



Comparing Adherent-Cell Technologies

Amplification of Virus Stocks and Viral Vectors

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FUJIFILM Diosynth Biotechnology (FDB) is a multifunctional commercial development manufacturing organization (CDMO) that manufactures virus-based therapeutics. Its site in Texas specializes in both adherent and suspension cell culture for production of virus vectors and for development of analytical assays to support production and expression of our clients' therapeutics. We work with a number of cell lines such as human embryonic kidney (HEK293) and human retinal cells (Johnson & Johnson/Crucell's proprietary PER.C6 line), which can grow in suspension. However, many of the cell lines we work with do not grow well in suspension and thus are difficult to work with at large scale.

When a client approaches us to develop a process for manufacturing a vaccine or viral-based gene therapy, we first evaluate the cell line that has been used to grow the virus or viral vector. Few cell lines are suspension adapted and able to take advantage of the smooth scale-up process from shake flasks or spinner flasks into bioreactors. Suspension cultures grown in bioreactors allow for tight control of cell nutrients and parameters such as dissolved oxygen (DO), as well as sampling for easy monitoring.

Many viruses selectively grow in polarized cells and take advantage of that polarization for transport in and out of those cells. Such viruses often are difficult to produce in suspension culture. Thus, there is a need for a system that can support adherent cultures and be scaled up easily for manufacturing viral vaccines and vectors. Below, we describe case studies in three adherent cell growth systems and offer an overview of some analytical methods we can use to characterize viral products.

ADHERENT CULTURE SYSTEMS

Adherent cell culture typically is preferred for viruses that grow selectively in polarized cells and often is difficult to translate into a suspension cell culture. To maximize virus infectivity — and ultimately production — we have compared three

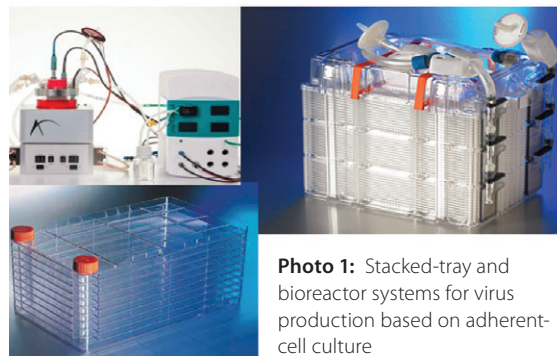


Photo 1: Stacked-tray and bioreactor systems for virus production based on adherent-cell culture

different technologies for adherent-cell culture: Corning® CellSTACK®-10 and HYPERStack®-36 two-dimensional culture systems and an iCELLis® Nano bioreactor from Pall Corporation. Scale-up of simple flask cultures can be problematic when large-volume processes are the goal. Traditional tissue-culture flasks and roller bottles have large footprints and must be grown in incubators, requiring substantial space in a manufacturing facility. CellSTACK-10 units can replace a number of flasks, and the HYPERStack system improves on the cell stack technology. The iCELLis system acts as a bioreactor to support adherent cell culture, offering many advantages over tray-based systems.

Stacked Trays: A CellSTACK-10 unit has a surface area of 6,350 cm² (equivalent to ~42 T-150 flasks) in 1.4 L of media. Polystyrene layers provide head space for CO₂ absorption. Trays are easy to fill and empty: Users simply pour media in or out, working with multiple units in a reasonable amount of space and with limited personnel resources. Limitations of this system include uneven cell growth throughout the layers because of restricted airflow inside the chamber. In addition, trays allow for only a limited field of view under a microscope. That is not ideal because we want to be able to ensure that cells are growing uniformly before infection, then be able to monitor the culture for cytopathic effect during infection to determine the appropriate time to harvest.

The HYPERStack system supplies 18,000 cm² of growth area (equivalent to 120 T-150 flasks) in 3.9 L of media. Each unit has a much smaller footprint than standard tissue culture flasks, and the growth surfaces can be coated with Corning's CellBind® coating. The major advantage of using this system over a standard CellSTACK unit is that each layer is gas permeable, which allows gas exchange directly to cells in culture (instead of through the chamber headspace and media). That allows cells to grow more evenly than in CellSTACK trays. However, microscopic viewing of the culture is limited to only the bottom two layers.

