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

Application Note

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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 introduced a new line of closed system modular vessels designed for growing adherent cells in large quantities. The HYPER*Stack* Cell Culture Vessel features Corning's HYPER (**H**igh **Y**ield **PER**formance) technology. The patented 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 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 T175 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 (or stackettes), 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) or the HYPER*Stack*-120 layer vessel (60,000 cm^2) (Fig. 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.

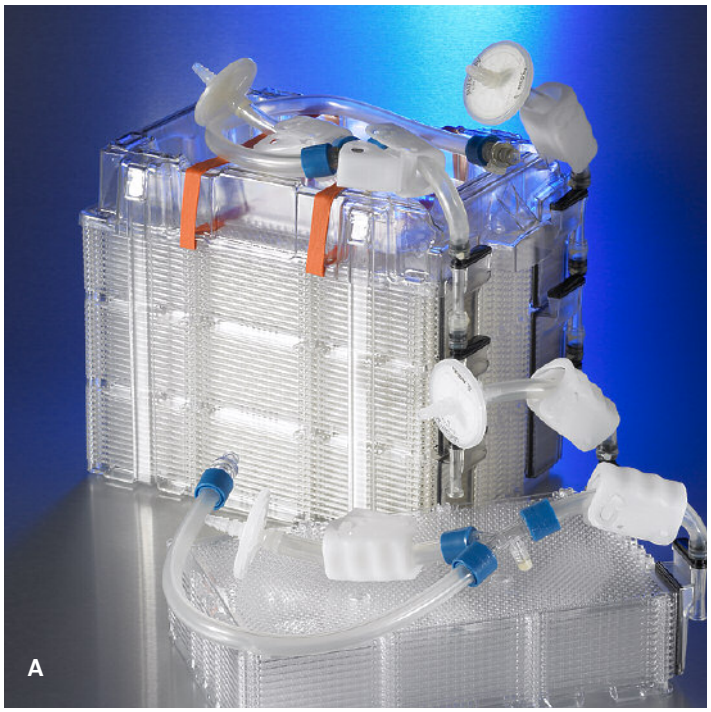


Figure 1. (A) HYPER*Stack*-12 and HYPER*Stack*-36 Cell Culture Vessels. (B) HYPER*Stack*-120.

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 gasses, 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 cryostorage and cell function assessment using the Corning® label-free 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.

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 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) (Mediatech, Inc. Cat. No. 10-016-CM), supplemented with 10% Fetal Bovine Serum (FBS) (PAA Laboratories, Inc Cat. No. A15-201) 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 Corporation) 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 (Olympus, Inc). Due to the HYPER*Stack* Vessel's construction, a HYPER*Viewer* device (Corning Cat. No. 10045) was used to assist with the visualization of cells. Cells were harvested after incubation for 96 hours using room temperature trypsin/EDTA (Mediatech Inc. Cat. No. 25-052-CV) containing 0.1% Pluronic® F-68 (Mediatech Inc. 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 (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 (Mediatech, Inc. Cat. No. 25-005-CI) and 10% FBS (PAA Laboratories, Inc. Cat. No. A15-201). 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 hour 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 otherwise monitored and harvested as described above for cell growth testing.

Functional Testing

Early passage HEK-293 cells were seeded into a HYPER*Stack*-12 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 re-suspended 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 re-suspended in fresh growth media to a final concentration of 6.0 x 10^5 cells/mL. A fibronectin coated 384 well Epic® plate (Corning

Table 1. Scale-up Product Information

Product	Cat. No.	Purpose	Surface Area (cm^2)	Seed Volume (mL)
2 Layer CellSTACK Chamber*	3310	Standard Control (for 12 layer vessel)	1,272	276
10 Layer CellSTACK Chamber**	3320	Standard Control (for 36 layer vessel)	6,360	1,380
HYPER*Flask* Vessel	10024	HYPER Technology Control	1,720	373 (+ 190 mL media to fill)
HYPER*Stack*-12 Vessel	10012	Test Vessel	6,000	1,300
HYPER*Stack*-36 Vessel	10036	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.

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	120	60,000

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 plate 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 plate 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 non-invasive biosensor system that is centered around resonant waveguide grating biosensors.

Results

Cell Growth and Viability

The capability of the 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 (in cm^2) per cubic footprint of the vessel, ultimately providing for better use of valuable incubator space. To illustrate, a HYPER*Stack*-12 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 (Fig. 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 stackettes and throughout the HYPER*Stack* Vessels (Fig. 3).

