WHITE PAPER
UPSTREAM BIOPROCESSING

Cultivation of CHO-K1 Cells for Human Enterovirus EV71 Virus-Like Particle (VLP) Production

Description

Tide Motion™ CelCradle-500A (CC-500A) provides a powerful cell culture tool to achieve high cell density and high productivity of target bioproducts because of its unique feature of offering high oxygen transfer and low shear stress culture environment. To evaluate this point, an interdisciplinary research collaboration was conducted in A*STAR's Institute of Molecular and Cell Biology (IMCB) looking into the quick development of a Hand Foot and Mouth Disease (HFMD) vaccine. Through this collaboration, Esco Aster hopes to address the need for more affordable vaccines through improved biomanufacturing processes. The implementation of production scale bioreactor will deliver robust and optimized conditions for HFMD virus-like-particles (VLP) production, while providing highly consistent batch productions to IMCB.

In this study, stable CHO-K1 clones expressing Human Enterovirus (EV71) VLP was provided by Assoc. Prof. Justin Chu, IMCB, A*STAR. The application of CC-500A for the growth of CHO-K1 cells and production of Human Enterovirus (EV71) Virus-Like Particle (VLP) is elucidated. CHO-K1 cells (2×10^8) were seeded in CC-500A or seeded in 10 cm petri dishes (4×10^6). On Day 3, the cell number per CC-500A reached 1.05×10^9. Conditioning of Human Enterovirus (EV71) VLP with serum free media was performed on Day 3 for CC-500A or day 2 for 10 cm petri dishes. Samples were collected after three days of conditioning and Human Enterovirus (EV71) VLP amount was determined using Western Blot. CelCradle achieved a 44-fold Human Enterovirus (EV71) VLP production when compared to 2D conventional cultures with 10 cm petri dish.

This study shows CC-500A can be applied to CHO-K1 cells cultivation for Human Enterovirus (EV71) VLP production. However, the optimal condition for cultivation of CHO-K1 cells and Human Enterovirus (EV71) VLP production can be further optimized.

Materials

Device	Cell Line /Product	Medium	Seed
CelCradle-500A	CHO-K1 cells / Human Enterovirus (EV71) VLP	DMEM/F12/10%FBS/400 µg/ml G418 DMEM/F12: (Gibco, Cat.: 12400-024) FBS: (Hyclone, Cat.: SV30160.03HI) G418: Invivogen ant-gn-5)	2×10^8

Methods

Phase 1A: Seeding of CHO-K1 Cells onto CelCradle

Seed	Seed Volume	Seeding Method
2×10^8 cells/bottle	120 ml	Inverted with white cap Swirl the bottle gently every 30 mins for first 2 h, every hour for 3 h.

Phase 1B: Expansion of CHO-K1 Cells

Media Volume	Tide Motion Parameters	Media Change Frequency	Growth Period
CC-500 A - 500 ml	Uprate: 1 mm/sec Up hold: 10 sec Downrate: 1 mm/sec Down hold: 30 sec	Every day after day 2	3 days
2D petri dish - 10 ml	-	-	1 day

Phase 2: Human Enterovirus (EV71) VLP Conditioning

Cell Density	Tide Motion Parameters	Conditioning Period
1.05 x 10^9 cells/ bt of CC-500A	Uprate: 1 mm/sec Up hold: 10 sec Downrate: 1 mm/sec Down hold: 1 min	3 days after day 3
~8-10 M cells / 2D petri dish	-	3 days after day 1

Protocol

4.1 Inoculation

2×10^8 cells were provided as seeding stock to seed into CC-500A bottles. 50 ml of inoculums containing 2×10^8 cells was pipetted into CC-500A bottle containing 70 ml of complete media to make up 120 ml final volume. The bottle was tightly capped with white cap and inverted. All BioNOC™ II carriers were submerged in 120 ml media and the bottles were swirled gently to evenly distribute the inoculums in the media. The CC-500A bottle was placed and incubated in 37°C, 5% CO_2 incubator. The bottle was swirled gently every 30 minutes for the first 2h, and 1h for the 3rd hour.

For 2D petri dishes, 4×10^6 cells were seeded into each plate for a total of 3 plates and incubated in 37°C, 5% CO_2 incubator.