Fixed-Bed iCELLis bioreactors come in a small-scale (Nano) version for process development laboratories and a newer, larger instrument (500 m² of cell-growth space) that can be used for good manufacturing practice (GMP) production. In this type of bioreactor, cells grow on woven, medical-grade polyester fibers. An iCELLis Nano bioreactor provides 5,300–40,000 cm² of growth space in a small area, depending on the packing density of the fibers. That is equivalent to 35–267 T-150 flasks. The system uses 900 mL of media at the smallest scale and can be scaled up with perfusion. Growing in a three-dimensional (3D) structure rather than the two-dimensional (2D) growth that occurs in traditional tissue-culture flatware, cells can reach higher densities in a relatively smaller space.

CASE STUDIES

To assess and compare their efficiency in virus production, we tested the three adherent culture systems described above using two virus-host cell systems. The first experiment used Madin-Darby canine kidney epithelial cells to amplify influenza B (Malaysia strain) from an egg stock. This virus is historically challenging to cultivate in tissue culture. The second experiment used human embryonic kidney (HEK-293) cells to amplify an E1- and E3-deleted adenovirus vector.

Finally, we evaluated virus production using a hemagglutination (HA) assay for influenza B and an immunostaining assay for adenovirus. Although the hemagglutination assay does not measure infectious virus, it is a rapid way to evaluate titer for daily monitoring of virus production. The immunostaining assay is a fast method to measure adenovirus titer, but it does not provide immediate results (which the HA assay does).

To evaluate the growth of those viruses before scaling into the larger systems, we performed pilot

Figure 1: Hemagglutination assay

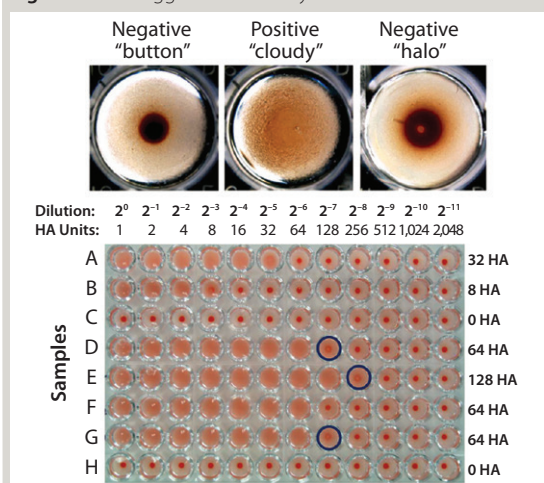
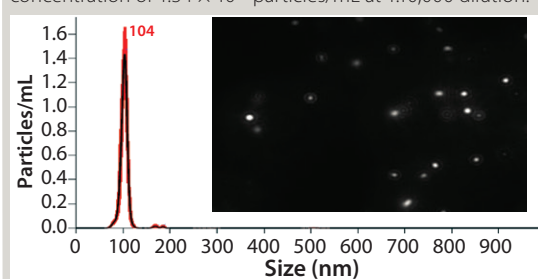


Figure 2: Nanoparticle tracking analysis shows a total concentration of 1.34×10^{12} particles/mL at 1:10,000 dilution.



experiments to determine the best media composition, the multiplicity of infection (MoI), and the proper amount of trypsin-like enzyme necessary to cleave the hemagglutinin protein of influenza. We chose to use 50 mPrU of Thermo Fisher Scientific's TrypLE™ synthetic trypsin-like enzyme with a MoI of 0.01 in serum-free Dulbecco's Modified Eagle's Medium (DMEM). For adenovirus, we found that a two-hour adsorption was optimal with a MoI of 10 in DMEM containing 2% fetal bovine serum (FBS).

Case Study Results: We assessed the amount of infectious influenza B virus produced in each of the three large-scale adherent cell culture systems using a hemagglutination assay. For this, we serially diluted the virus-containing supernatant resulting from each vessel in twofold increments using a 96-well microplate. Each dilution then was seeded with 0.5% chicken red blood cells and incubated for an hour at room temperature. The 96-well plate was scored for evidence of agglutination, which causes the red blood cells to remain suspended in solution. That makes for a diffuse, cloudy ring throughout a well, indicating that sialic acid residues on those cells have bound

Table 1: Influenza virus production results

	T-150 Flask	iCELLis	Cell- Stack	Hyper- Stack
Cell density	379,000 cells/cm ²	343,000 nuclei/cm ²	NA	NA
Harvest time	120 hpi	144 hpi	96 hpi	120 hpi
Equivalence	1 flask	35 flasks	42 flasks	120 flasks
HA titer	1:512 dilution	1:1,024 dilution	1:32 dilution	1:512 dilution
Estimated PFU/cell	4,000	4,879	1,410	9,245

to virus particles. If the red blood cells are not agglutinated, they will settle out and form a “button” on the bottom of a plate well.

