

# Monitoring Adenovirus Infections with On-Line and Off-Line Methods

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Several known process monitoring methods were tested for their efficacy in the detection of adenovirus infections. The methods that we explored include several indirect indications of viral infections, including metabolic rate analysis, secondary gauges of respiration, cell size measurement, cell number and cell viability determination, and changes in capacitance. Direct indications of the adenovirus infection were also applied, including total viral particle and infectious particle measurements, as well as a flow cytometry method for detecting infected cells. All of the methods tested in the study provide some positive indication of an adenovirus infection. Many of the methods require repeated sampling, which may limit their utility in a manufacturing process. All of the indirect measures of viral infection may be limited by the fact that they do not uniquely identify an infection. The simplest monitoring methods appear to be detection of changes in respiration or the capacitance of the culture, both of which seem to provide a clear indication of an infection. Further work will be required to demonstrate that these indications are characteristic of only a successful and productive adenovirus infection.

## Introduction

Adenoviruses are one of the common gene delivery systems used for gene therapy. The large scale production of adenovirus vectors can be facilitated by monitoring methods that can qualitatively or quantitatively indicate successful and consistent infections.

Adenovirus infections have a fairly well understood infection cycle (1), which includes receptor binding (CAR receptor followed by integrin interaction), intake of virus, disassembly of the virus particle, penetration of viral DNA into the nucleus, viral commandeering of the cellular metabolism, production of the viral protein and nucleic acid components, and then a final assembly of the virus particles in the nucleus in inclusion-like structures. The entire process of a primary infection can be completed in approximately 2 days.

There have been a number of prior studies on monitoring of viral production processes (2–11), most of which are concerned with the infection of Sf9 insect cell cultures. A few studies have concerned mammalian cell culture infections (2, 6), but there seems to be only one study to date that has focused on adenovirus infections (4). The prior studies either exploit features of viral infectious cycles, such as changes in cell metabolism and physical structure, or directly assay for the presence of virus or viral transgene. Several papers have reported monitoring changes in the respiration of infected cultures (6, 9–11) or changes in the rates of metabolite consumption (2, 3, 8, 9). Two studies have looked at the green

fluorescent protein as a marker transgene for studying infections (4, 5), and one study has looked at the ABER capacitance probe as a tool for monitoring infections (7).

In this work, some of the process monitoring approaches described in these previous studies were compared to determine which methods would serve as simple and robust means for monitoring an adenovirus infection. The methods evaluated for monitoring infections included monitoring the respiration of the culture (by tracking the response of the dissolved oxygen control loop), the use of the ABER capacitance probe, cell size analysis, metabolic rate analysis, monitoring of cell viability, direct measurement of total and infectious virus titers, and a FACS assay for assessing the number of infected cells.

## Methods

**Cell Culture.** All cell culture was conducted using a proprietary human cell line transfected with the adenovirus 5 (Ad5) E1 gene region. The proteins expressed by this section of the adenovirus genome act in trans and support the replication of E1-deleted adenoviruses. These cells were cultured in suspension in a custom serum-free medium formulation. Cell culture and infections occurred either in controlled bioreactors (see below) or in shake flasks maintained in 37 °C incubators. Cells were counted using a hemocytometer, and viability was assayed by Trypan blue staining.

**Adenovirus Vectors.** Several virus constructs were used in the course of this study. All of the constructs had similar backbones, and in all cases the E1 region of the Ad5 genome was replaced with a transgene. A variety of transgenes were used in these studies.

**Bioreactors.** Applikon 3 and 10 L working volume bioreactors were used for cell culture and viral infections.

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Custom-made Braun DCU control systems (B. Braun Biotech, Allentown, PA) maintained the temperature, dissolved oxygen, pH, and other parameters of the system. Air and oxygen flows into the reactor were controlled by mass-flow controllers. The reactors were monitored by an Intellution FIX supervisory control and data acquisition (SCADA) system interfaced to the DCUs. Samples were removed from the reactors on a periodic basis for the assays described. Seed reactors were maintained in perfusion mode at  $2\text{--}4 \times 10^6$  cells/mL, using an external spin filter for cell retention as described previously (12).

Bioreactors were inoculated from the seed reactors or shake flasks at  $0.5\text{--}0.8 \times 10^6$  cells/mL, and the cells were cultured for up to 24 h, prior to virus seed additions at multiplicity of infections (MOIs) of 10–50. Bioreactors were harvested at  $\sim 48$  hpi by isolation of the infected cells from the culture supernatant, followed by cell lysis and clarification prior to purification. Harvesting at  $\sim 48$  hpi prior to significant cell lysis allows a concentration step by removal of low viral titer supernatant from the virus-containing cells, whereas later harvest times require processing of both streams for good recovery.