4.2 Attachment Efficiency Monitoring

After 3 h into inoculation, 10 ml of supernatant was collected and centrifuged to determine the attachment efficiency. Number of cells remaining in the supernatant was counted using hemocytometer. Formula used to calculate attachment efficiency is as below:

Attachment Efficiency calculation:

(1-(cells in supernatant that did not attach/ total inoculation cells)) × 100%

4.3 Cell Cultivation

The CC-500A bottle was topped up with 390 ml of warmed complete media and before placing and locking it on the CelCradle Stage in 37°C, 5% CO_2 incubator. The tide motion parameters was set as below:

Tide Motion Parameters
Uprate: 1 mm/sec Uphold: 10 sec Downrate: 1 mm/sec Downhold: 30 sec

Full media change was performed on day 2.

4.4 Cell Culture Monitoring on CC-500A

4.4.1 Staining of Carriers

One carrier was randomly sampled. To observe cells distributed on carrier, the carrier was transferred to 24 well plates. 500 µl of DMEM/F12 was added to the carrier containing 1 µg/ml of Hoescht 33342 (Thermo Fisher, H3570), and 1 µg/ml propidium iodide (PI, Sigma P4170). Carriers were incubated for 30 min at 37°C, 5% CO_2, before adding 1 µg/ml of fluorescein diacetate (FDA, Thermo Fisher F1303). Microscope images were then taken with their respective filters.

4.4.2 Cell Count Via Trypsinization (3 carriers)

Three carriers were transferred into a 1.5 ml micro-centrifuge tube from CC-500A. The carriers were washed gently with 1 ml PBS for 3 times before performing enzymatic dissociation with trypsin. 1 ml of 0.05% Trypsin-EDTA was added and the carriers were incubated at 37oC for 15 min. 1 ml of media was added to neutralize the action of trypsin, before flicking tube against a metal rod for 10-20 times. The solution was collected, and 1 ml of PBS was added to wash the cells out. Flicking of carriers were repeated for 3 more times and the cell suspension was collected. Cells were then counted using a hemocytometer.

4.4.3 PrestoBlue® Cell Viability Reagent for Cellular Metabolism of Live Cells

Three carriers were transferred from CC-500A and each carrier was placed in each well of a 24-well plate. Blank carriers were added as control. 450 µl of media with 50 µl of PrestoBlue® Cell Viability Reagent (Thermo Fisher, Cat No: A13261) was added into each well, and incubated at 37 °C, 5 % CO_2 for 1 hour. 200 µl of media was then transferred to a 96 well plate and fluorescence values of excitation 560 nm and emission 590 nm was read with a spectrophotometer or absorbance of 570 nm and OD 600 was used as reference wavelength. Background values were corrected with control wells.

4.4.4 pH and Glucose Monitoring

2 ml of supernatant was collected every day from the CC-500A bottle. pH reading was monitored using SevenCompact pH meter S210-Std-Kit (Mettler Toledo, Cat No: 30130863). The CO_2% for the incubator was decreased from 5% to 3% on day 1 and 0% on day 2. It was maintained at 0% for the rest of the duration.

Glucose reading was measured using GlucCell® Glucose Monitoring System (VacciXCell, Cat No: 1400009). 1.5 µl of media was pulsed into the glucose strip to check leftover glucose in media (Glucose TN). Fresh media was used as a baseline for initial glucose value (Glucose T0). Glucose consumption was calculated with glucose at T0 – glucose TN.

4.5 Conditioning of Human Enterovirus (EV71) VLP with Serum Free DMEM/F12

Human Enterovirus (EV71) VLP conditioning was performed on day 3 for CC-500A, when PrestoBlue® assay show a plateau in cellular metabolism. For 2D culture, cells were conditioned the next day after seeding into 10 cm petri dish. For CC-500A and 2D culture, the media was removed and 10 ml and 500 ml of PBS was added into the petri dish and bottle respectively to wash off remaining serum. PBS wash was performed twice before adding DMEM/F12 media alone. Samples were collected after 3 days of conditioning in serum free DMEM/F12, for both CC-500A and 2D culture, and passed through a 0.22 µm filter.

Cells from 2D petri dish were harvested by trypsinization, neutralized, washed with PBS and stored as a cell pellet. Cells from 17 BioNOC™ II carriers were harvested via trypsinization and stored as cell pellet. Both cell pellets were added with 1X LDB and boiled at 95oC for 5 min before storing in -20oC in preparation of gel electrophoresis.

