COMMUNICATION TO THE EDITOR

New Disposable Tubes for Rapid and Precise Biomass Assessment for Suspension Cultures of Mammalian Cells

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Abstract: We present a new approach for biomass assessment in cell culture using a disposable microcentrifuge tube. The specially designed tube is fitted with an upper chamber for sample loading and a lower 5 μL capillary for cell collection during centrifugation. The resulting packed cell volume (PCV) can be quantitatively expressed as the percentage of the total volume of the sample. The present study focused on the validation of the method with mammalian cell lines that are widely used in bioprocessing. Using several examples, the PCV method was shown to be more precise, rapid, and reproducible than manual cell counting. © 2006 Wiley Periodicals, Inc.

Keywords: packed cell volume; CHO DG44; HEK 293; NS0; cell growth; PCV tube; cell volume

INTRODUCTION

Biomass is a key parameter for the evaluation of bioprocesses. The precise monitoring of this variable is required to establish the specific growth rate and the maximal or final cell concentration. Moreover, the accurate assessment of biomass is a prerequisite for the determination of metabolic shifts and for the establishment of control strategies for fed-batch and continuous cultures (Konstantinov, 1996). Numerous methods that allow the quantification of biomass have been suggested. Optical density and the gravimetric determination of dry mass are widely used for the monitoring of growth kinetics in microbial systems, but these methods are not sensitive enough for cultures of mammalian cells due to the relatively low densities to which these cells grow in vitro. Cell counting using dye exclusion remains the most common approach in many academic laboratories. However, the reproducibility and accuracy of this method are limited, and the microscopic examination is highly subjective, tedious, and particularly time-consuming for multiple samples. Automated particle counters offer a faster and more reproducible approach to cell counting. Such instruments use sampling devices, vital dyes, and a counting chamber combined with an integrated microscope or they operate without dyes and use electrical or optical properties to distinguish living and dead cells. However, these systems often require the dilution of the cell suspension within a defined cell density range, the staining of cells, and the use of specific suspension buffers. Also, cell aggregates can induce major biomass determination errors. Although some of these systems are already applied in industrial bioprocesses, they represent a major cost factor. For instrumented bioreactors the rates of oxygen uptake or glucose consumption can be employed for the indirect on-line determination of mammalian cell concentration (Ducommun et al., 2001; Lubenova et al., 2003; Schoenherr et al., 2000). Common intracellular enzymatic assays have also been utilized to quantify the number of viable cells. These methods demand carefully controlled incubations and the establishment of a standard curve for each cell type.

Faced with the limitations of current biomass determination methods we tested an approach that is widely employed for blood analysis. Typically, an aliquot (50 μL) of blood is loaded into a capillary and then centrifuged. The height of the cell pellet within the capillary is measured instrumentally and expressed as the packed cell volume (PCV) or hematocrit, meaning the fraction of blood occupied by the cells. Typical PCV values in this case range from 30–50%. For cultivated cells the PCV is much lower and as a consequence the sensitivity of the hematocrit tube is not sufficient. We therefore designed and tested a novel disposable microcentrifuge tube that overcomes this limitation. The geometry of the tube was chosen so that up to 1 mL of cell suspension can be loaded into the upper chamber

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having an external diameter that is identical to a standard 1.5 mL microcentrifuge tube. The lower chamber is a graduated capillary with a volume of 5 μL. During a brief centrifugation, the cells in the sample are concentrated within the capillary. Thus the method uses the occupied volume of the cells and centrifugable cell fragments in a sample as a measure of biomass.

The method described here has the advantage of being more precise, more reproducible, and faster than microscopic cell counting. In the case of multi-parameter testing of a large number of process conditions, the PCV method allows a comparison of biomass proliferation in hundreds of different cultures at any given time. Typically, the assessment of 50 samples takes less than 30 min, which is clearly faster than any other currently available biomass assessment method. The precision and ease of this method even makes it convenient to measure and analyze with high precision the growth kinetics within a large cell culture vessel in a time interval of only a few hours.

Here, we present the first description of a novel disposable mini PCV tube for the biomass assessment of suspension cultures of mammalian cells. This study focused on the reproducibility, precision, and reliability of the PCV method as compared to manual cell counting. We also present examples for the successful application of mini PCV tubes in typical bioprocesses with Chinese hamster ovary (CHO), human embryo kidney 293 (HEK 293), and mouse NS0 myeloma cells.

