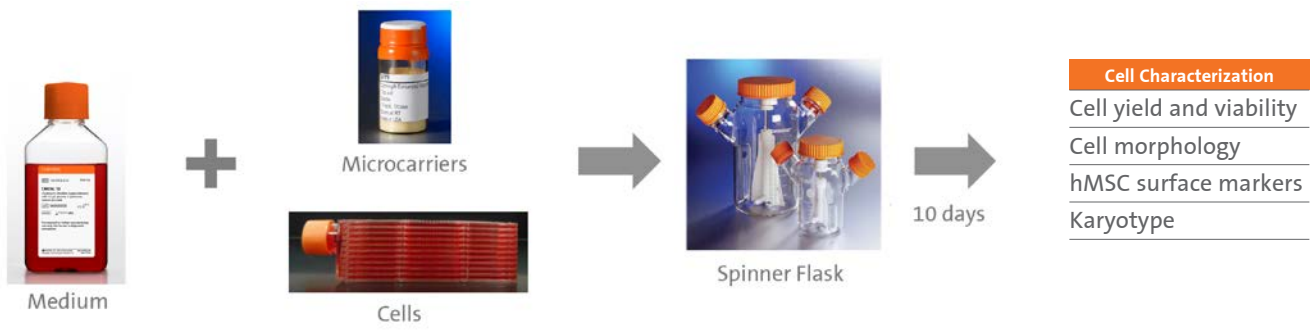

Figure 2A. Experimental workflow for optimization of microcarrier culture conditions

B

Attachment	
Cell seeding density	6,000 cells per cm^2
Serum concentration	10%
Microcarrier surface	Enhanced attachment
Microcarrier concentration	4 cm^2/mL (11 g/L)
Agitation rate	Continuous at 15 rpm for 20 hours
Working culture volume	400 mL
Vessel format	1L glass spinner flask
Expansion	
Replenish medium	50% volume; days 3, 5, 7, 9, and 11
Agitation rate	Continuous at 30 rpm to 80 rpm
Microcarrier clump size	2 to 3 microcarriers/clump
Agitation rate to control microcarrier clump size	Increase by 10 rpm on day of re-feed
Duration of culture	10 days (>40,000 cells/cm^2)

Figure 2B. Optimized conditions for hMSC attachment and expansion on Corning enhanced attachment microcarriers in 1L glass spinner flask.

After 10 days, cells were removed from microcarriers using TrypLE™ and re-seeded onto Corning® CellBIND® surface 6-well plates at 5,000 cells/cm^2. As shown in Figure 3C, cells retained typical elongated morphology, normal 46, XY karyotype, and high expression of hMSC surface markers, CD73, CD90, and CD105, after culture on enhanced attachment and Collagen-coated microcarriers.

Validation of the Continuous Agitation Protocol Across Multiple hMSC Donors

hMSCs isolated from different bone marrow donors often have different recovery and expansion rates after cryopreservation. To test the robustness of our protocol, hMSCs from 3 bone marrow donors were thawed and expanded on Corning enhanced attachment microcarriers as shown in Figure 1A (scale-up) and Figure 2B (microcarrier culture conditions). For each hMSC donor, two independent experiments were performed from thaw to microcarrier expansion. As shown in Figure 4, high cell yields (>40,000 cells/cm^2) were observed for all donors, confirming the robustness of this protocol. As anticipated with the variability in hMSCs from different donors, a higher rate of cell expansion was observed for donor 4880 compared to donors 4853 and 4415.

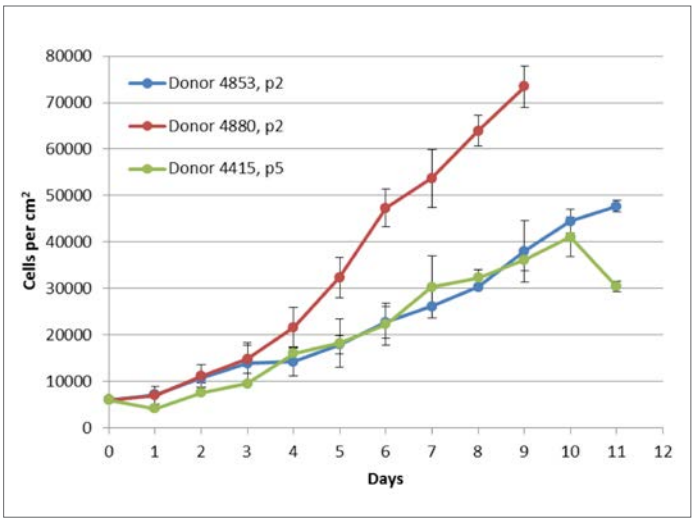

Figure 4. Protocol consistency was demonstrated across three hMSC bone marrow donors.

Conclusions

- hMSCs attached to and expanded on Corning enhanced attachment microcarriers using continuous agitation in 1L glass spinner flasks (from 6,000 cells/cm^2 to >40,000 cells/cm^2 in 10 days).
- Microcarrier clump size was maintained at <5 microcarriers per clump by increasing the agitation rate after culture re-feeds every 2 to 3 days.
- hMSCs cultured on Corning enhanced attachment microcarriers maintain standard morphology, a typical phenotypic marker expression profile, and normal karyotype.

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Amplifying Adenoviral Particles in the Corning® HYPER*Stack*® Cell Culture Vessel

Application Note

CORNING

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Introduction

Adenoviruses and other viral systems are routinely used in research and industrial applications[1,2]. Reports indicated that the use of adenoviruses for the delivery of transgenes is one of the most commonly used tools in both *in vitro* and *in vivo* research[2]. Additionally, adenovirus transductions have been recently used at both the preclinical and clinical stages in cell therapy and vaccine production leading to an increase in demand to produce more virus as efficiently as possible[1,2].

To allow researchers and vaccine manufacturers the opportunity to produce even higher yields in the same spatial footprint as stacked vessels, Corning offers the HYPER*Stack* cell culture vessel. The Corning HYPER*Stack* vessel features Corning's HYPER (**H**igh **Y**ield **PER**formance) technology which consists of a gas permeable film as the attachment surface, eliminating the air headspace requirement in traditional vessel types. This approach provides an increase in the number of layers and corresponding cell growth surface area compared to traditional rigid single or multi-layered culture vessels.

The focus of this study was to determine the efficacy of generating amplified virus using the unique Corning HYPER technology. Standard methodologies utilize traditional stacked vessels for virus generation. Utilizing the HYPER*Stack* vessel, researchers can generate similar 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 HYPER*Stack* vessel led to similar titers but larger yields compared to a standard 2-layer stacked vessel.

Methods and Materials

Cell Culture

HEK-293AD cells (Cell BioLabs Cat. No. AD-100) were maintained in DMEM without sodium pyruvate (Corning Cat. No. 10-017-CM), 10% FBS, and 1X MEM Nonessential Amino Acids (Corning Cat. No. 25-025-Cl).

Transduction of HEK-293AD Cells

Cells were seeded onto a Corning CellBIND® surface 2-layer CellSTACK® (Corning Cat. No. 3310) or Corning HYPER*Stack* 12-layer vessel, Corning CellBIND surface treated (Corning Cat. No. 10012) at 45,000 cells/cm^2 (0.217 mL/cm^2) and incubated overnight at 37°C, 5% CO_2, 98% relative humidity. The following day, the medium was removed and combined with adenovirus encoding Green Fluorescent Protein (GFP) (Multiplicity of Infection [MOI] 10). The medium was then added back to each vessel. The amount of virus (mL) added to each vessel was calculated using the following formula:

$$\frac{(\text{Cells/cm}^2)\ [\text{cm}^2 \text{ of Well}])\ \times\ (\text{MOI 10 [IFU/Cells]})}{(\text{IFU/mL})}$$

An MOI of 10 was selected to reach the desired cytotoxic effect (<50% cells remained) in 72 hours. The crude adenovirus added to each vessel was prepared as described in Generating Crude Adenoviral Particles in the Corning HYPER*Flask* Vessel (CLS-AN-213). GFP expression and cell morphology were monitored throughout the course of the experiment using the Olympus IMT-2 inverted fluorescence microscope. For tips on cell visualization in a HYPER*Stack*-12 vessel, see Corning Application Note: Options to Visualize Cells in Corning HYPER*Stack*-12 Vessels (CLS-AN-174).

Adenovirus Harvest

The cells and medium were collected 72 hours post-transduction (Figure 1). To collect the cells from the HYPER*Stack* vessel, PBS (without Ca^{2+} and Mg^{2+}) (Corning Cat. No. 21-040-CM) was added to each vessel (0.033 mL/cm^2) and incubated at 37°C for 3 to 5 minutes. To collect cells from the stacked vessel, 2 to 3 PBS washes were necessary to remove all cells. To minimize volume during freeze-thaw cycles, the cells were pelleted in a centrifuge

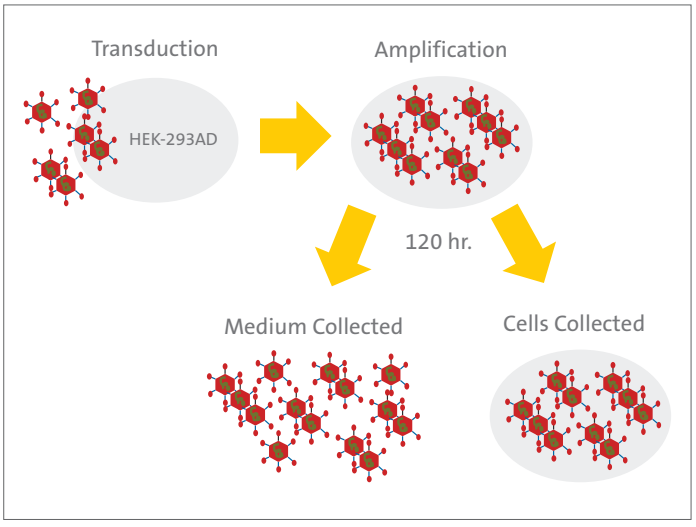

Figure 1. Schematic of adenovirus production.

at 500 x g for 10 minutes at 4°C, and the cell pellet was resuspended in 10 mM Tris, pH 8.0, 100 mM NaCl (0.023 mL/cm^2). The medium was retained, aliquoted into 50 mL centrifuge tubes (Corning Cat. No. 430921), and stored at -80°C to be titered later. The total volume of the medium was also recorded. The cell suspension was then subjected to three freeze-thaw cycles (-80°C/37°C), then centrifuged at 3,000 x g for 10 minutes at 4°C to pellet the cell debris and the supernatant containing the adenoviruses released from the cell suspension was collected. The recovered adenovirus encoding GFP was also aliquoted and stored at -80°C.