4.6 Human Enterovirus (EV71) VLP Quantitation

Human Enterovirus (EV71) VLP yield was determined using Western Blot to compare Human Enterovirus (EV71) VLP amount between 2D culture and CC-500A. 30 ml of media from 2D petri dish and CC-500A was concentrated with Vivaspin Turbo 4, 100 kDa centrifugal concentrator (Sartorius, Cat No: VS04T42) to 90 µl. The media was added with 4x LDB to a total volume of 120 µl and boiled at 95oC for 5 min. 20 µl of samples (both cell lysate and supernatant) were added to 10% SDS-PAGE for gel electrophoresis. Samples were then transferred to PVDF membranes and blocked in blocking buffer (BioRad, Cat No: 1706404) before incubating primary antibodies overnight at 4oC. Secondary antibody was incubated at 2 h after washing with TBST thrice the next day.

The expression of each protein was detected using the following primary antibodies: anti-mouse Human Enterovirus (EV71) (1:1000, Merck MAB979), anti-mouse ß-tubulin (1:1000, Abcam), and secondary mouse antibody (1:1000, GE Healthcare, NA931V).

Clarity™ Western ECL substrate (BioRad Laboratories, Cat No: 1705061) was used to detect all protein signals before capturing images with Amersham™ Imager 680 (GE Healthcare). Analysis of protein intensity was performed by Amersham™ Imager analysis software version 2.0.

Results

CHO-K1 Cell Growth

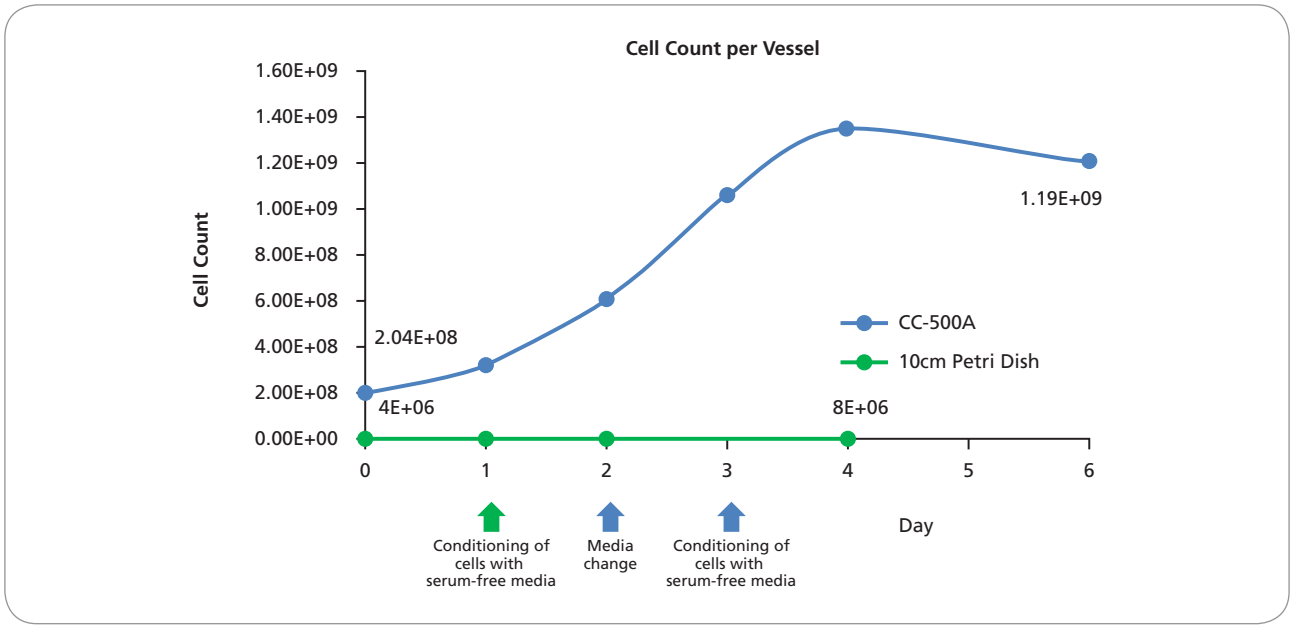

Figure 1. CHO-K1 cell growth in CC-500A. Cell numbers reached 1.05 x10^9 per bottle on Day 4