Additional assessment was performed once cultures reached confluence and the cells were harvested. Trypan blue exclusion was used to determine cell density and 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% (Fig. 4 and Fig. 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 (Fig. 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

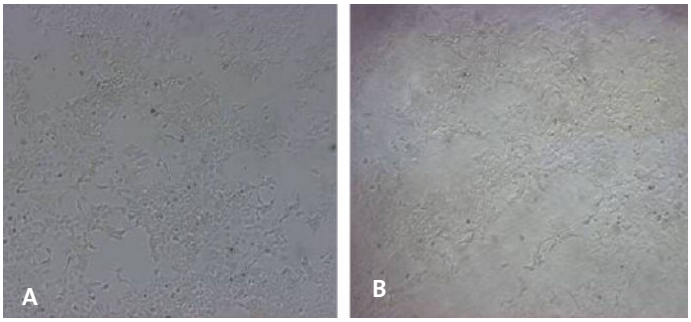


Figure 2. Visual Analysis. Micrographs of HEK-293 cultures at 4x magnification using an inverted microscope. (A) 10-layer CellSTACK and (B) HYPER*Stack*-12 using the HYPER*Viewer* device. Overall similar cell densities and morphology were observed.

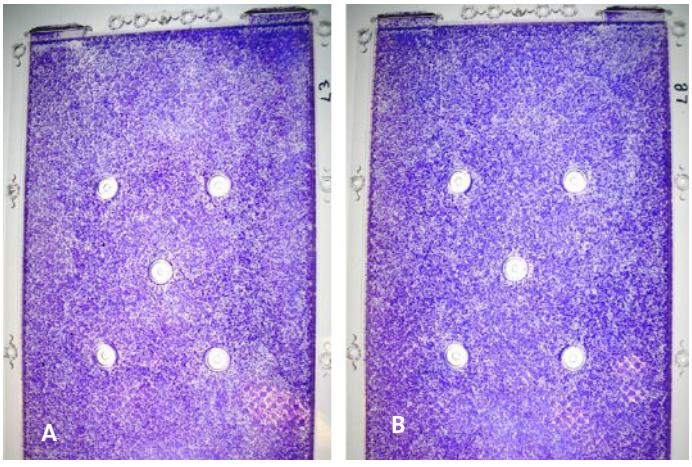


Figure 3. Cell distribution analysis. Crystal violet stained MBDK cultures after 96-hour incubation, 3rd layer (A) and 8th layer (B) stackettes from HYPER*Stack*-12 Vessel. Staining demonstrates equal cell distribution throughout layers and within the vessel.

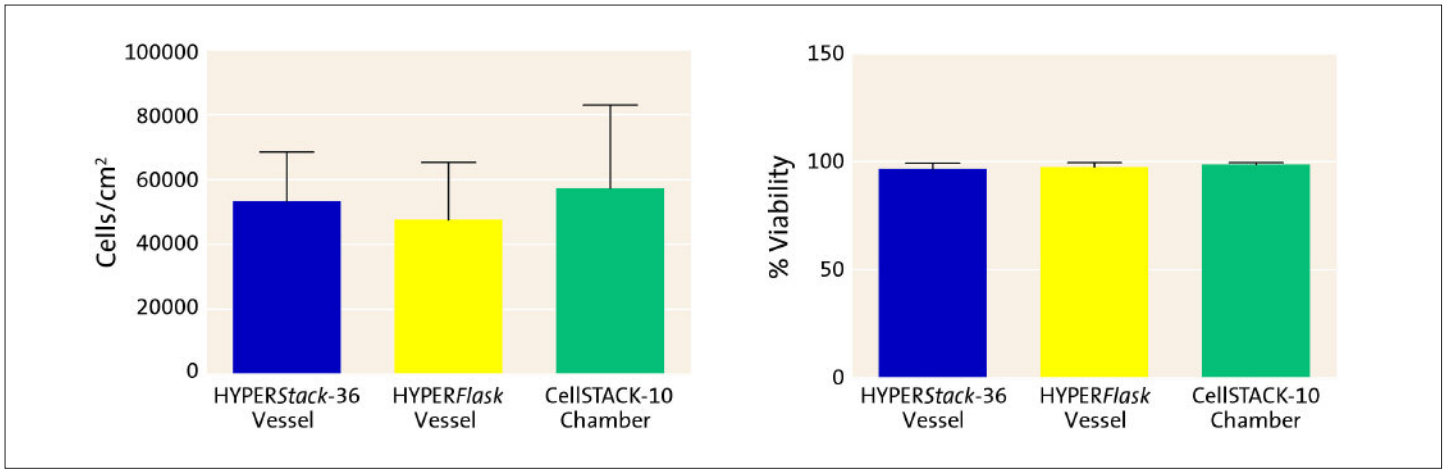


Figure 4. Cell Growth Analysis. HEK-293 cells cultured in HYPER*Stack*™-36, 10 layer CellSTACK® and HYPER*Flask*® vessels. 96-hour incubation in a humidified incubator at 37°C and 5% CO_2 Paired T-test (0.166 and 0.678 values) indicates no statistical difference in cells/cm2 between the vessels. Data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