**Capacitance Probe.** The capacitance probe (ABER Instruments Ltd., Aberystwyth, U.K.) functions by measurement of the capacitance of the cell culture (13, 14). The probe signal was zeroed in a medium-filled, cell-free reactor, and then the slope of the signal was set to the initial reactor seeding concentration.

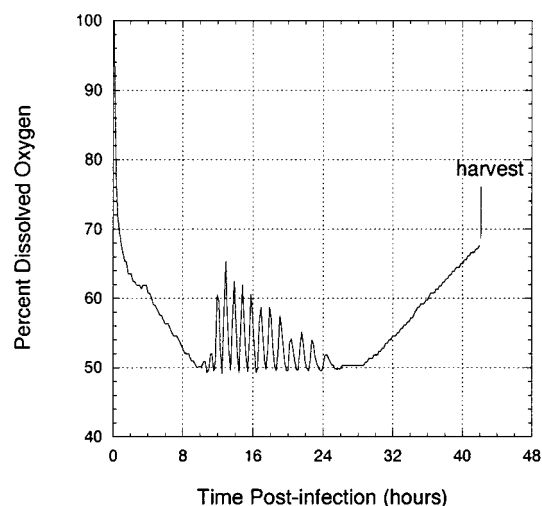
**Glucose and Lactate Analysis.** Glucose and lactate were measured with a YSI biochemistry analyzer (Yellow Springs Instruments Inc., Yellow Springs, OH). The YSI instrument employs membrane-bound glucose and L-lactate oxidases to produce hydrogen peroxide, which is electrochemically oxidized at a platinum electrode to produce a signal proportional to the chemical concentration.

**Flow Cytometry Assay.** The flow cytometry assay used in this study was developed in-house (15). An approximately 6 mL sample of cells was removed from infected or uninfected cultures and centrifuged at  $\sim 190 \times g$  for  $\sim 4$  min. The pellets were resuspended in  $\sim 100 \mu\text{L}$  of phosphate buffered saline (PBS). These cells were fixed for 10 min at ambient temperature with  $100 \mu\text{L}$  of 10% paraformaldehyde (Polysciences, Warrington, PA), with occasional mixing. The fixed cells were diluted in 13 mL of PBS, and centrifuged at  $\sim 190 \times g$  for  $\sim 4$  min. The pelleted cells were resuspended in  $200 \mu\text{L}$  of  $0.5 \times$  Power Block (Biogenex, San Ramon, CA), 0.1% Tween 80 (Sigma, St. Louis, MO), and  $10 \mu\text{g/mL}$  of fluorescein isothiocyanate (FITC)-labeled antiadenovirus penton a virus coat protein monoclonal antibody (Biodesign Int. Saco, ME). The samples were incubated for  $\sim 25$  min at ambient temperature and then diluted in 13 mL of PBS. The cells were centrifuged at  $\sim 300 \times g$  for 5 min and resuspended in 0.5 mL of PBS. These samples were then analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

**Cell Size Assay.** Cell size was measured with the Coulter Multisizer II (Coulter Electronics LTD, Luton Beds, England), which determines the particle size distribution of a cell suspension by measurement of changes in impedance as cells pass through an aperture.

**Total Virus Particles.** Determination of the total viral particles (p) was performed by analytical anion exchange HPLC (AIX-HPLC) following an adaptation of a published method (16).

**Infectious Virus Particles.** Infectious virus particles (ip) were determined using a variation of the end-point



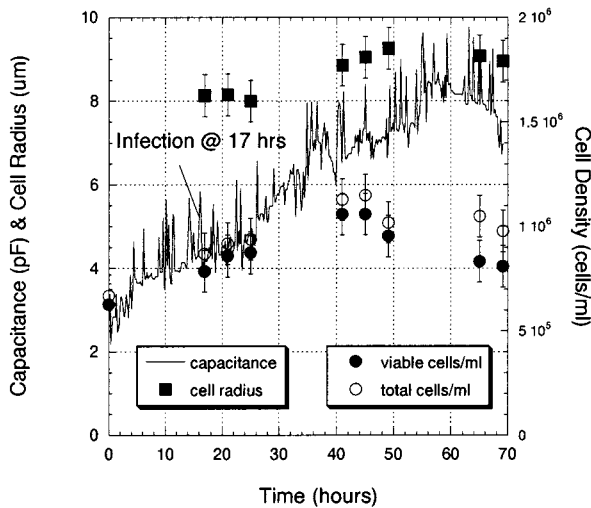
**Figure 1.** The dissolved oxygen profile of a batch bioreactor infection. For this infection, the cells were seeded into the reactor just prior to infection. The percent dissolved oxygen (%DO<sub>2</sub>) gradually drops to the setpoint, where it is then controlled by the system. In this particular case, the control loop was tuned to overshoot the setpoint in proportion to the oxygen demand, so the subsequent oscillation of the %DO<sub>2</sub> value demonstrates a significant demand for oxygen early in the infection, which tapers off after the first 24 h. The harvest of this infection is indicated at  $\sim 43$  h postinfection.