MATERIALS AND METHODS

Cell Culture

Suspension adapted CHO DG44, HEK 293, and NS0 cells were grown in chemically defined ProCHO5-CDM (Cambrex Bio Sciences, Verviers, Belgium), EX-CELL 293 Serum-Free Medium (SAFC Biosciences, Andover, UK), and HyQ-CDM4NS0 (HyClone, South Logan, UT), respectively. Unless otherwise mentioned, the cells were cultivated in either 50 mL single-use TubeSpin® bioreactors (Techno Plastics Products AG, Trasadingen, Switzerland) (De Jesus et al., 2004) or 500 mL round- or square-shaped bottles (Muller et al., 2005). The cells were incubated in a CO_2- and humidity-controlled atmosphere at 37°C (ISF-4-W, Adolf Kühner AG, Birsfelden, Switzerland) with orbital agitation (Muller et al., 2005). The cells were passed every 3–4 days at densities of $2–5 \times 10^5$ cells/mL. Additionally, a recombinant NS0 cell line was cultivated in a 20 L pilot-scale bioreactor (Bioengineering, Wald, Switzerland) in a fed-batch culture for more than 250 h.

Samples of CHO DG44 cells with different viabilities were produced by mixing viable and non-viable cells. To produce non-viable cells, a cell suspension was centrifuged at 200*g* for 5 min. After removal of the supernatant, the cell pellet was incubated at 37°C for 4 h. Manual cell counting by the Trypan blue exclusion method (see below) demonstrated that the cells lost their viability but retained their size and shape. By mixing aliquots of viable and non-viable cells at different ratios, samples with different viability percentages were obtained.

Cell Counting and PCV Determination

Cell density and viability were assessed with a hemocytometer using the Trypan blue exclusion method. The cell density included both viable and non-viable cells and was therefore referred to as total cell density. For PCV measurements, aliquots of a well-mixed suspension culture (100–1,000 μL in multiples of 100 μL) were transferred into a mini PCV tube (Techno Plastics Products AG, Trasadingen, Switzerland) (Fig. 1A). The tubes were centrifuged in a microcentrifuge (Model 5417C, Eppendorf AG, Hamburg, Germany) fitted with a swinging-bucket rotor (Model A-8-11, Eppendorf AG) for 1 min at 2,500*g* (5,000 rpm) unless otherwise mentioned. For determining the height of the cell pellet, a visual assessment was made using the capillary graduation (Fig. 1B). Alternatively, an in-house image analysis device was employed with non-graduated tubes provided by the manufacturer (Fig. 1C). An image of the lower part of the mini PCV tube was acquired with a webcam (Watchport/V2 USB camera, Inside Out Networks, Austin, TX) and automatically analyzed with a program that was specifically developed for this purpose using Java technology. The software was optimized to measure the height of the cell pellet and calculate the corresponding volume as a percentage of the total volume loaded into the tube.

RESULTS AND DISCUSSION

The mini PCV tubes were validated by determining the optimum centrifugation conditions. First, samples from cultures of either CHO DG44 or HEK 293 cells were centrifuged in mini PCV tubes at centrifugation forces ranging from 100 to 6,000*g* while the centrifugation time was kept constant at 1 min. The PCV varied from approximately 1%–2% depending on the centrifugal force with a sharp decrease as the force increased from 100 to 1,000*g* (Fig. 2A).

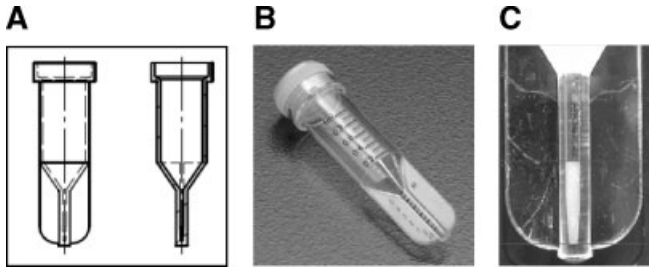

Figure 1. Mini PCV tube geometry (**A**), commercial version of the mini PCV tube with graduation (**B**), and image analysis of the cell pellet (**C**) (technical drawing and photo courtesy of Techno Plastic Products AG, Switzerland). The transparent tube has a diameter of 13 mm and a height of 43 mm. The maximal volume of the capillary is 5 μL.