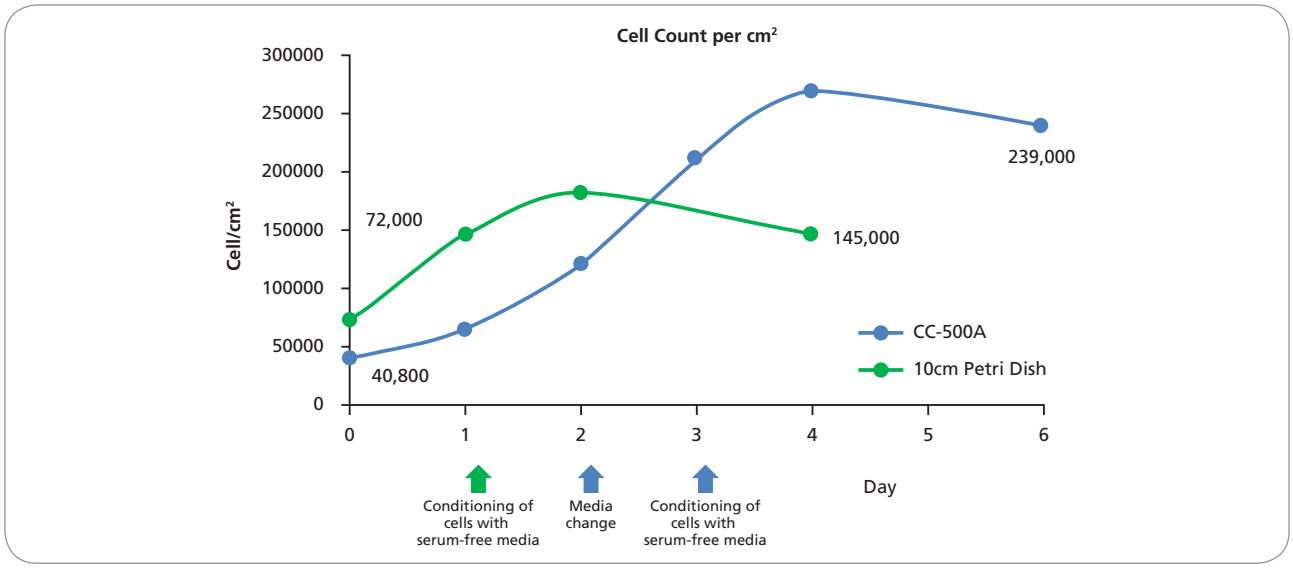

Figure 2. Comparison of cell count per cm² between CC-500A and 10 cm petri dish

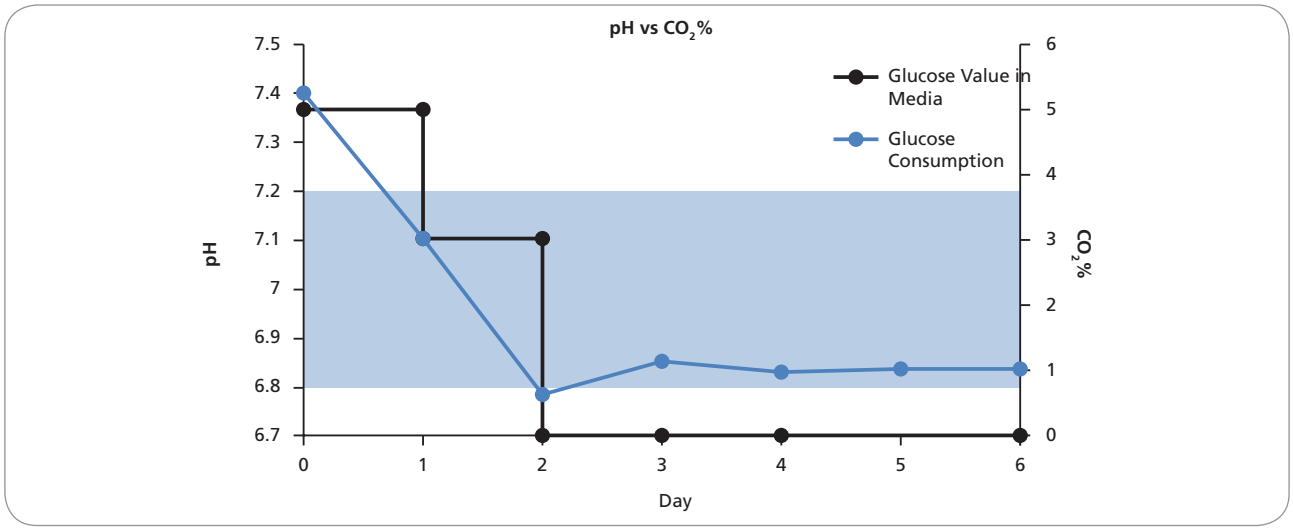

Figure 3. pH was well maintained within the acceptable range to ensure optimal condition for cell proliferation and Human Enterovirus (EV71) VLP conditioning

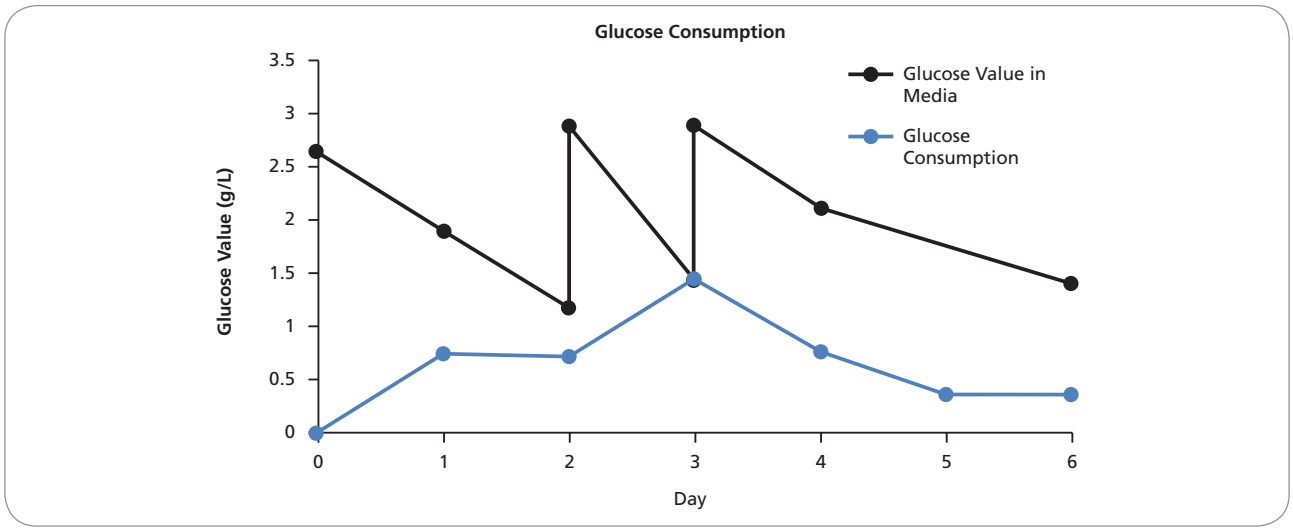

Figure 4. Glucose was maintained at 1 g/L and above throughout the period of cell growth and Human Enterovirus (EV71) VLP conditioning

pH and Glucose Monitoring during CHO-K1 Cell Growth and Human Enterovirus (EV71) VLP Production

During the entire Human Enterovirus (EV71) VLP production period, pH was maintained within 6.8 to 7.2 to ensure optimal cell growing condition (Figure 4). CO_2% of the incubator was reduced from 5% to 3% on day 1 and 0% on Day 2.

Apart from maintaining pH, glucose value was also monitored to ensure sufficient nutrients were supplied.

Human Enterovirus (EV71) VLP Production (Western Blot Results)

Cells can achieve high confluence on BioNOC™ II on CC-500A. However, the amount of Human Enterovirus (EV71) VLP produced per cell number from CC-500A is slightly lower compared to 10 cm petri dish. This may be due to cells changing morphology from spindle shapes to rounded and squeezed when a high confluence is obtained on BioNOC™ II. Therefore Prestoblue™ Assay is a better measure of cell metabolism and health and the ability of CHO-K1 cells to produce Human Enterovirus (EV71) VLP.