Figure 1 illustrates the hemagglutination assay results for multiple samples in which HA titer varies from zero (rows C and H) to 128 (row E). The hemagglutination assay reports in HA units, which can be used to estimate infectious titer assuming that 1 HA $\approx 1 \times 10^6$ infectious viral particles (1, 2).

Table 1 summarizes data from the influenza experiments. Because cell density cannot be directly measured in CellSTACK or HYPERStack systems, we used T-150 flasks as controls to estimated cell density at the time of infection. That estimate in turn was used to calculate the amount of virus required for infection to achieve a target MoI. By contrast, the iCELLis bioreactor allows for removal of its woven cell supports to release cell nuclei for counting (assuming that each cell has just one nucleus) and thus quantification of approximate cell numbers. Harvest time varied based on how cultures appeared after infection. Each format supported cell growth and virus production. We observed the highest virus yield per cell with the HYPERStack system and the next highest with the iCELLis bioreactor. However, further studies with additional controls will be needed to confirm that observation.

We measured the ability of the HYPERStack and iCELLis systems to produce adenovirus and compared them to T-150 flask controls using an immunostaining titration assay. For this, HEK-293 cell monolayers were grown in 12-well plates and infected with 10-fold dilutions of freeze-thawed samples potentially containing adenovirus recovered from each culture system. After 48 hours of infection, those cells were fixed and permeabilized. An antibody directed against the adenovirus hexon protein was used to detect infected cells, which were then visualized using chromogenic staining and counted using light microscopy to calculate infectious titers for each sample.

Table 2: Adenovirus production results

	T-150 Flask	iCELLis Bioreactor	HyperStack System
Cell density	420,000 cells/cm ²	343,000 nuclei/cm ²	NA
Harvest time	48 hpi	48 hpi	48 hpi
Equivalence	1 flask	35 flasks	120 flasks
Particle yield	76.2/cell	203/cell	2,244/cell
Infectious titer	0.764/cell	2.37/cell	1,085/cell
Particle/ infectious ratio	99.7	85.7	2.07

Table 2 summarizes data from the adenovirus experiment. As with the influenza B virus experiments, we used control flasks to estimate cell density at time of infection for the HYPERStack system. In this experiment, all cultures were harvested at 48 hours after infection, and we measured virus particle yield/cell and infectious titer/cell. The resulting numbers were then used to calculate the particle/infectious ratio for each sample. That ratio indicates the quality of virus produced: The more infectious virus units are produced, the more potent a virus-based product will be. The HYPERStack system gave the lowest and best infectious particle to total particle ratio.

One problem that might have affected yield in the iCELLis® system is that we used HEK 293 cells that originally came from a suspension culture but were adapted to grow adherently in our laboratory. Despite the fact that the adherence-adapted cells grew well in stacked trays and flasks, the cells initially adhered well to their supports in the bioreactor but soon began to come off and revert back to a suspension-like phenotype. The resulting difficulty in getting good cell numbers might have affected yield. Further investigation will be necessary to assess the cause of the differences in those ratios in each system.

Table 3 summarizes our observations on these different systems. One feature that we prefer is to be able to view cells to evaluate and monitor their health over the course of an experiment. The iCELLis bioreactor allows for visualization of its cell supports under a microscope, but with stacked-tray systems visualization is limited to just one layer — and even that requires a microscope that can accommodate a tall cell culture vessel.

Another feature that we find important in a cell culture system is the feasibility of assessing cell density. The iCELLis bioreactor allows for lysis of cells from their supports to estimate the total number of cells present, whereas the stacked-

Table 3: Comparing operating parameters across adherent-cell production systems

	iCELLis Bioreactor	CellStack System	HyperStack System
Microscope	Cell supports are viewable under a microscope.	Bottom layer only	Top layer only; microscope must be equipped with a detachable lamp and condenser unit.
Cell counting	Cell supports are removable for nuclei counting.	Cell counts must be estimated using a satellite flask.	Cell counts must be estimated using a satellite flask.
Sampling	Sample port	Media can be removed.	Media cannot be removed.
Controls	Bioreactor controls (no incubator) for pH, O ₂ , CO ₂ , and temperature	Incubator can control CO ₂ and temperature.	Incubator can control CO ₂ and temperature.
Lytic viruses	Harvest media only	Harvest media only	Harvest media only
Cell-associated viruses	Cells on cell supports must be lysed in buffer.	Cells can be dissociated, concentrated, and lysed.	Cells can be dissociated, concentrated, and lysed.
Virus adsorption	Media volume can be reduced.	Media volume can be reduced.	Media volume cannot be reduced.
Addition of media components	Media components can be added easily.	Media components can be added.	Addition of media components requires a complete media change.

tray systems require use of a satellite flask to estimate cell density.