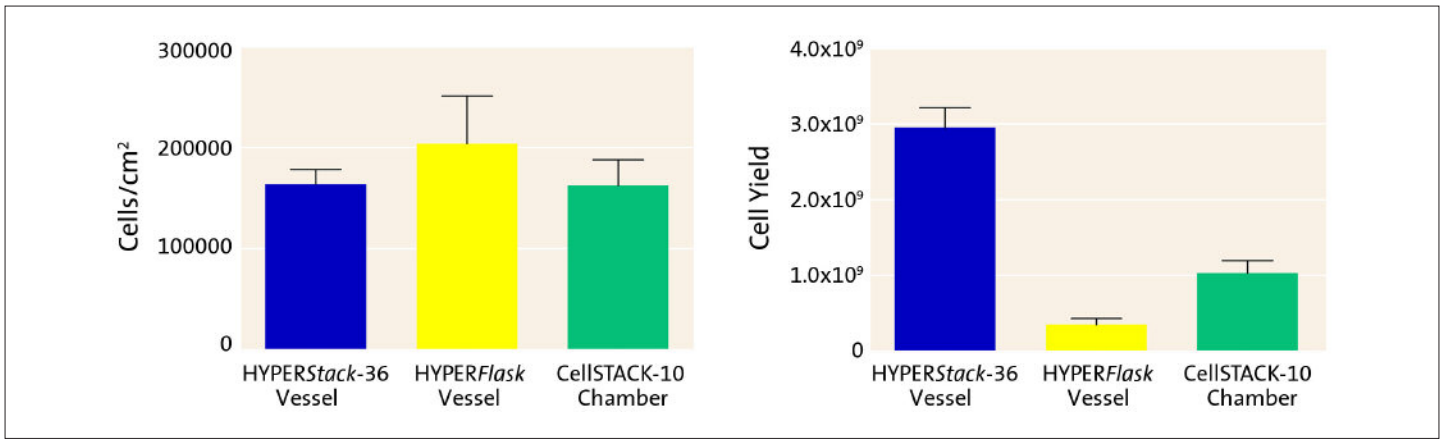


Figure 5. Cell Growth Analysis. CHO-K1 cells cultured in HYPER*Stack*-36, 10 layer CellSTACK and HYPER*Flask* vessels. 96 hour incubation at 37°C and 5% CO_2. Paired T-test (0.09 and 0.881 values) indicate no statistical difference in cell/cm² between the vessels. Total cell HYPER*Stack*-36 yields are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

metabolite concentrations, dissolved gasses, and electrolyte values. For example, Fig. 6 show the results from CHO-K1 growth environments in HYPER*Stack*-12, HYPER*Flask* and 2-layer 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.

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 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