Adenovirus Titer

The QuickTiter™ Adenovirus titer ELISA kit was purchased from Cell BioLabs (Cat. No. VPK-110). The ELISA assay was performed as described previously (CLS-AN-213) and the signal in the wells was measured utilizing a PerkinElmer EnVision® Multilabel Reader.

Functional Analysis of Adenovirus

MDBK and Vero cells were transduced with adenovirus obtained from both vessels and analyzed via flow cytometry as described previously (CLS-AN-213).

Results

Cell Morphology and GFP Expression

To assess adenoviral production on a 2-layer stacked vessel compared to a Corning® HYPER*Stack*® vessel, HEK-293AD cells were transduced with adenovirus encoding GFP. GFP expression and cell morphology were monitored throughout the course of the experiment. The cells and medium were collected when less than 50% of the cell population remained attached to each vessel. Similar cell morphology and GFP expression were observed in both vessels (Figure 2) at the time of harvest.

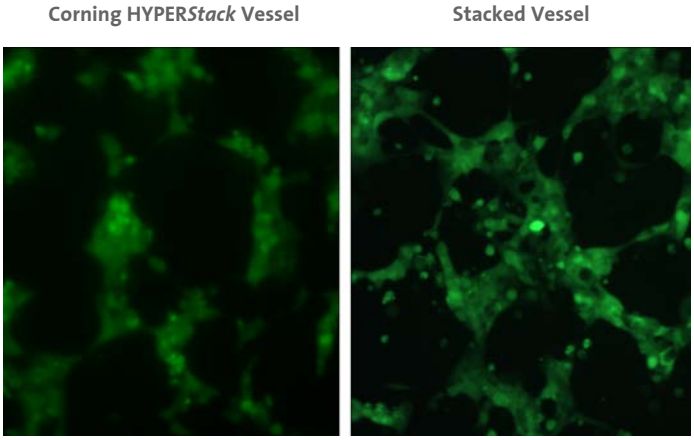

Figure 2. Similar cell morphology and GFP expression was observed between vessels. Representative images from the same experiment demonstrating morphology/GFP expression on the day of harvest of the HEK-293AD cells. Cells and medium were collected 72 hours post-transduction. Images obtained using an Olympus IMT-2 inverted fluorescence microscope. Magnification 100X.

Adenoviral Production

The viral particles obtained from either the cells or medium remained in two different fractions throughout the course of the study to (i) minimize processing and (ii) demonstrate the viral yields obtained from both fractions. For large-scale production, the cells may be lysed by either lowering the ionic strength (hypotonic shock) or with the aid of mild pressure changes that can be induced by a microfluidizer® (Microfluidics) or cross-flow filtration system[3]. Once collected, the titer of adenovirus encoding GFP from each vessel (either from cells or medium) was determined using the QuickTiter ELISA kit to quantitate infectious forming units (IFU)/mL. Similar titers were obtained from both vessels (Figure 3). The average titer obtained from

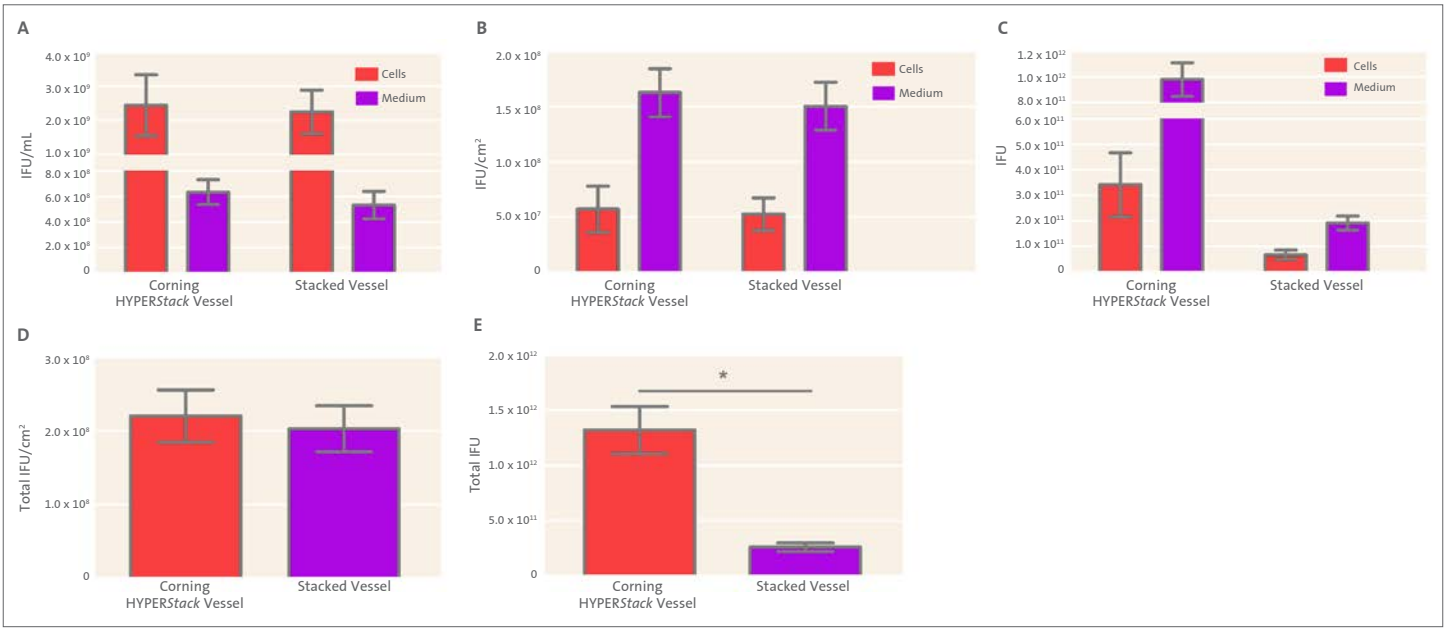

Figure 3. The Corning HYPER*Stack* vessel leads to equivalent viral production per cm² compared to a stacked vessel. (A) Direct comparison between the HYPER*Stack* vessel and stacked vessel titers obtained using the QuickTiter ELISA Adeno kit. (B, D) When normalized on a per cm² basis, the HYPER*Stack* yielded similar infectious adenoviral particles. (C, E) The HYPER*Stack* vessel generated a significantly higher amount of total infectious adenoviral particles. (D, E) Total infectious adenoviral particles were calculated based on titers and the volume of each fraction (cells and medium) Paired t-test, * p <0.05, N=3.

the adenoviruses recovered from the cells were 2.4 x 10^9 IFU/mL and 2.2 x 10^9 IFU/mL from Corning® HYPER*Stack*® and stacked vessels, respectively (Figure 3A). The average titer obtained from the medium was 6.3 x 10^8 IFU/mL and 5.3 x 10^8 IFU/mL from HYPER*Stack* and stacked vessels, respectively (Figure 3A). When normalized based on surface area, similar IFU/cm^2 were observed with both vessels (Figures 3B and 3D). However, since the HYPER*Stack*-12 vessel has a larger surface area compared to a 2-layer stacked vessel there was a significant increase in total viral yield (>5 times) (Figures 3C and 3E). These results indicate that adenovirus particles may be generated in the HYPER*Stack* vessel with similar titers but larger yields compared to the standard 2-layer stacked vessel.

GFP Expression in Vero and MDBK Cells

To verify that the virus obtained from the HYPER*Stack* vessel was as functional as virus obtained from the 2-layer stacked vessel, Vero and MDBK cells were transduced with amplified adenovirus encoding GFP. Each cell type was transduced with virus obtained from either the HYPER*Stack* or stacked vessel at MOI of 100. After 72 hours, the cells were collected and analyzed via flow cytometry. After three independent experiments, the average GFP fluorescence in each cell line with each adenovirus was greater than 95% (Figure 4). Cells were transduced at MOI of 100 to ensure high expression. Previous results also demonstrated equal GFP expression regardless of the vessel when transduced

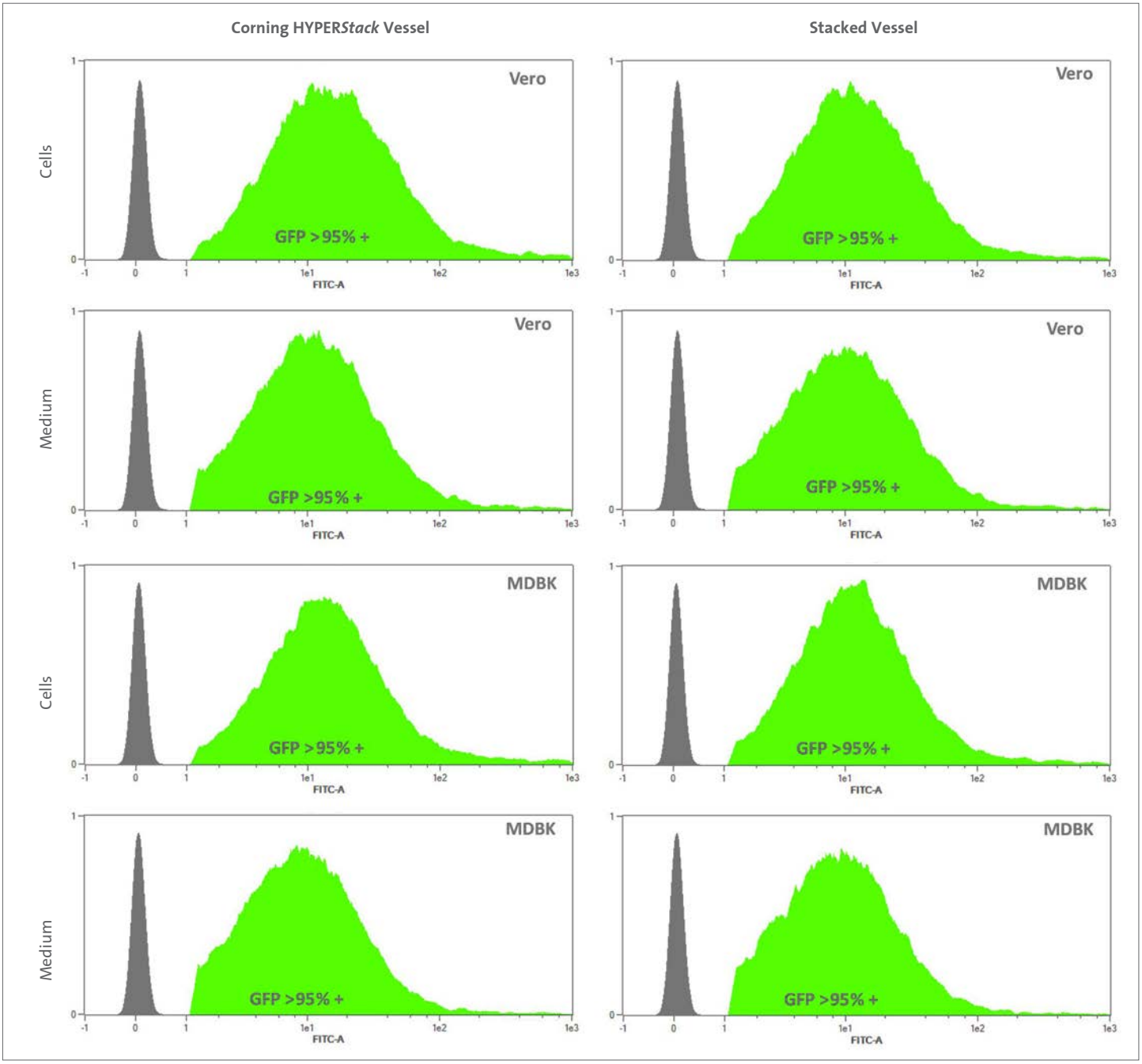

Figure 4. Vero and MDBK cells transduced with adenovirus exhibit similar GFP expression levels. Representative flow cytometry data shows the expression of GFP (green) compared to a negative control of non-transduced cells (black). After three independent experiments, the GFP expression in both the Vero or MDBK cells was greater than 95% regardless of which vessel or fraction the virus was generated in.