dilution (EPD) assay (17). In brief, a virus sample is serially diluted into wells containing permissive cells, and the infectivity is determined from the limiting infectious dilution.

## Results

**Dissolved Oxygen Control Data.** Bioreactor dissolved oxygen control is configured such that a low level flow of air is continually sparged into the reactors, and control demand is met by an increasing flow of oxygen. With this scheme, the percent dissolved oxygen (%DO<sub>2</sub>) level of each infection gradually drops from an initial value of 100% (when cells are seeded prior to infection) to the control level of  $\sim 50\%$  and then is maintained near this point by the control loop. The rate of decrease in %DO<sub>2</sub> during the first 10 min of the infection is a direct reflection of oxygen demand as there is no control demand flow when the %DO<sub>2</sub> is above the 50% setpoint. The control loop for this run was tuned to overshoot the setpoint in proportion to the oxygen demand, so the height of the increase in %DO<sub>2</sub> during the oscillations is at least somewhat reflective of the level of oxygen consumption (Figure 1). These data then suggest that the rate of oxygen consumption gradually increases to a maximum near 15 h postinfection, indicating a possible maximum in respiratory metabolism at that time. At 20–30 h into each infection, however, the dissolved oxygen value rises above the control point and continues to rise until the end of the infection (see Figure 1). This effect is seen with all bioreactor infections that have been run and seems to reflect a subsequent decrease in respiratory metabolism in the culture. The decrease in respiratory metabolism is probably due to the death of the cells after completion of viral synthesis and seems to be an indication of a successful infection.

**Cell Size Measurements.** Samples from infected cultures were assayed at a number of time points during an infection using a Coulter Counter. There was an increase in average cell radii at 24 h or more postinfection (Figure 2). For this particular infection, the cell radii



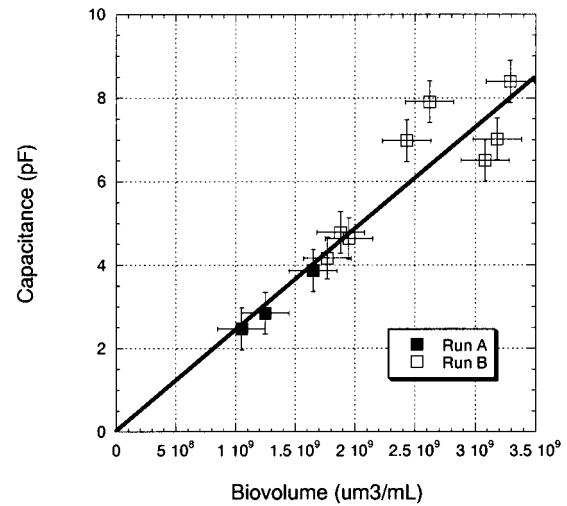
**Figure 2.** ABER capacitance signal, viable and total cell counts, and average cell radii for a batch bioreactor infection. Cells were seeded at time zero and infected at 17 h. The capacitance increases significantly above the signal per cell for the uninfected portion of the culture where the average signal is linear. The viable cell count and total cell count increase up to ~24 h postinfection, and then begin to decrease. The average cell size increases by greater than 10% at 30 h postinfection. The error bars represent the estimated standard deviations for the assays.

increased by up to 15% at 24 hpi. This increase in cell size correlates in part with an increase in capacitance (Figure 3).