Ease of sample collection is another desired feature in a cell culture system. The iCELLis bioreactor includes a sample port for culture monitoring. Media samples can be removed easily from a CellSTACK tray; however, the task is more difficult to perform with a HYPERStack unit. The bioreactor allows for control of pH, O₂, CO₂, and temperature. Using the stacked trays, you control CO₂ and temperature through an associated incubator.

When choosing an adherent cell culture system to support large-scale virus production, it is important to note that for lytic viruses the media are harvested, making all three systems relatively easy to work with. However, cell-associated viruses require cell lysis for virus recovery. But one must consider whether lysing cells directly in the system or using a cell-dissociation method before lysis is more appropriate. Also, better infection is obtained in many cases by concentrating virus in a low volume of media during the absorption stage. The iCELLis and CellSTACK systems allow for media reduction during that period, whereas the HYPERStack requires full media volume.

ADDITIONAL VIRAL ANALYTICAL CAPABILITIES

At FDB, our goal is to use the best tools available for virus production as well as for virus sample analysis. Our ever-growing toolbox of virus sample analytics currently includes assays to measure total particles, infectious titer, genome copy, and potency of all viruses that come to our facility. Below we discuss some additional assays in our repertoire for characterizing virus particle number and infectious titer.

Virus Particle Determination: NanoSight technology from Malvern Instruments uses nanoparticle tracking analysis to characterize 10-nm to 2,000-nm nanoparticles in solution. Through direct observation and measurement of diffusion events, it can measure size and concentration of virus or virus-like particles. In addition, a fluorescence mode can differentiate labeled or naturally fluorescing particles.

Figure 2 shows NanoSight nanoparticle tracking results. Particles are measured as they move past a detector that images them to produce a plot of particle number and size. The adenovirus is about 100 nm in diameter. Figure 2 shows a sample with a clean, sharp peak, confirming the presence of pure adenovirus particles. This technique detects larger particles such as virus aggregates, which is helpful for stability studies. We can determine whether viruses are aggregating or breaking down by looking at the peak size. Smaller peaks that appear in degradation studies can help us identify the conditions under which that could occur.

Infectious Titer Determination: Photo 2 shows a representative image, and Table 4 lists data for an example of our immunostaining results using the immunotiter assay. In the photo, black dots indicate cells that are infected with adenovirus and thus express the adenovirus hexon protein. The table shows that after multiple runs, a consistent titer can be obtained with high precision as indicated by the <20% CV across runs.

In addition to measuring infectious titer by immunostaining as described in our case study, we also use plaque assays and fluorescence-activated cell sorting (FACS) to measure infectious titer. The plaque assay is the gold standard in virology for

Photo 2: 10x micrograph of immunostained cells infected with virus

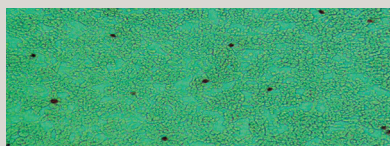


Table 4: Comparing immunotiter results from three different experimental runs

Average + Cells/Field	Titer	Average Titer	Titer SD	%CV
37.3	9.83×10^8	1.09×10^9	1.89×10^8	17.36%
49.6	1.31×10^9			
37.0	9.74×10^8			

determining infectious virus titer. In this assay, cell monolayers are infected with virus-sample dilutions and then overlaid with agarose or coaxial methyl cellulose. After a period of days, plaques (holes) in the monolayer can be visualized by staining to differentiate between live and dead cells. This assay also helps to detect differences in virus populations because viruses that grow very well will give rise to larger plaques, thus enabling isolation of a particular type of variant in a population. Figure 3 shows an example of such an assay.

We also use FACS assays to evaluate virus titer. Here, a known amount of cells is incubated with different dilutions of a virus-containing sample, then after a period of time stained for a viral protein. A FACS instrument provides visualization of the percentage of cells that are positive for expression of the targeted protein. By comparing the number of infected cells with the total number of cells, one can calculate the virus titer. This rapid method provides results in 24 hours or less, making it valuable for in-process testing (e.g., during purification). A FACS assay also allows for dual color staining to differentiate between different virus populations (e.g., those expressing a gene of interest and not).