at lower MOIs (10, 50, and 100) for shorter time periods (24 and 48 hours). GFP expression from each experiment varied between 30% to 95% depending on a) MOI and b) time (data not shown). These data indicate that the virus obtained from the Corning® HYPER*Stack*®-12 vessel is as infectious as virus obtained from a 2-layer stacked vessel.

Summary

- This study demonstrates the utility of the HYPER technology in adenovirus production.
- Adenoviral particles can be amplified in the Corning HYPER*Stack* vessel at similar titers compared to traditional tissue culture vessels, allowing for greater virus production in a smaller footprint.
- Adenoviral particles generated on the HYPER technology platforms also exhibit similar levels of infectivity as in a traditional vessel.

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Generating Lentiviral Particles in the Corning® HYPER*Stack*® 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 HYPER*Stack* cell culture vessel. The Corning HYPER*Stack* vessel features Corning's HYPER (**H**igh **Y**ield **PER**formance) technology, which consists of a gas permeable film that serves as the cell attachment surface and eliminates the headspace within a vessel (Figure 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 HYPER*Stack* 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 Cat. No. 10-013-CM), 10% FBS (Corning Cat. No. 35-010-CV), and 1X MEM Nonessential Amino Acids (Corning Cat. No. 25-025-CI).

DNA Preparation

GC10 competent cells (Sigma 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 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 Axygen® AxyPrep Plasmid Maxiprep kit (Corning Cat. No. AP-MX-P-25) and quantified with the EnVision® multimode plate reader (PerkinElmer).

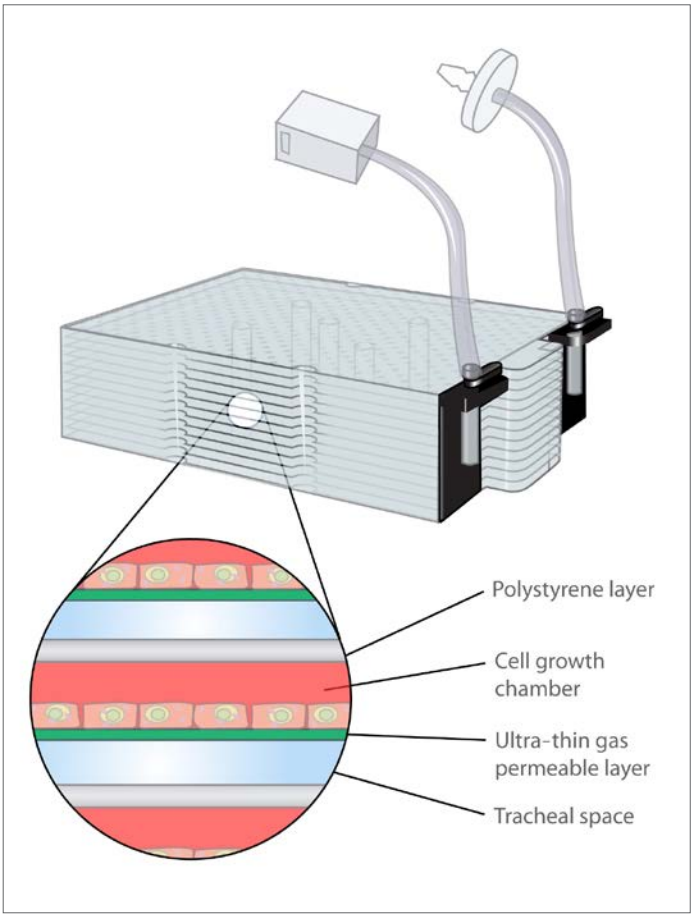

Figure 1. Corning HYPER technology eliminates the headspace found in traditional cell culture technology.

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 7,400 cm^2 (6,000 cm^2 [Corning® HYPER*Stack*®-12 vessel] + 1,272 cm^2 [comparison 2-layer Corning CellSTACK® chamber] + 128 cm^2 [extra]). To prepare the mix, 48 mL of freshly made 2X HBS buffer (50 mM HEPES, 250 mM NaCl, 1.5 mM Na_2HPO_4, 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/cm^2:

- 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 Cat. No. 25-055-CV) and 1 M $CaCl_2$ (Sigma 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 $CaCl_2$-DNA solution was added, the mixture became cloudy. Each experiment was performed three independent times. **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 Corning CellBIND® surface (Corning Cat. No. 3310) and a Corning HYPER*Stack*-12 cell culture vessel (Corning Cat. No. 10012) at 100,000 cells/cm^2 (0.217 mL/cm^2) and incubated overnight at 37°C (5% CO_2, 98% relative humidity). The following day, the media was removed 8 hours before the transfection and replaced with fresh media (0.217 mL/cm^2) containing 25 µM chloroquine (Sigma Cat. No. C6628). **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., HYPER*Stack*-36 vessel), the medium containing chloroquine may need to incubate longer (e.g., >8 hr.) 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:$CaPO_4$ solution prepared above was added drop by drop at a rate of approximately 7 mL/min. The medium containing the DNA:$CaPO_4$ solution was added 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, then collected 48 hours later. GFP expression was monitored throughout the course of the experiment using the AMG EVOS® Fl 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 multivesicular 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 3,000 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.

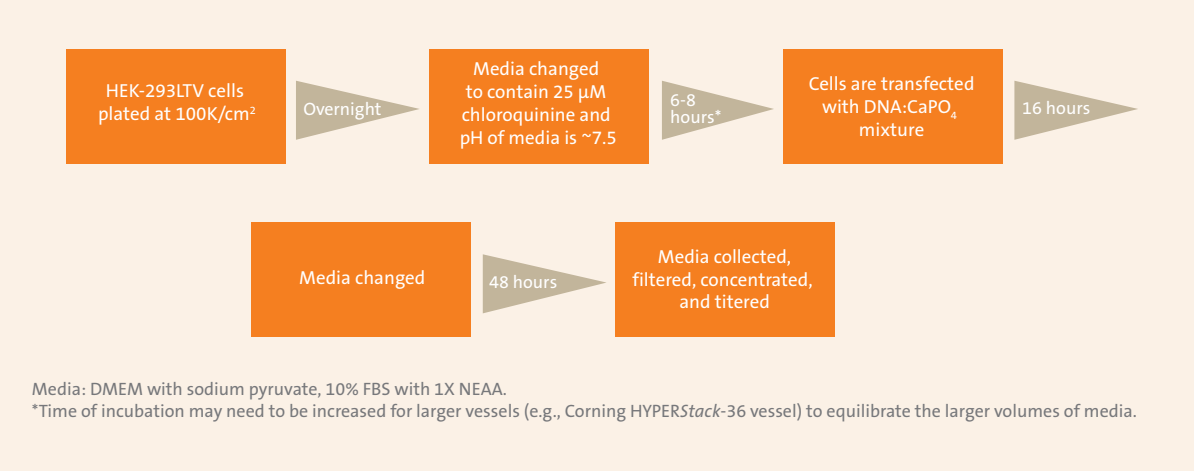

Figure 2. Protocol for $CaPO_4$ transfections using the Corning HYPER*Stack*-12 cell culture vessel.

Lentivirus Titer

The Lenti-X™ qRT-PCR titration kit (Clontech Cat. No. 631235) was used to perform the assay 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, 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/cm^2 and incubated overnight at 37°C (5% CO_2, 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 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] * [cm^2 of well] * (MOI 10 [TU/cells]) / (TU/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 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® HYPER*Stack*®-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 (Figure 3) throughout the course of the experiment.