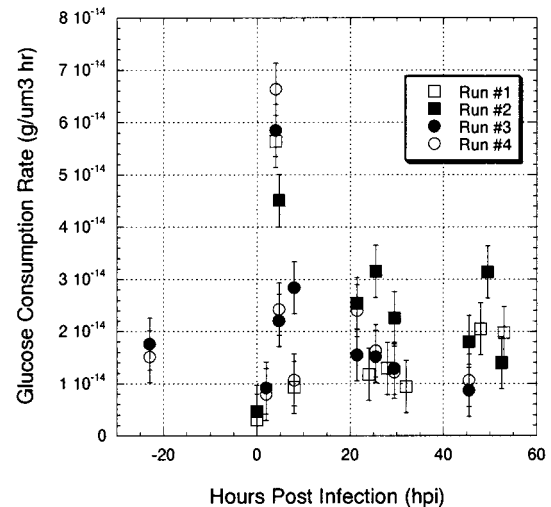
**Capacitance Probe Measurements.** A capacitance probe was used to monitor adenovirus infections in bioreactors, as was demonstrated previously for Sf9 infections (7). In our hands, the probe signal has previously been shown to correlate linearly with cell number in long-term uninfected mammalian perfusion cultures (data not shown). When the probe was used to monitor a bioreactor infection, however, there was a substantial increase in the probe signal after the infection, which did not correspond to an equivalent increase in viable cell number (see Figure 2). This increase appears to be due to an increase in cell volume upon infection (see Cell Size Measurements). The change in capacitance that is seen with infection correlates best with the total viable biovolume, as was demonstrated by Zeiser et al. (7), with an  $r^2$  value of 0.875. (see Figure 3). The absence of a better correlation may imply that other factors (i.e., lysed cell membranes, adenovirus proteins and particles) may have some impact on the capacitance of the culture. The capacitance from infections does not correlate well with the total or viable cell density.

**Cell Viability.** In most infections, there was a notable drop in cell viability from a preinfection value of  $\geq 95\%$  to  $\sim 75\%$  at 48 hpi (see Figure 2, as an example of the decrease in viable cells). This drop is significantly lower than that reported for 293 cells adapted to serum-free medium and suspension culture at 48 hpi (7). Total cell counts were less consistent in that typically there was a small reduction in total cell number at 48 hpi of approximately 5% (data not shown). Cell viability continues to decrease after 48 hpi, and since this process consists of harvesting virus from the cell fraction and not the supernatant (see Methods), viral yields begin to decrease with harvest times greater than  $\sim 48$  hpi.

**Glucose Consumption Rates.** Analysis of the consumption of glucose indicates a substantial increase upon infection (Figure 4). At around 2 hpi, the glucose consumption rate per biovolume increased substantially over



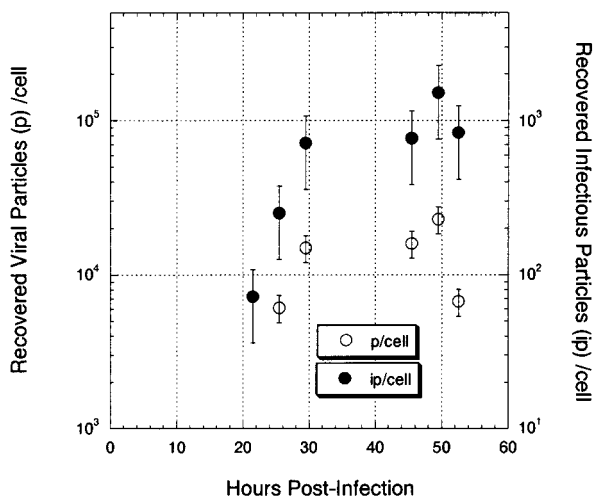
**Figure 3.** Correlation of capacitance (pF) with biovolume ( $\mu\text{m}^3/\text{mL}$ ) from two infections. Cell diameter ( $\mu\text{m}$ ) was measured off-line on samples taken from infected bioreactors at various time points over 48 h. The biovolume ( $\mu\text{m}^3/\text{mL}$ ) was calculated as the viable cell density (cells/mL) times the calculated volume per cell ( $\mu\text{m}^3$ ), assuming a spherical cell. These numbers are plotted against the measured capacitance from these bioreactor runs at these time points. A linear regression yields capacitance =  $2.47 \times 10^{-9} \times (\text{biovolume})$ ;  $r^2 = 0.875$ . The error bars represent the estimated standard deviations for both assays.



**Figure 4.** Glucose consumption rates over the course of four batch bioreactor infection. In both runs, the cells were seeded  $\sim 24$  h prior to infection, and the rates at or before time zero represent the uninfected cultures. The calculations are based on the difference in concentrations and the average cell density between time points. The rate of glucose consumption increases significantly within a few hours of infection, and decreases to near the typical level thereafter. The error bars represent the estimated standard deviations for the assay.

the normal rate of uninfected cultures and then quickly returned to a level at or near the normal rate. This sudden increase in rate is repeatable, but detection requires frequent samplings. Lactate generation per cell did not consistently increase over background (data not shown).