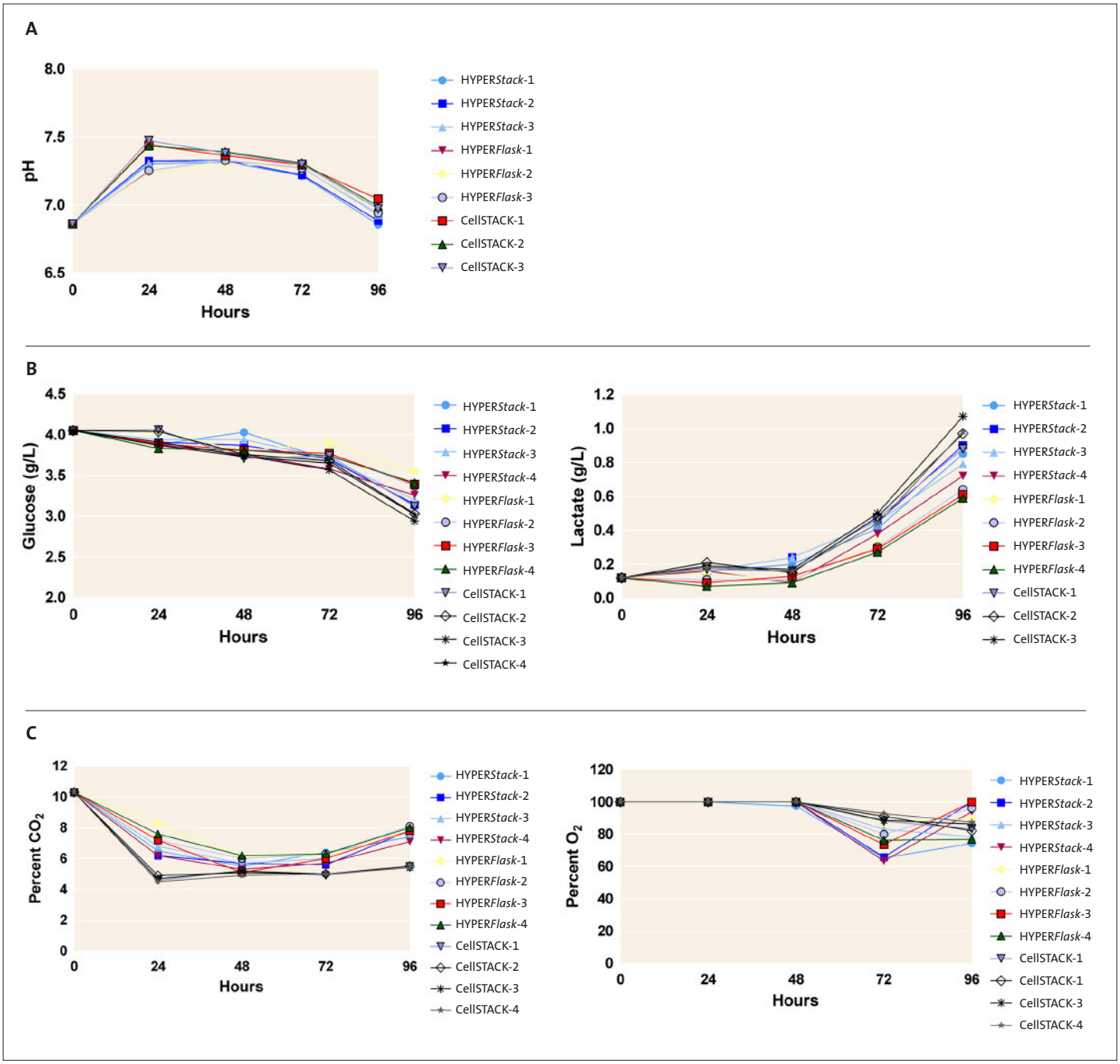


Figure 6. Analysis of the cell growth environment. Representative graphs of CHO-K1 cultures over a 96-hour period. (A) Electrolyte monitoring. pH from all three cultures equilibrated similarly after 48 hours and maintained for the duration of the study. (B) Nutrient and metabolite monitoring. Similar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

analysis of this data using the paired T-test, there was no significant difference (p-test values; 0.643 to HYPER*Flask*® and 0.660 to CellSTACK®) in the rate of cell growth between the three cell culture vessels (Fig. 7A). Total HYPER*Stack* Vessel yields were 4x higher than stacked control vessel of similar volumetric footprint (Fig. 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. 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 from two different vessel environments. Using the 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 HYPER*Stack* vessels or CellSTACK vessels. HEK-293 cell were cryo-preseved, 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 (Fig. 8A and 8B). Additionally, the data demonstrate no difference in two important metrics of assay reliability; %CVs and Z' values (Fig. 8C). If either vessel impacted overall cellular health and physiology, both metrics could be indirectly altered.