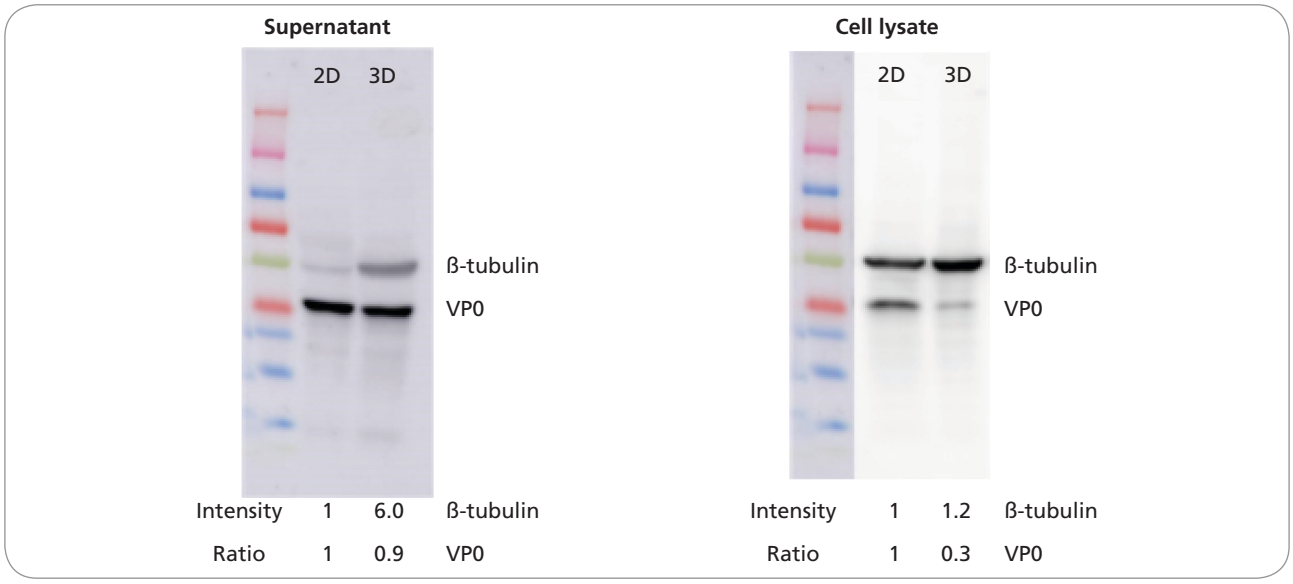

Figure 5. Comparison of Human Enterovirus (EV71) VLP (VP0) and housekeeping protein (ß-tubulin) in supernatant and cell lysate between 10 cm petri dish and CC-500A

Table 1. Comparison of total Human Enterovirus (EV71) VLP produced and normalized to petri dish

Condition	Signal of VP0	Amount of Human Enterovirus (EV71) VLP Normalized against Petri Dish
Petri dish - 10 ml	391912	1
CelCradle - 10 ml	347889	0.9
CelCradle - 500 ml		44.4