**Total and Infectious Viral Particle Analysis.** Normal development and production assays for viral particles include AIX-HPLC and EPD. These assays can be used to detect the presence of particles and infectious particles from an infection, usually after harvest. Tracking these values during infections indicates the titer of virus per cell over the course of the infection (Figure 5), as the process harvests only the cells and not the



**Figure 5.** Virus particle and infectious particle titers assayed during the course of a batch bioreactor infection. As described in Methods, cells are isolated from the cell culture supernatant, so recovered virus particles and infectious particles are representative of particles and infectious particles per cell. The error bars represent the normal error range for both assays.

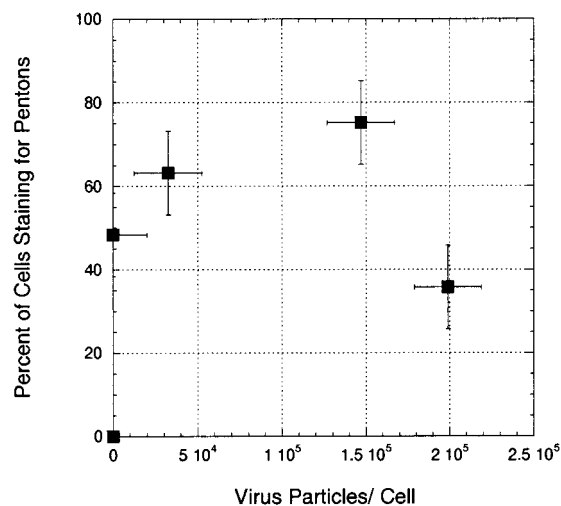
supernatant. As a result of this harvest procedure, the yields of virus decrease after ~48 hpi, as cell lysis increases and more virus is released into the supernatant. The viral content of the supernatant is significantly lower than the content of the cells at times prior to and including 48 hpi (data not shown).

**Flow Cytometric Analysis.** The flow cytometry assay allows the detection of cells that are actively producing virus. Uninfected cells are run as a control, and all cells with a fluorescence greater than the control are counted as positive for the presence of pentons (data not shown). There is no detectable staining beyond background up to 8 h after infection, indicating that cells do not stain unless they are actively producing adenovirus proteins (i.e., the virus used for infection is not detectable). In the range of 24 h, approximately half of the cells of a normal infection stain positive for pentons. By 30–40 h, the percentage of positive cells approaches 75%. At later time points we begin to see a decrease in the number of cells staining positively in this assay (~36% at 48 h in the infection data used for Figure 7), despite the fact that we see no decrease in virus production per cell in this same region of time. (As the method of harvest isolates cells from the culture supernatant, so assayed viral titers reflect intracellular virus content. See Methods and Figure 5). As a result, the percentage of cells staining positive for virus does not correlate with the total number of virus particles per cell at these time points, as computed from the yield of virus and the viable cell count at harvest (see Figure 6). This could indicate that as the assembled virus accumulates in the nucleus (1), they become less accessible to the antipenton antibody.

### Conclusions

The purpose of this study was to evaluate both on-line and off-line methods that might be consistent indicators of a successful adenovirus infection.

The off-line methods evaluated included measurement of cell size, measurement of cell viability, metabolite assays, a flow cytometry assay for the presence of adenovirus proteins, an AIX-HPLC assay for total viral particles, and an EPD assay for total infectious viral particles. These various off-line methods as we conducted them have the disadvantage that they require additional



**Figure 6.** Correlation of the percentage of cells labeled positive for adenovirus pentons at various time point samples from a batch infection, compared with the total adenovirus particles per cell recovered at the same time points. The error bars represent the estimated standard deviations for both assays.

work from operators or lab staff for sampling, and they can be slow to provide feedback on the process.

Measurement of cell size shows that there is an increase upon infection that is reproducible and simple to assess. Measurement of cell viability shows that a decreasing percentage is an indicator of a successful infection. Both an increase in cell size and a drop in cell viability could also be driven by other factors, however, such as stress upon the culture, so it would be difficult to rely on only these measurements as the determinant of a successful infection.

Assays of the metabolites during an infection shows a brief but consistent jump in glucose consumption rates upon infection, and similar results have been noted by others (2, 3). The fact that the large change in consumption happens very shortly after infection limits its usefulness; additional sampling would be required close to the time of infection, and the rates seem to drop to normal several hours after infection.