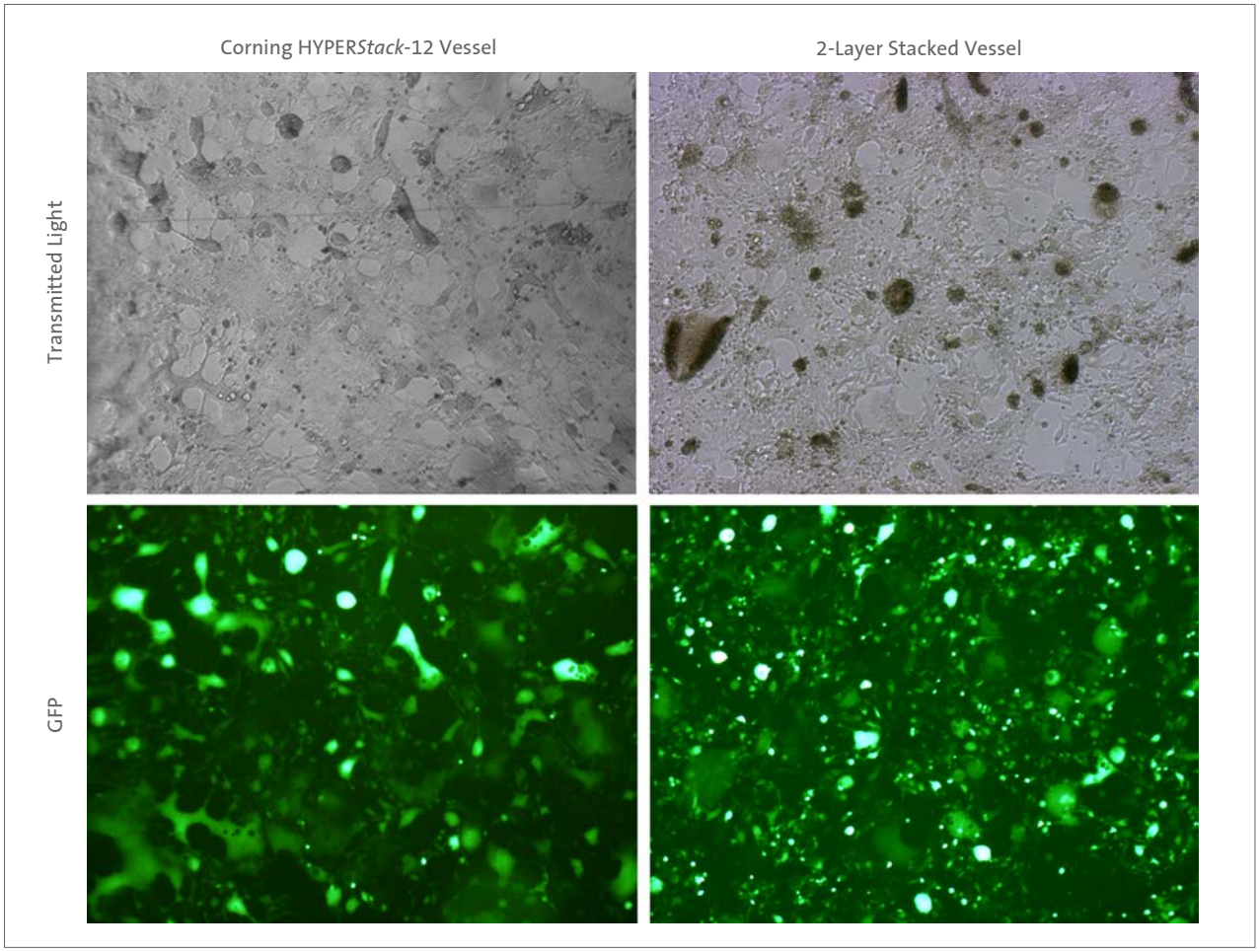

Figure 3. Analysis of GFP expression in the Corning HYPER*Stack* vessel. Representative images from the same experiment demonstrating similar GFP expression in the HYPER*Stack* vessel and 2-layer stacked cell culture vessel. These trends were observed with all experiments. Images obtained using an Olympus 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® HYPER*Stack*® vessel yielded similar copies/mL (Figure 4A), similar copies/cm² (Figure 4B), and approximately 5 times more copies (Figure 4C) compared to lentivirus obtained from the 2-layer stacked vessel. These results indicate that lentivirus particles may be generated in the HYPER*Stack* 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 HYPER*Stack* 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% (Figure 5, two independent experiments). Taken together, these results indicate that the HYPER*Stack* vessel produces similar infectious lentiviral particles per cm² compared to a traditional stacked vessel.

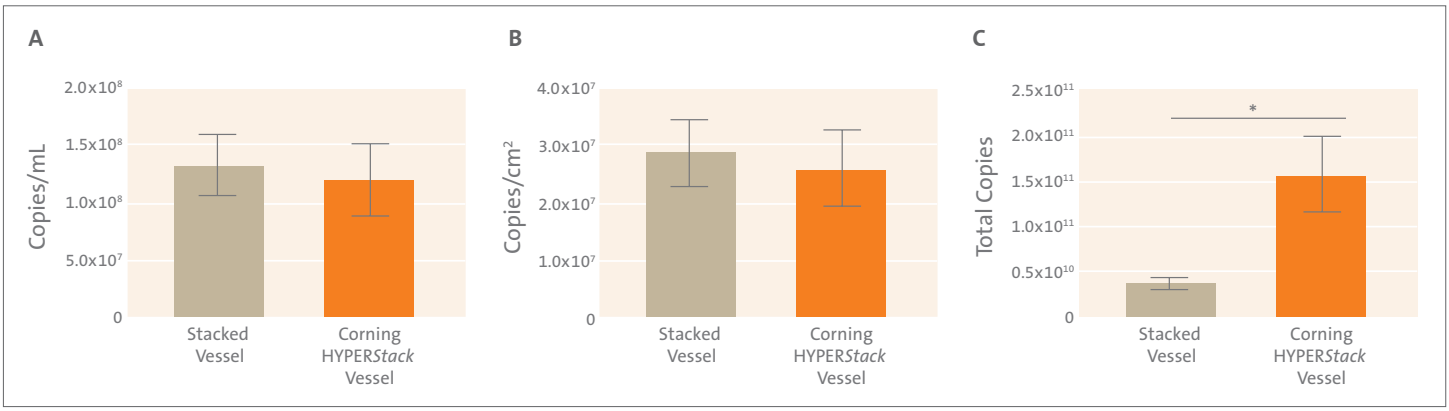

Figure 4. The Corning HYPER*Stack* vessel supports comparable viral production with a higher yield of total virus compared to a 2-layer stacked vessel. (A) Titers obtained from the Lenti-X™ qRT-PCR titration kit. (B) When normalized on a per cm² basis, the HYPER*Stack* vessel yields similar amount of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

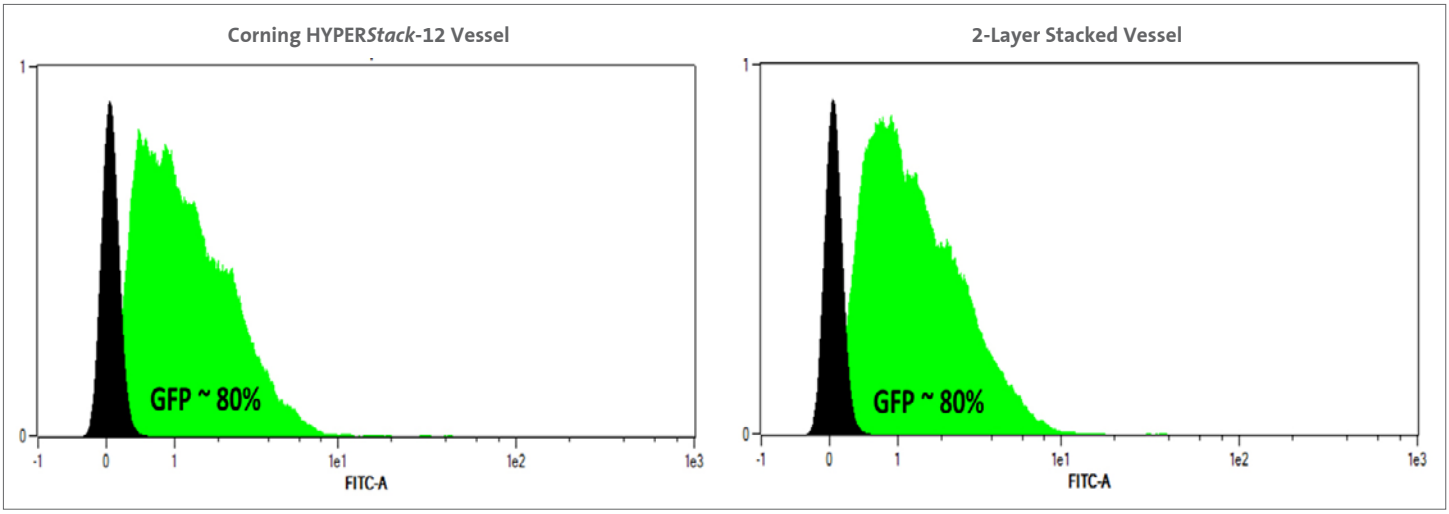

Figure 5. MDBK cells transduced with lentivirus exhibit comparable levels of GFP expression. Representative flow cytometry data show the expression of GFP (green) compared to a negative control of non-transduced cells (black). After two independent experiments, the GFP expression in MDBK cells was greater 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® HYPER*Stack*® 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.

Using the Corning® *HYPERStack*® Cell Culture Vessel and the Enhanced Attachment Microcarriers for Scale-up and Production in the Vaccine Industry

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Introduction

Viral transductions are used at the preclinical and clinical stages in the vaccine industry leading to an increase in demand to produce more virus more efficiently. The Corning® HYPER technology and microcarrier beads offer the ability to increase efficiency by increasing surface area without increasing spatial footprint. The focus of this study was (i) to determine the efficacy of generating Adeno- and lentiviral particles using the unique Corning HYPER technology, and (ii) to evaluate the use of the Enhanced Attachment microcarrier beads on cell lines typically used in the vaccine-producing industry (e.g. Vero, and HEK-293).

To assess viral production on the HYPER technology HEK-293AD (adenovirus) or HEK-293LTV (lentivirus) were transduced (adeno) or transfected (lenti) on the HYPER*Stack*-12 to produce the viral particles. The results demonstrated that adeno- and lenti-viral particles can be generated in the Corning HYPER*Stack*® vessel at similar titers compared to traditional tissue culture vessels, while allowing for greater virus production in a smaller footprint.

To optimize cell scale-up on the Corning microcarrier beads various conditions were evaluated using multiple vaccine producing cell types. The data presented here demonstrate Vero and HEK-293AD cell expansion on Corning Enhanced Attachment microcarriers.

Methods and Results

Using the Corning HYPER*STACK* for lentiviral production

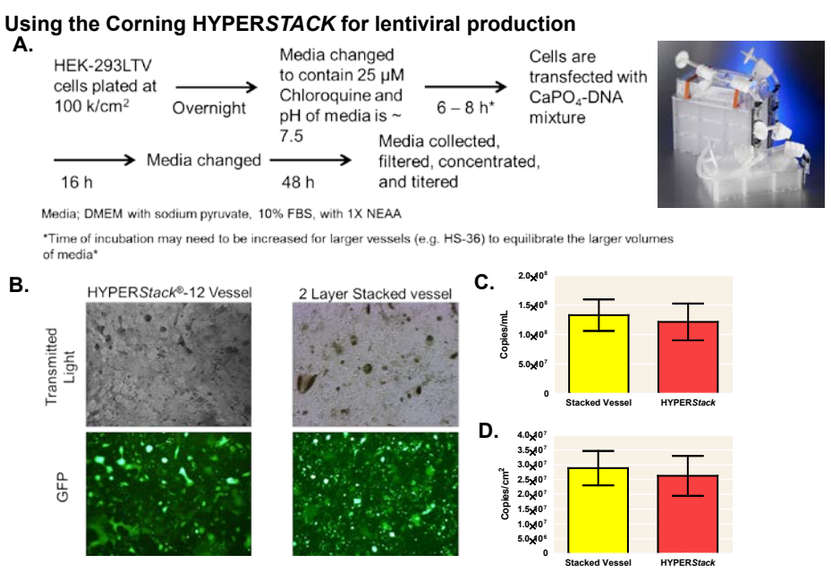

Figure 1. The Corning HYPER*Stack* -12 vessel supports comparable viral production, with a higher yield of total virus compared to a 2 layer stacked cell culture vessel. (A) Experimental outline. Media components purchased from Corning cellgro® and chloroquine from Sigma-Aldrich™. (B) Representative images demonstrating morphology/GFP expression on the day of harvest of the HEK-293LTV cells. Medium was collected 48 h post transfection. Images obtained using an Olympus® IMT-2 inverted fluorescence microscope. Magnification, 10X. (C - D) 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. Similar titers were obtained between vessels (C) and when normalized on a per cm² basis the HYPER*Stack* vessel yields similar amount of lentiviral particles (D).