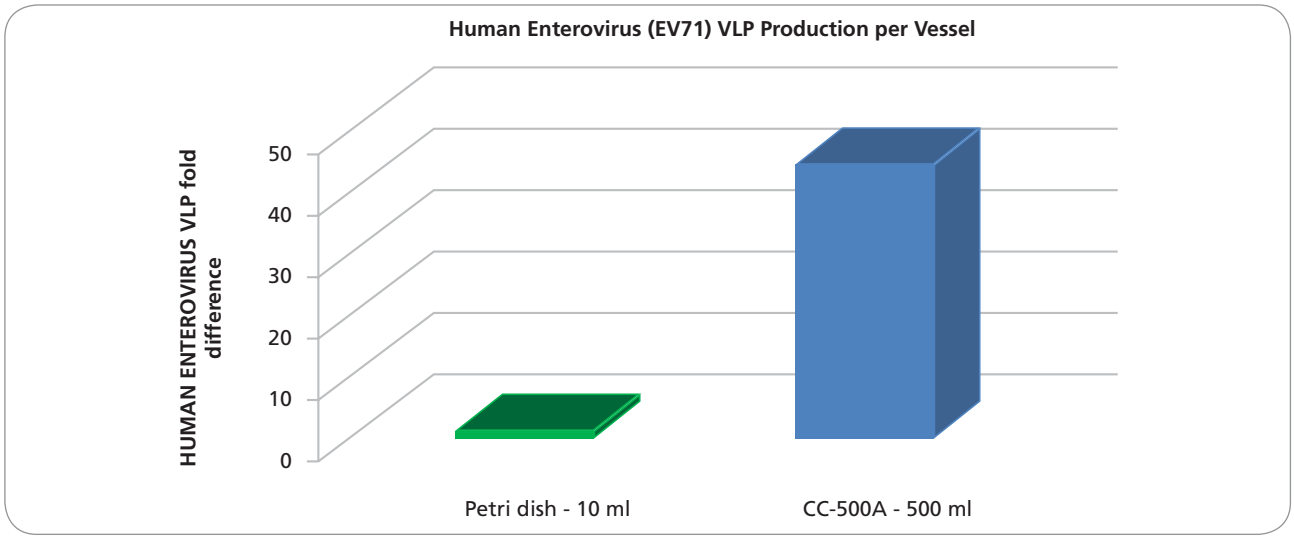

Figure 6. Comparison of Human Enterovirus (EV71) VLP production between petri dish and CC-500A

Table 2. Comparison of Human Enterovirus (EV71) produced from each vessel and normalized to housekeeping gene

Type	Condition	Marker	Intensity from Western Blot	Normalized VP0/ß-tubulin
2D 10cm petri dish	Supernatant	VP0	391912	3.1
	Cell lysate	ß-tubulin	125224	
3D CC-500A	Supernatant	VP0	347889	2.3
	Cell lysate	ß-tubulin	150117	

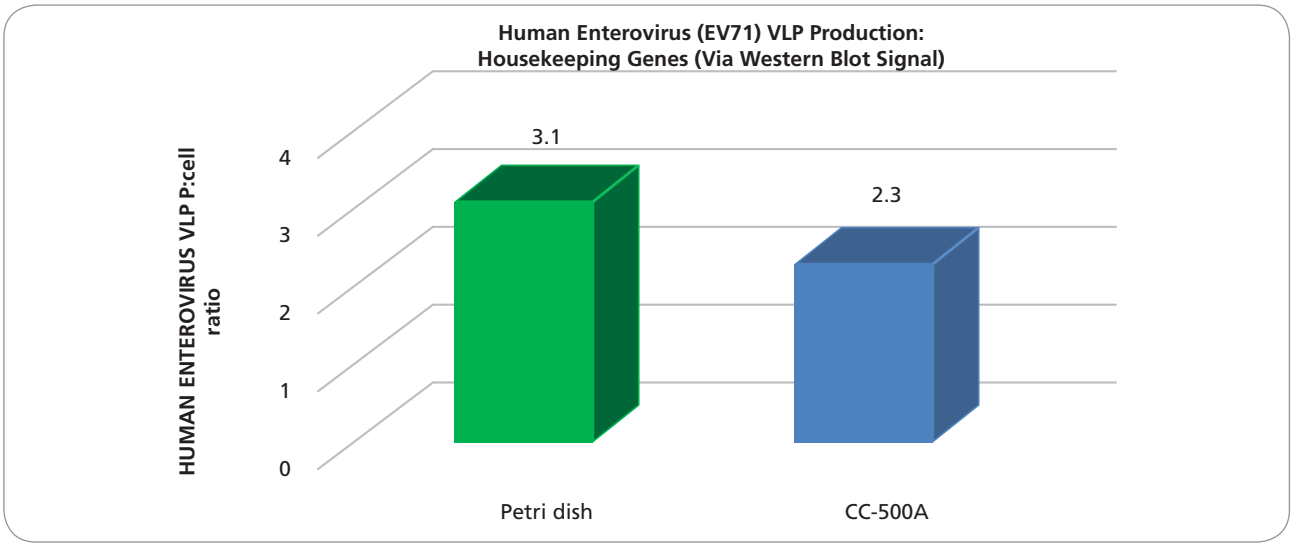

Figure 7. Comparison of Human Enterovirus (EV71) VLP per cell between 10 cm petri dish and CC-500A. Results extrapolated from Western Blot signals using Amersham™ Imager software.

Conclusion

In conclusion, CHO-K1 cells were successfully grown on CC-500A with high Human Enterovirus (EV71) VLP production. Total Human Enterovirus (EV71) VLP yield in CC-500A is 44-fold higher compared to a 10 cm petri dish. Further optimization processes are recommended to obtain higher Human Enterovirus (EV71) VLP production.

VacciXcell Technical Support

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