Direct assessment of the number of virus particles produced or, better, the number of infectious particles produced is the standard means to quantitate the production of virus in the process. Although the AIX-HPLC method can have signal-to-noise problems with low yielding infections, it is normally a fast and reliable off-line method for analyzing an infection. The EPD method takes a few weeks to run, so it cannot provide feedback to ongoing infections. Simpler cytopathic effect methods can be used to estimate infectivity in about 2 days, but that is by nature as long as the infectious process.

The flow cytometric analysis is a valuable development tool in that it directly shows the number of cells that are producing virus in a culture, and it has been used previously with viruses producing green fluorescent protein (4, 5). Flow cytometry may be too labor intensive, however, to be used to analyze every production run. Also, we have found that the number of cells staining positive for penton does not correlate with viral recovery and decreases near what appears to be the end of the primary infection.

The two on-line methods described are the ABER capacitance probe and the profile of a %DO<sub>2</sub> probe used with our particular control scheme. These methods have the advantage of not requiring additional sampling or operator effort. The %DO<sub>2</sub> profile of these infections is

an outcome of the specific control scheme used and is not a general approach. This profile clearly shows a decrease in consumption at the end of an infection, but the possible increase in consumption in the beginning of the infection is debatable. The use of an OUR measurement instead of this simple profile tracking, should provide better information, as has been demonstrated previously for other viral infections (6, 9–11). OUR measurements are currently being designed into our bioreactor system.

The ABER capacitance probe seems to provide an equally reliable means for indicating that an infection has occurred and correlates fairly well with the increase in biovolume seen upon infection, as has been shown previously for baculovirus infections (7). The increase in capacitance is larger than would be expected by normal cell growth in the reactor and is easily compared to the normal cell counts from sampling that would be performed on a production reactor. The deviation from a linear correlation between capacitance and biovolume (see Figure 3) may indicate that other factors in the infected culture are impacting the capacitance signal.

The capacitance probe and some measure of the oxygen consumption rate (e.g., the oxygen profile from these reactors or preferably an OUR measurement) seem to be the best approaches for monitoring adenovirus infections, in that they require little operator intervention beyond calibration and provide what seems to be unambiguous indicators of a successful infection. It is possible, however, that stresses to the cells could cause an increase in cell size and thus capacitance or an increase in oxygen utilization (e.g., by contamination). The ABER probe has consistently shown this response for the few infections that we have studied, and this same response has been reported by others (7). The oxygen profile that we see is reproducible for every bioreactor infection we have run where we have a record of these data.

The adenovirus infections described in this work follow an interesting dynamic, starting with an early increase in glucose metabolism and possibly oxygen consumption rate, followed by a later increase in cell size and the percent of cells producing pentons, and ending with a decrease in viability, a decrease in respiration, and an increasing level of recoverable virus. These results seem to follow the known life-cycle of adenovirus (1), where the virus first commandeers the cell metabolism and cycle and then synthesizes its proteins and DNA, followed by virus assembly, and DNA packaging and clustering of the virus in the cell nuclei, before the final stage of cell lysis and virus release (The production scheme described in this work attempts to avoid this final step).

This study has evaluated some tools that can be applied to monitor adenovirus infections both at a development and a production scale. All of these methods have advantages and disadvantages, which will need to be evaluated for each situation. The ideal situation would be the use of simple accurate on-line probes or measurements that require little operator labor or a fast and reliable off-line assay, where either provides an unambiguous measure of the infection. Unfortunately, all of the simple assayed described are secondary indicators of the adenovirus life cycle and do not uniquely identify an adenovirus infection. Only direct measurements of the virus particles unequivocally demonstrate an infection. The online measurements of the %DO<sub>2</sub> (which may be improved by direct OUR measurements) or the ABER capacitance probe are two potential solutions for monitoring adenovirus vector production that should provide a clear indication of an infection under normal conditions. Either of these assays would be best supported with a

particle analysis (AIX-HPLC), which provides the fastest direct measurement of the outcome of the process. Future work in this area might include studies to determine the mechanisms that drive cells infected with adenovirus to demonstrate such behavior. Other studies should confirm that secondary indications of an infection, such as the %DO<sub>2</sub> profile or the capacitance profile, indicate a complete infection and not either a low MOI infection where little virus is produced or a high MOI infection where the cells are killed by a toxic effect, or some other noninfectious cell stress.

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