Using the Corning HYPER*STACK* for adenoviral production

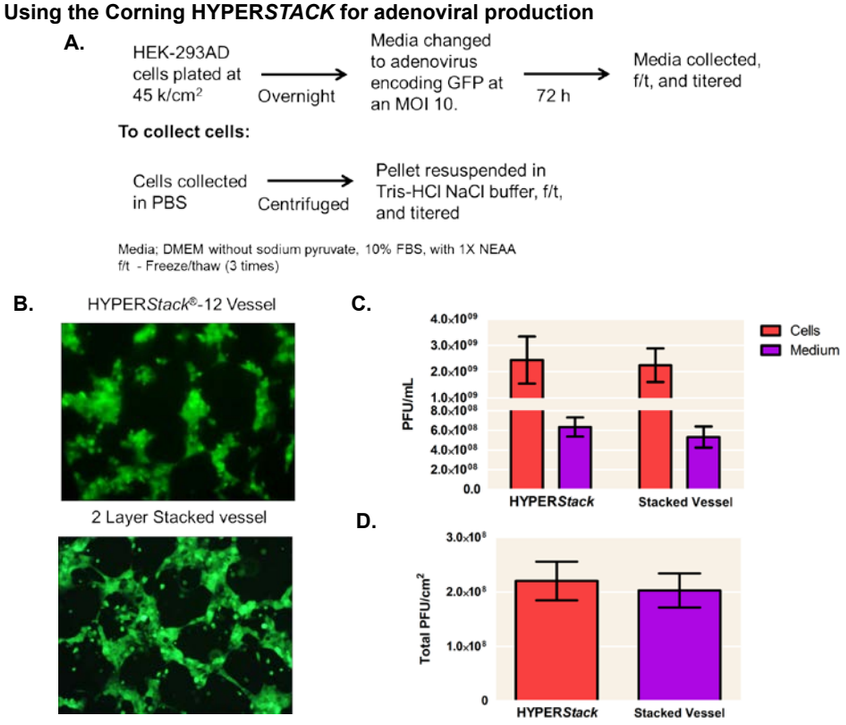

Figure 2. The Corning HYPER*Stack* -12 vessel supports comparable viral production, with a higher yield of total virus compared to a 2 layer stacked cell culture vessel. (A) Experimental outline. Media components purchased from Corning cellgro®. (B) Representative images demonstrating similar morphology/GFP expression on the day of harvest of the HEK-293AD cells. Medium was collected 72 h post transfection. Images obtained using an Olympus® IMT-2 inverted fluorescence microscope. Magnification, 10X. (C) Direct comparison between the HYPER*Stack* vessel and Stacked Vessel titers obtained using the QuickTiter Elisa Adeno kit (Cell Biolabs). (D) When normalized on a per cm² basis the HYPER*Stack* yielded similar infectious adenoviral particles.

Expansion of Vero cells with Corning microcarrier beads

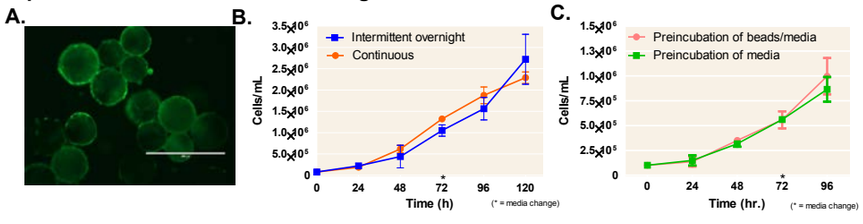

Figure 3. Vero cell expansion on Corning Enhanced Attachment microcarriers. (A) Vero cells on microcarriers and stained with Calcein AM. Image was captured using the AMG EVOS® FI microscope. Scale bar represents 400 µm. (B – C) Vero cells were expanded in a 125 mL disposable spinner flask (DSF), 0.1 mLs/cm², 10,000 cells/cm² and agitated at 30 rpm. Cells were cultured in DMEM with 5% FBS, 1X NEAA (Corning cellgro). The data suggest similar cell growth in a 125 mL DSF. Current studies are ongoing evaluating cell growth in a 1 L DSF.

Expansion of 293-AD cells with Corning microcarrier beads

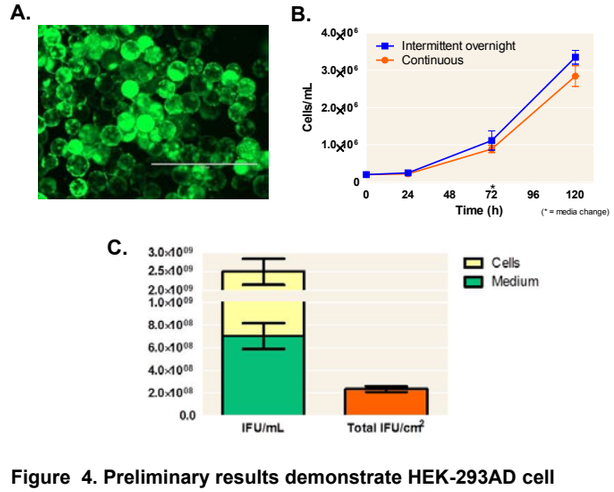

Figure 4. Preliminary results demonstrate HEK-293AD cell expansion and viral production on Corning Enhanced Attachment microcarrier beads. (A) HEK-293AD cells on microcarriers stained with Calcein AM. Image was captured using the AMG EVOS® FI microscope. Scale bar represents 1000 µm. (B) Cells were expanded in a 125 mL DSF, 0.1 mLs/cm², 20,000 cells/cm² and agitated at 30 rpm. Cells were cultured in DMEM with 5% FBS, 1X NEAA (Corning cellgro) (N=2). (C) Cells were cultured in a 125 mL DSF, 0.2 mLs/cm², 50,000 cells/cm² with intermittent agitation overnight at 30 rpm. The following day cells were transduced at a MOI = 15 and changed to continuous agitation. Cells cultured in DMEM with 10% FBS, 1X NEAA (N=2, in duplicate).

Summary

- Lentiviral and adenoviral particles can be generated in the Corning HYPER*Stack* vessels at similar titers compared to normal tissue culture vessels, allowing for greater virus production in a smaller footprint. Additionally, the viral particles generated on the HYPER technology platforms also exhibit similar levels of infectivity as in a traditional vessel (data not shown).
- Corning microcarrier beads can be used to expand Vero and HEK-293AD cells. Additionally, preliminary studies demonstrate the use of these beads for adenovirus production.

Future Work

- Evaluate cell growth and viral production in 1L DSF and larger.
- Compare cell growth on Corning microcarriers to other commercially available microcarriers.

Corning® HYPER*Stack*® Cell Culture Vessel: Performance Analysis

Application Note

CORNING

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Introduction

Production quantities of recombinant proteins, antibodies, viruses, vaccines, and cells are commonly required by researchers and manufacturers in biotechnology and pharmaceutical industries. Corning has a line of closed system modular vessels designed for growing adherent cells in large quantities. The Corning HYPER*Stack* cell culture vessel features Corning's HYPER (**H**igh **Y**ield **PER**formance) technology. The proprietary HYPER technology, using gas permeable film, eliminates the air gap within the vessel resulting in an increased cell growth surface area compared to traditional cell culture vessels. The first example of this technology was demonstrated with the Corning HYPER*Flask*® cell culture vessel, which utilized the HYPER technology to provide 1,720 cm^2 cell growth surface area (10 layers) in the same spatial footprint as a T-175 vessel. The HYPER*Stack* cell culture vessel line is available in approximately the same spatial footprint as the 2-, 10-, and 40-layer traditional stacked cell culture vessels exhibited by the Corning CellSTACK® cell culture chambers or the Nunc® Cell Factories. Each HYPER*Stack* module is composed of 12 individual chambers featuring the Corning CellBIND® surface treatment for optimal cell attachment. An individual module provides 6,000 cm^2 cell growth surface area. The modules are joined together to form the HYPER*Stack* 36-layer vessel (18,000 cm^2) (Figure 1). The closed system, compact design, and optimized performance of the HYPER*Stack* vessel make it ideal for use in large-scale production of cells, vaccines, and protein therapeutics.

To demonstrate the capabilities of the HYPER*Stack* vessel, several commonly used cell lines were assessed for growth, viability, and morphologic health. In addition, the cell growth environment was analyzed by monitoring dissolved gases, electrolytes, nutrients, and cellular metabolites. Analysis of both CO_2-dependent and CO_2-independent growth environments were investigated to determine the utility of the HYPER*Stack* vessel under different growth conditions. Recovery from cryogenic storage and cell function assessment using the label-free Corning Epic® technology were also measured for cells derived from HYPER*Stack* vessels. Finally, crystal violet staining confirmed there was uniform cell distribution, adherence, and upon harvesting, complete cell recovery from vessels.