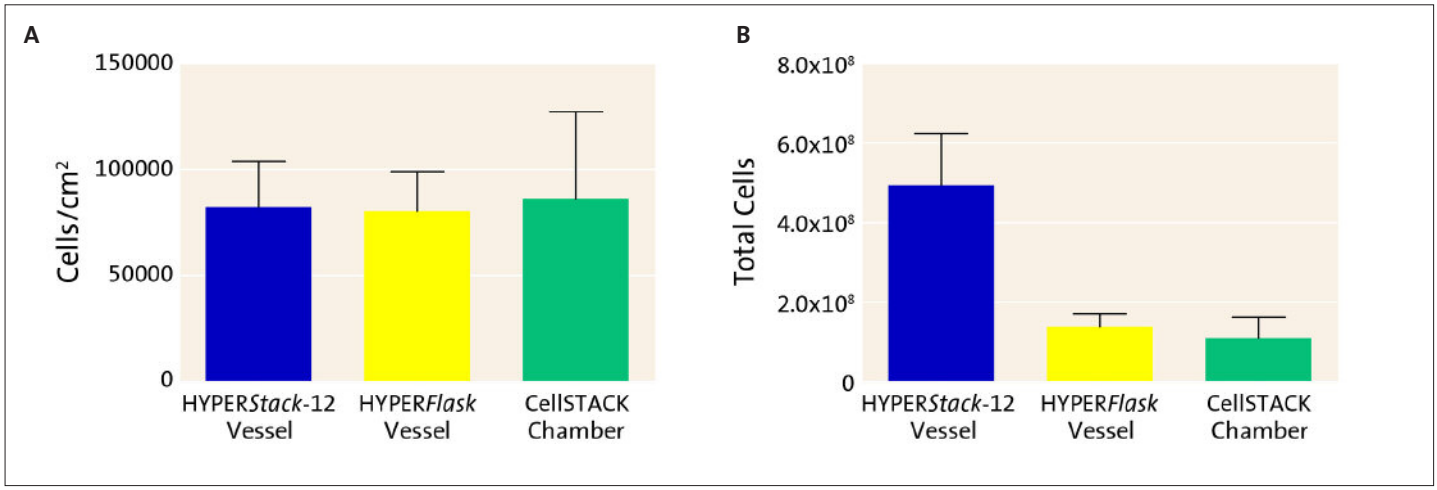
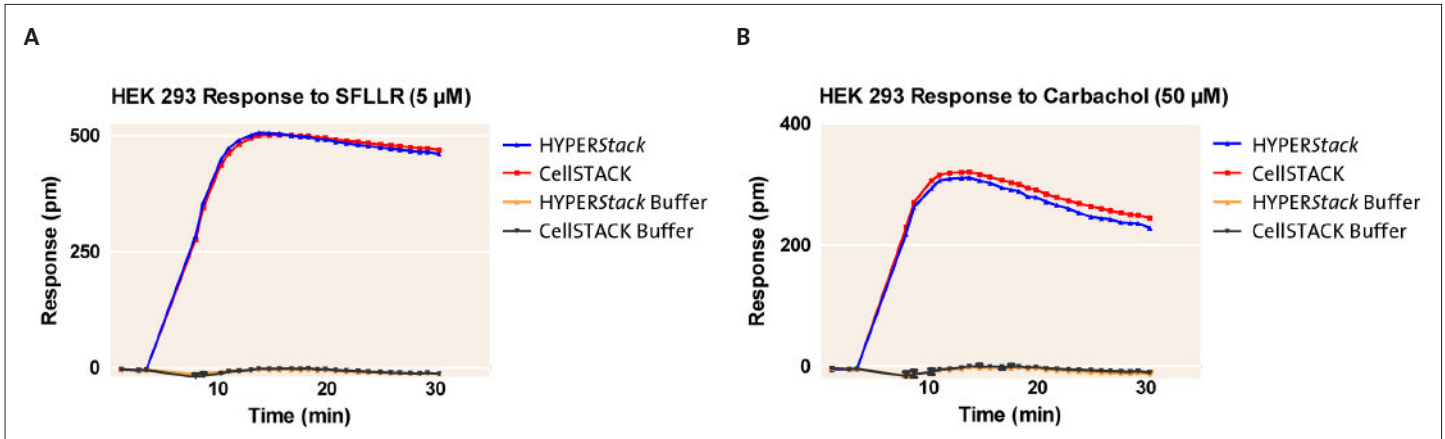


Figure 7. CO_2-Independent Growth Analysis. (A) Vero cultures incubated in warm room under atmospheric conditions for 96 hours. Paired T-test indicates no significant difference in cells/ cm^2 between the three products tested. Data shown is the average of three independent experiments (n = 9). Error bars represent ± S.D. (B) Total HYPER*Stack* Vessel yields were 4X greater than yields from CellSTACK vessel with similar cubic footprint.



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Summary of Fixed Dose Responses

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

Figure 8. Functional Analysis. Epic label-free analysis of HEK-293 cultures derived from HYPER*Stack* and CellSTACK vessels.(A) No detectable difference in cellular response SFLLR (5 µM) between the two conditions. (B) No difference in cellular response to carbachol (5 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/cm^2 yield, the HYPER*Stack*™ Vessel performance was equal to that of the HYPER*Flask*® and CellSTACK® control vessels.
- Based on total cell yield, the HYPER*Stack* Vessel produced 2.5 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.

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