Reporting titers in exponential form, Table 5 compares the accuracy of the immunostaining, plaque, and FACS assays. The results are similar for all methods evaluated. And in virology, anything less than half a log is not considered to be a meaningful difference, so all three methods gave essentially the same titers.

Genome Copy Determination: Infectious titer can be estimated based on genome copy number using quantitative polymerase chain reaction (qPCR). We use this method both to monitor in-process samples (it takes only a few hours to get results) and when working with vectors such as adenoassociated virus (AAV) that don't have a classical titration assay available. A reference standard is used to set up a standard curve and analyze unknown samples against it to estimate the genome copy number.

CONCLUSION

We have assessed and compared three novel technologies that facilitate large-scale culture of adherent cells in virus production. Using two different virus-host cell systems, we showed that all three technologies support growth of MDCK and HEK293 cells and production of an influenza B virus and an adenovirus vector, providing yields that are higher than or at least comparable to the typical cell culture flask platform. Herein, we also demonstrate our capabilities in cell culture using multiple platforms and cell lines to produce virus as well as in performing diverse analytical assays to quantify and monitor virus production.

ABOUT FDB

FUJIFILM Diosynth Biotechnologies an industry-leading biologics contract development and manufacturing organization (CDMO) with locations in Billingham and Redcar, UK; Research Triangle Park, NC, and College Station, TX. The company

Figure 3: Plaque assay results — cells stained for two hours at 37 °C, 22 plaques counted for virus dilution 10^{-8} (titer = 2.9×10^9 pfu/mL); representative plaque sizes are more easily observed with staining.

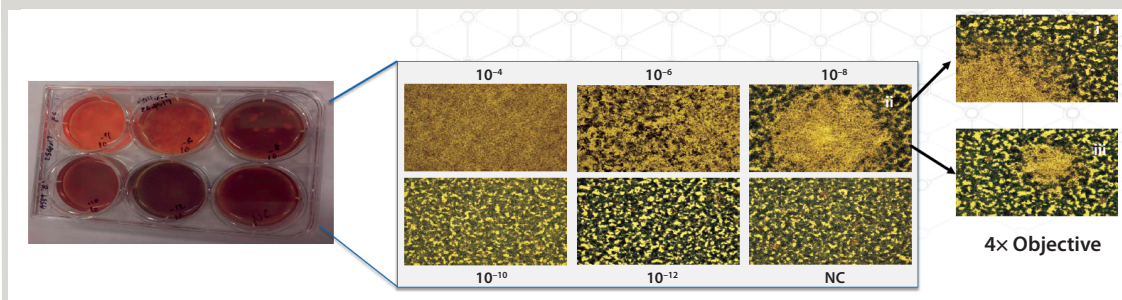


Table 5: Virus sample titer evaluation — comparing immunostaining and FACS with gold-standard plaque assay

	Immuno-staining	Plaque Assay	FACS Run 1	FACS Run 2
Virus sample	7.50×10^8	6.90×10^8	5.00×10^8	6.45×10^8
Log	8.87	8.83	8.6	8.81
Log difference	-0.04	NA	+0.23	-0.02

Photo 3: NCTM facility uses modular cleanrooms.



has over 25 years of experience in development and manufacturing of recombinant proteins, vaccines, and monoclonal antibodies, among other large molecules, viral products and medical countermeasures expressed in a wide array of microbial, mammalian, and host/virus systems. FDB offers a comprehensive list of services from cell-line development using its proprietary pAVEway™ microbial and Apollo™ cell line systems to process and analytical development, clinical and FDA-approved commercial manufacturing. FDB is a partnership between FUJIFILM Corporation and Mitsubishi Corporation.

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FUJIFILM Diosynth Biotechnologies is a world leading cGMP Contract Development and Manufacturing Organization supporting our partners in the biopharmaceutical industry in the development and production of their therapeutic candidates. We bring extensive process and analytical development as well as cGMP manufacturing experience to meet your needs at every stage of the product lifecycle.

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- Cell Culture capacity in both Stainless Steel (650L and 2,000L) and Single Use (200L - 2,000L)
- Microbial Fermentation capacity (50L - 5,000L)
- High Containment manufacturing with Mobile Clean Room Technology BSL-2+ ready and BSL-3 capable
- Dedicated suites for production of High Potency Biologics
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