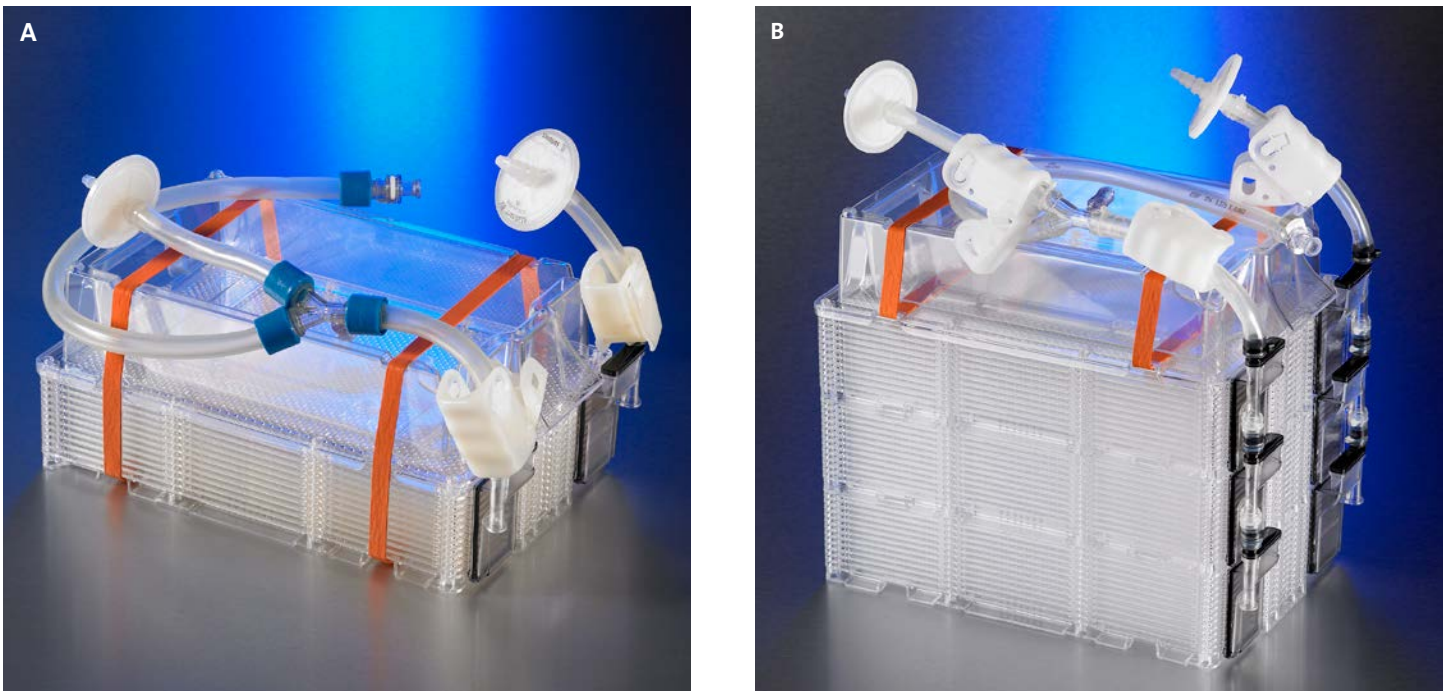


Figure 1. (A) Corning HYPER*Stack* 12-layer cell culture vessel and (B) HYPER*Stack*-36-layer cell culture vessel.

Materials and Methods

The following methods all included Corning® HYPER*Flask*® cell culture vessels and Corning CellBIND® surface CellSTACK® culture chambers as controls for the assessment of the Corning HYPER*Stack*® vessels.

Cell Growth and Viability

Chinese Hamster Ovary (CHO-K1; ATCC Cat. No. CCL-61) and Madin Darby Bovine Kidney (MDBK; ATCC Cat. No. CRL-22) cells were seeded at 3,000 cells per cm^2, while Human Embryonic Kidney 293 (HEK-293; ATCC Cat. No. CRL-1573) cells were seeded at 5,000 cells per cm^2. The cell lines were cultured using Iscove's Modified Dulbecco's Medium (IMDM) (Corning Cat. No. 10-016-CM), supplemented with 10% Fetal Bovine Serum (FBS) pre-warmed to room temperature. Cell suspensions were prepared and seeded in triplicate for each product type (Table 1) at a volume of 0.217 mL/cm^2 to achieve equivalent seeding densities for each vessel. Cultures were maintained in a humidified incubator set to 37°C and 5% CO_2. Daily samples of medium were taken and evaluated using the Nova BioProfile Flex® analyzer (Nova Biomedical) to monitor electrolytes, gas saturation, nutrient, and metabolite contents of the cultures. Cell morphology and overall growth was monitored visually using an Olympus inverted microscope. Due to the HYPER*Stack* vessel's construction, a device was used to assist with the visualization of cells. Cells were harvested after incubation for 96 hours using room temperature trypsin/EDTA (Corning Cat. No. 25-052-CV) containing 0.1% Pluronic® F-68 (Corning Cat. No. 13-901-CI). To ensure that all cells were removed from the vessels, an additional phosphate buffered saline (PBS) wash was performed and collected. An additional vessel was set up for each study and stained with a crystal violet solution (Thermo Fisher Cat. No. 23-750025) to assess equal cell distribution within individual layers. Studies were repeated three times independently.

CO_2-independent Growth

Vero cells (ATCC Cat. No. CRL-1586) were adapted to CO_2-independent conditions by growing them in Leibovitz L-15 medium (L-15), (Lonza Cat. No. 12-700Q) supplemented with 4 mmol L-glutamine (Corning Cat. No. 25-005-CI) and 10% FBS. Cells were seeded into each vessel at 3,000 cells per cm^2 in 0.217 mL/cm^2 (Table 1) using the controls stated above. To maintain a CO_2-independent system during culture, CellSTACK culture chambers were fitted with solid caps. Cultures were maintained for 96 hours in a warm room at 37°C and 20% relative humidity (RH). Daily samples of medium were acquired and evaluated using the Nova BioProfile Flex analyzer. Cultures were monitored and harvested as described above for cell growth testing.

Functional Testing

Early passage HEK-293 cells were seeded into a HYPER*Stack* 12-layer vessel or 2-layer CellSTACK culture chamber following the method described above. Upon reaching 90% confluence, vessels were harvested and total cell yields determined. Cell counts and viability were obtained through the trypan blue exclusion method using the Nova BioProfile Flex analyzer. Following analysis, the cells were concentrated by centrifugation at 270 x g for 7 minutes. The cell pellets were resuspended in freezing media (10% DMSO + 90% growth media) to yield a final concentration of 5.0 x 10^6 cells per vial and frozen down. To assess cellular function, cells from each test vessel were thawed into 3 mL of pre-warmed complete growth medium (10% FBS, IMDM). Cell concentration and viability were determined following centrifugation at 220 x g for 5 minutes to remove traces of DMSO. Cell pellets were resuspended in fresh growth media to a final concentration of 6.0 x 10^5 cells/mL. A Fibronectin-coated 384-well Corning Epic® microplate (Corning Cat. No. 5042) was pre-warmed to room temperature, filled with 10 µL/well of growth medium then spun briefly in the centrifuge to remove trapped air. Half of the Epic microplate was filled with 30 µL/well of HYPER*Stack*-12 cell suspension and the other half with 30 µL/well of CellSTACK cell suspension. After brief centrifugation, the microplate was incubated overnight in a humidified incubator at 37°C and 5% CO_2. The following day, the HEK-293 cellular response to SFLLR (5 µM) (Bachem Cat. No. H-2938.0025), a Par-1 agonist, or Carbachol (50 µM) (Sigma Cat. No. C4382), an acetylcholine receptor agonist, was evaluated using the label-free Epic assay. The Epic technology is a label-free noninvasive biosensor system that is centered around resonant waveguide grating biosensors.

Table 1. Scale-up Product Information

Corning Cat. No.	Description	Purpose	Surface Area (cm^2)	Seed Volume (mL)
3310	2-layer Corning CellSTACK chamber*	Standard control (for 12-layer vessel)	1,272	276
3320	10-layer Corning CellSTACK chamber**	Standard control (for 36-layer vessel)	6,360	1,380
10024	Corning HYPER*Flask* vessel	HYPER technology control	1,720	373 (+ 190 mL media to fill)
10012	Corning HYPER*Stack*-12 vessel	Test vessel	6,000	1,300
10036	Corning HYPER*Stack*-36 vessel	Test vessel	18,000	3,900

*Used as control when setting up HYPER*Stack*-12 studies.
**Used as control when setting up HYPER*Stack*-36 studies.

Results

Cell Growth and Viability

The capability of the Corning® HYPER*Stack*® cell culture vessel to perform as a viable tool for closed system, large scale, adherent cell production was evaluated. Utilizing Corning's gas permeable technology enables compact vessel construction and provides a higher growth surface area (cm^2) per cubic footprint of the vessel, ultimately providing for better use of valuable incubator space. To illustrate, a HYPER*Stack* 12-layer vessel has a volumetric footprint similar to that of a 2-layer stacked cell culture vessel, but offers the growth surface area closer to that of a 10-layer stacked cell culture vessel (Table 2). Initial comparison of the HYPER*Stack* vessel to control vessels was assessed through cell morphology, phenotype, distribution, and growth. Microscopic observation indicated no visible difference in morphology or distribution of cells and there were no phenotypic abnormalities observed (Figure 2). To evaluate the effect on cell morphology, density, and distribution of cells, the vessels were stained with crystal violet. Crystal violet is a histological dye that works by fixing cells to the growth surface and staining the cells purple. Crystal violet staining of confluent vessels revealed uniform cell distribution within the individual stacks and throughout the HYPER*Stack* vessels (Figure 3).

Additional assessment was performed once cultures reached confluence and the cells were harvested. Trypan blue exclusion was used to determine cell density, viability, and cell counts were converted to cells/cm^2 to uniformly compare the three different vessels types. Based on cells/cm^2, the HYPER*Stack* vessels performed equally to the control vessels, with respect to cell viability which was measured above 95% (Figures 4 and 5). In addition, there was typically approximately 3X higher total cell yields from the HYPER*Stack* vessel when compared to control vessels of similar volumetric footprint (Figure 5).

Cell Growth Environment

To assess the performance of the HYPER*Stack* vessel the growth environment was monitored daily. Media samples were taken from all vessels and analyzed for nutrient and metabolite concentrations, dissolved gases, and electrolyte values. For example, Figure 6 shows the results from CHO-K1 growth environments in the Corning HYPER*Stack* 12-layer, Corning HYPER*Flask*®, and 2-layer Corning CellSTACK® vessels over a 96-hour time period. Figure 6A shows a typical initial electrolyte equilibration of the growth medium which is usually completed in 24 to 48 hours. Figure 6B shows the normal nutrient/metabolite environment observed over a 96-hour growth period. The results show nutrients depleted and metabolites increased with cell proliferation. Figure 6C indicates the saturation of $\%CO_2$ and $\%O_2$ in the growth environment. Similar to the electrolytes, CO_2 equilibration of the media is complete after 24 to 48 hours. The HYPER*Stack* vessel's growth environment mimics that of the HYPER*Flask* vessel, which is constructed using the same gas permeable technology. Once complete equilibration of the growth media occurs, both HYPER technology vessels behave comparably to the CellSTACK control, which uses the standard liquid gas interface within the vessel.

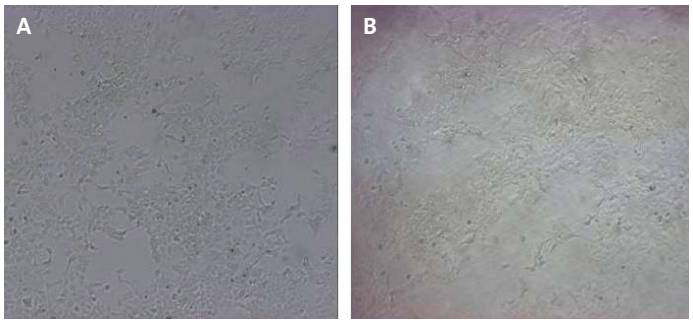


Figure 2. Visual analysis. Micrographs of HEK-293 cultures at 4X magnification using an inverted microscope. (A) 10-layer Corning CellSTACK and (B) Corning HYPER*Stack* 12-layer vessel using a viewing device. Overall similar cell densities and morphology were observed.

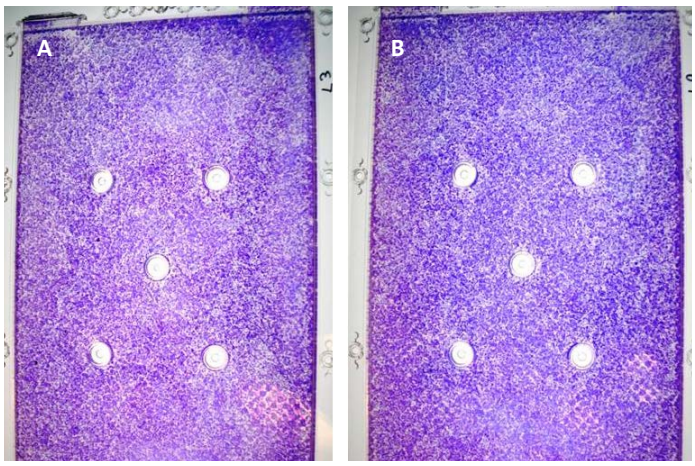


Figure 3. Cell distribution analysis. Crystal violet stained MBDK cultures after 96-hour incubation, third layer (A) and eighth layer (B) stacks from Corning HYPER*Stack* 12-layer vessel. Staining demonstrated equal cell distribution throughout layers and within the vessel.

Table 2. Stacked Vessel Comparison

	Corning CellSTACK Chambers		Nunc® Cell Factory Chambers		Corning HYPER*Stack* Vessels	
Footprint	**No. Layers**	**Surface Area (cm^2)**	**No. Layers**	**Surface Area (cm^2)**	**No. Layers**	**Surface Area (cm^2)**
2-stack	2	1,272	2	1,264	12	6,000
10-stack	10	6,360	10	6,320	36	18,000
40-stack	40	25,440	40	25,280	–	–

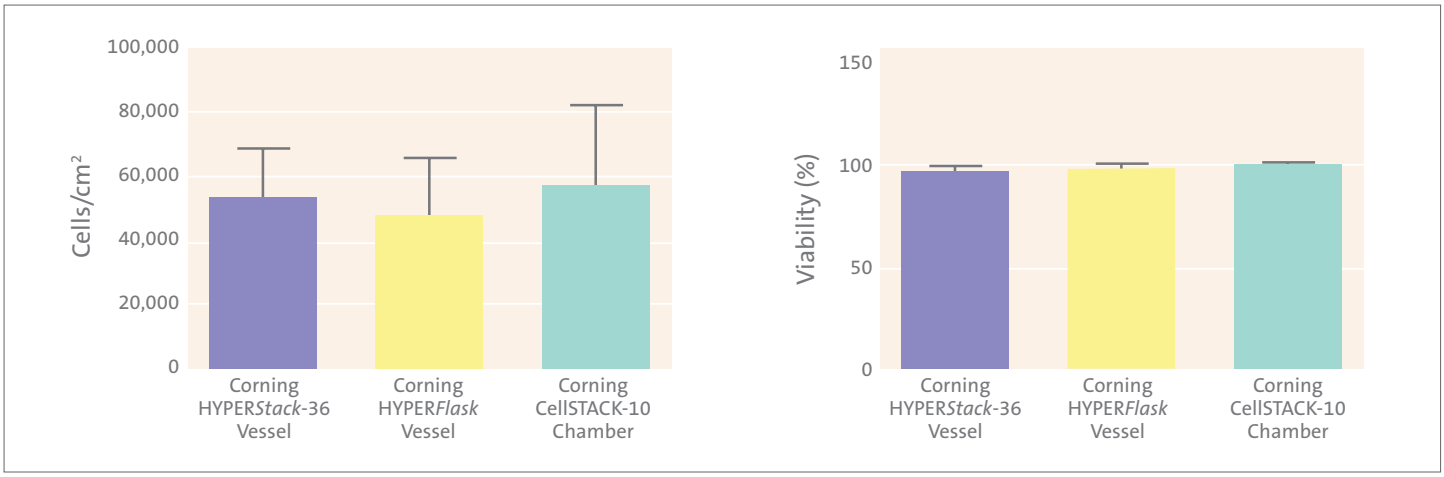


Figure 4. Cell growth analysis. HEK-293 cells cultured in Corning HYPER*Stack* 36-layer, Corning CellSTACK 10-layer, and Corning HYPER*Flask* vessels. 96-hour incubation in a humidified incubator at 37°C and 5% CO_2. Paired T-test (0.166 and 0.678 values) indicated no statistical difference in cells/cm² between the vessels. Data encompassed nine vessels/condition from 3 independent studies.

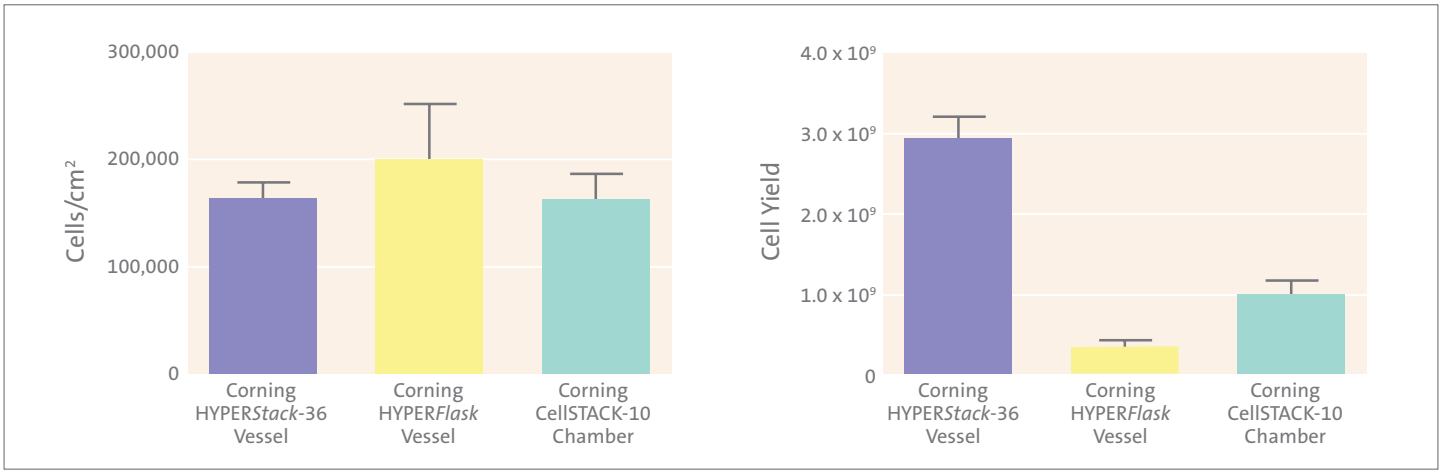


Figure 5. Cell growth analysis. CHO-K1 cells cultured in Corning HYPER*Stack* 36-layer, Corning CellSTACK 10-layer, and Corning HYPER*Flask* vessels. 96-hour incubation at 37°C and 5% CO_2. Paired T-test (0.09 and 0.881 values) indicate no statistical difference in cell/cm² between the vessels. Total cell HYPER*Stack*-36 yields are 3X greater than the 10-layer CellSTACK chamber. Encompassed data are from 3 independent studies.

CO_2-independent Growth

For many large-scale bioprocess environments, vessels used to culture cells are incubated in warm rooms under atmospheric conditions, due to the size and scale of the cultures. The ability to use the Corning® HYPER*Stack*® vessel in this environment can increase the value to the end-user by increasing cell production and eliminating the need for additional incubator space. Because Corning's HYPER technology uses gas permeable film, it is necessary to use CO_2-independent media, formulated to buffer without the use of sodium bicarbonate and compatible with atmospheric conditions. To evaluate the performance of the HYPER*Stack* vessel in a warm room environment, HYPER*Stack* and control vessels were seeded with Vero cells adapted to grow in L-15 medium. L-15 medium is specifically formulated to sustain cell growth in an atmospheric CO_2 environment by buffering the environment with phosphates, free base amino acids, galactose, and sodium pyruvate. Based on cells/cm² recovered per vessel and analysis of this data using the paired T-test, there was no significant difference (p-test values; 0.643 to Corning HYPER*Flask*® and 0.660 to Corning CellSTACK®) in the rate of cell growth between the three cell culture vessels (Figure 7A). Total HYPER*Stack* vessel yields were 4X higher than stacked control vessel of similar volumetric footprint (Figure 7B).

Functional Testing

Due to the unique environment found in the HYPER technology, where cells grow directly on the gas permeable film, it is important to understand any changes in cell behavior and physiology. Corning Epic® label-free technology, a highly sensitive method for detecting changes in cellular response using an optical biosensor, was employed to evaluate functional changes in cells cultured

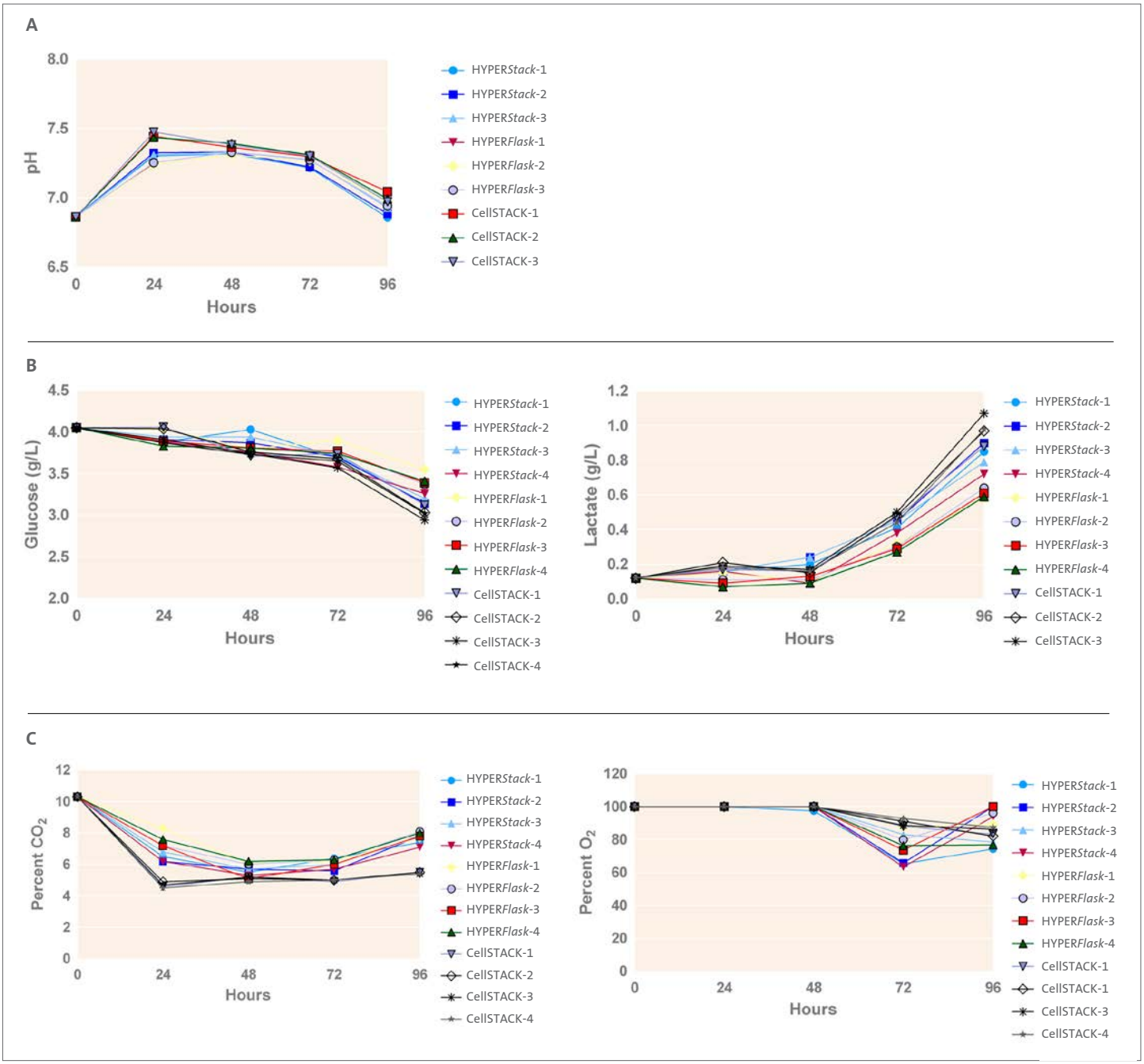


Figure 6. Analysis of the cell growth environment. Representative graphs of CHO-K1 cultures over a 96-hour period. (A) Electrolyte monitoring. pH from all three cultures equilibrated similarly after 48 hours and maintained for the duration of the study. (B) Nutrient and metabolite monitoring. Similar depletion of glucose (g/L) and buildup of lactate (g/L) by all three products. (C) Gas monitoring. Similar equilibration of CO_2 after 24-hour incubation and saturation of $\%O_2$.

from two different vessel environments. Using the Corning® Epic® label-free technology, changes in HEK-293 cell physiology were evaluated after challenge with either SFLLR (5 µM), a Par-1 agonist and carbachol (50 µM), an acetylcholine receptor agonist. After the addition of the test compound, the responses to these stimuli were compared after initial growth in either Corning HYPER*Stack*® vessels or Corning CellSTACK® vessels. HEK-293 cell were cryopreserved, thawed, and immediately used in a cellular response assay. The results indicate no statistically significant changes in cellular function in response to SFLLR or carbachol challenge between cells derived from the HYPER*Stack* or CellSTACK vessels (Figures 8A and 8B). Additionally, the data demonstrate no difference in two important metrics of assay reliability; %CV values and Z' values (Figure 8C). If either vessel impacted overall cellular health and physiology, both metrics could be indirectly altered.

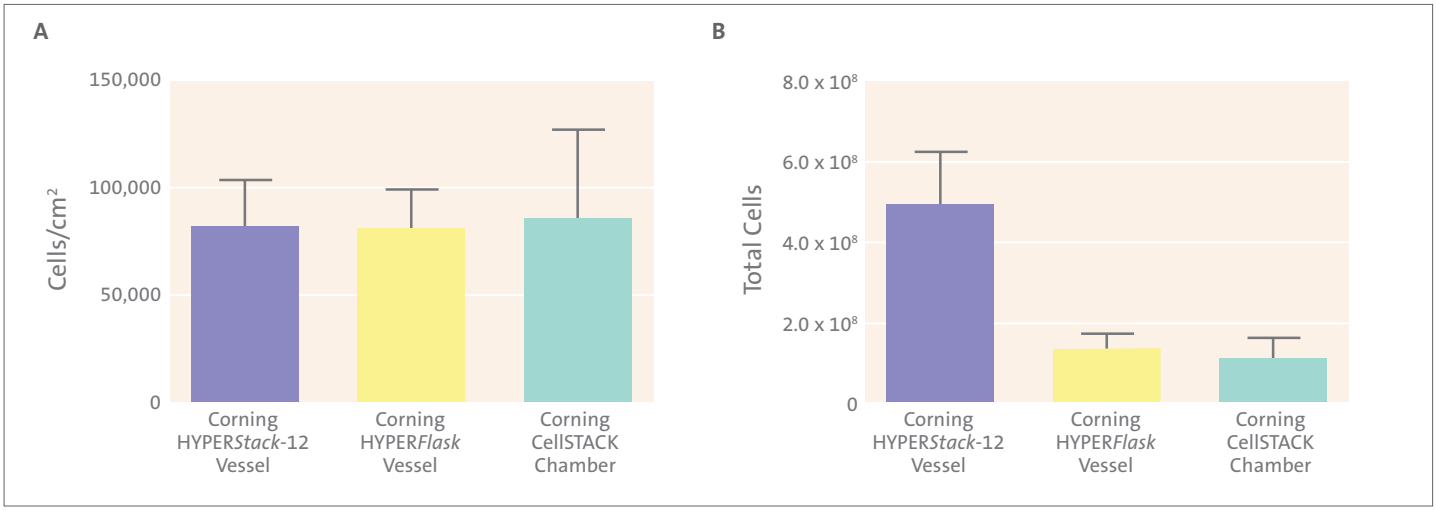
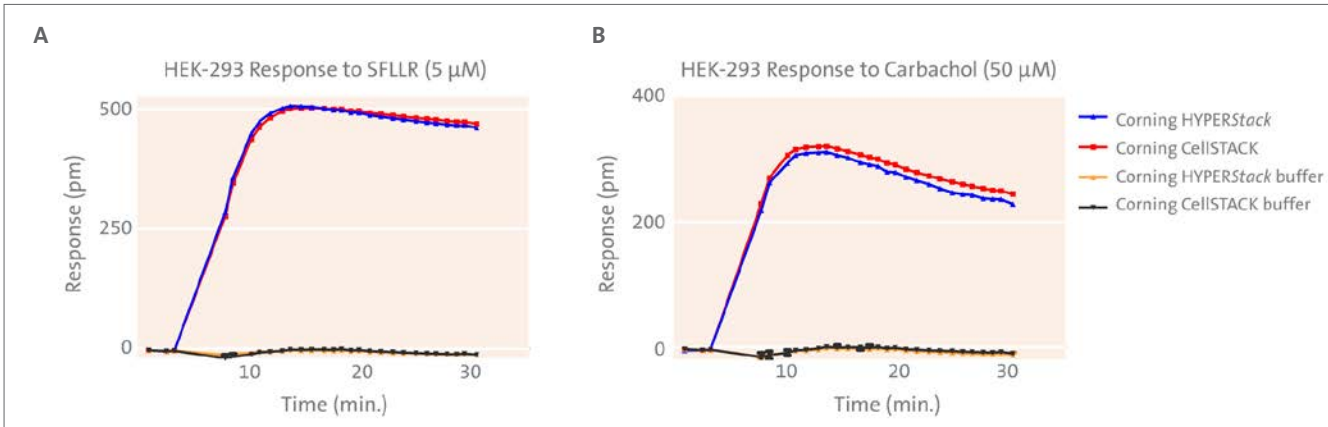


Figure 7. CO_2-independent growth analysis. (A) Vero cultures incubated in warm room under atmospheric conditions for 96 hours. Paired T-test indicated no significant difference in cells/cm² between the three products tested. Data shown is the average of three independent experiments (n = 9). Error bars represent ± S.D. (B) Total Corning® HYPER*Stack*® vessel yields were 4X greater than yields from Corning CellSTACK® vessels with similar cubic footprint.



C

Summary of Fixed Dose Responses

HEK-293	Compound	Z'	CV (%)	Avg. Signal (pm)	Sigma
Corning HYPER*Stack* 12-layer vessel	SFLLR	0.91	2.57	491.2	12.61
	Carbachol	0.914	2.21	317.95	6.967
2-layer Corning CellSTACK chamber	SFLLR	0.91	2.57	492.5	12.526
	Carbachol	0.907	2.24	328.46	7.293

Figure 8. Functional analysis. Corning Epic® label-free analysis of HEK-293 cultures derived from Corning HYPER*Stack* and Corning CellSTACK vessels. (A) No detectable difference in cellular response SFLLR (5 µM) between the two conditions. (B) No difference in cellular response to carbachol (50 µM) between the two conditions. (C) Summary data of Epic label-free analysis.

Summary of Analysis

- Based on analysis of growth environment, visual inspection of cell morphology and cells/cm² yield, the Corning® HYPER*Stack*® vessel performance was equal to that of the Corning HYPER*Flask*® and Corning CellSTACK® control vessels.
- Based on total cell yield, the HYPER*Stack* vessel produced 2.5X to 3X higher cell numbers than control vessels with similar spatial footprints.
- When used with the CO_2-independent medium, HYPER*Stack* vessels can be successfully used in warm room environment for production of large numbers of adherent cells or cell products.
- Based on cellular response obtained by using the Corning Epic® label-free system, cells derived from a HYPER*Stack* vessel performed comparably to those derived from a CellSTACK culture chamber.

For more specific information on claims, visit the Certificates page at **www.corning.com/lifesciences**.

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