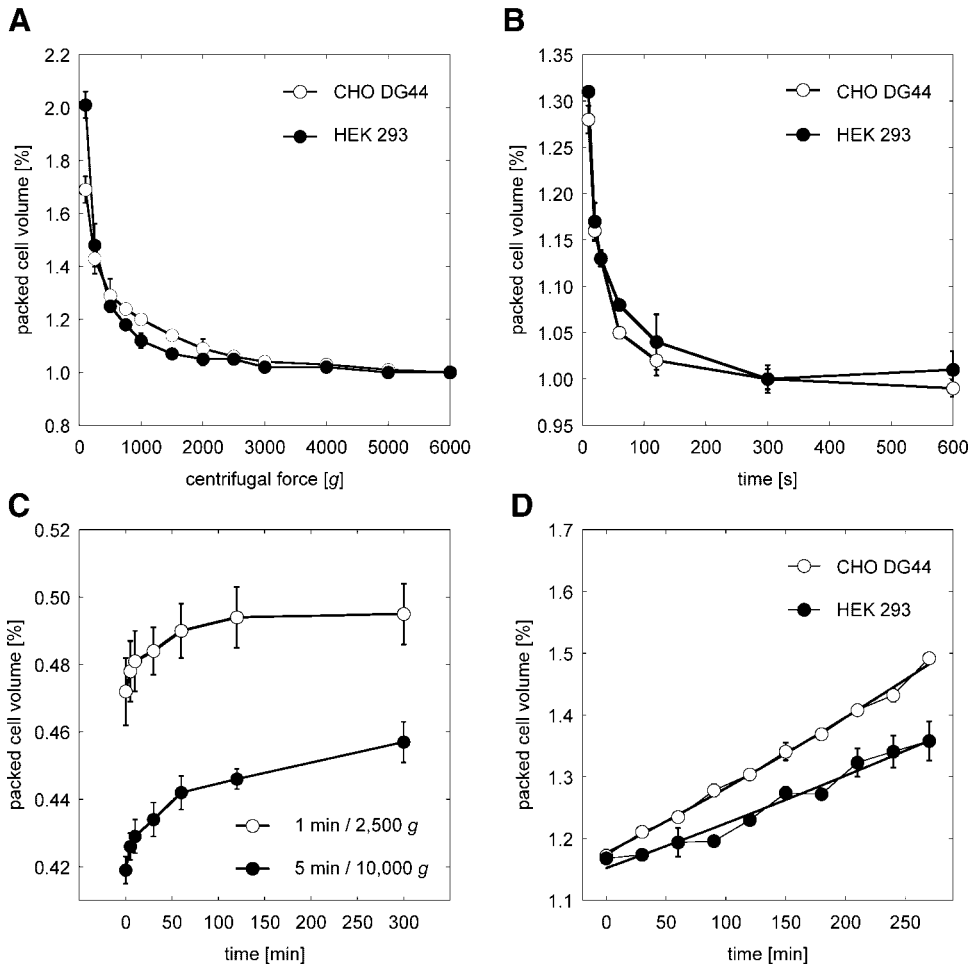

Figure 2. Variation of the PCV as a function of the centrifugation force (**A**) and centrifugation time (**B**) for suspension CHO DG44 and HEK 293 cells. The cells were centrifuged for 1 min in mini PCV tubes. Average PCV values are reported ($n = 3$). (**C**) Increase of the packed cell volume post-centrifugation in mini PCV tubes at different time and speed combinations. PCV measurements were made from the same batch of CHO DG44 cells. Average PCV values are reported ($n = 8$). (**D**) Packed cell volume measurements at 30 min time intervals of suspension CHO DG44 and HEK 293 cells in exponential growth phase. Average PCV values are reported ($n = 3$).

At centrifugation forces greater than 2,500*g*, the PCV tended toward a horizontal asymptotic value. At centrifugation forces less than 2,500*g*, the profiles of the curves for CHO DG44 and HEK 293 cells were clearly different. At the lower centrifugation forces tested, even small differences in medium viscosity may explain such variation by acting on the drag forces. CHO DG44 and HEK 293 cultures, even at roughly the same biomass concentration, might have different viscosities due to different medium compositions and to the release of cell-line specific metabolic compounds. The packing of the cells at lower centrifugation forces may also have differed because of cell-line specific size distribution. This may have resulted in different void or liquid fractions between the packed cells, especially at low-centrifugation forces. At higher forces, due to increased packing of the cell pellet, these differences appeared to be less important.

Next, the centrifugation force was kept constant at 2,500*g* and the time of centrifugation was varied from 10 to 600 s. The resulting PCV values for CHO DG44 and HEK 293 cells varied from about 1%–1.3% (Fig. 2B). A centrifugation time of 1 min appeared to be a good compromise between the need for rapidity and the amount of variation in cell pellet size. From these observations, the standard conditions for PCV measurements were defined as a centrifugation force of 2,500*g* for 1 min. Further increases in both variables reduced the level of the error because of a denser cell packing process. However, it was observed that as the density of cell packing increased, a higher degree of post-centrifugation cell pellet expansion was observed (Fig. 2C). Cell culture samples were centrifuged in mini PCV tubes under two different conditions. Then the expansion of the packed cells was monitored over a period of 5 h. It was shown that after a 5 min centrifugation at 10,000*g* the expansion of the cell pellet at 5 h post-centrifugation was 8.2%. This was almost twice as much as the expansion measured with the standard centrifugation conditions (4.7% at 1 min and 2,500*g*). Moreover, at the higher centrifugation force and with the longer centrifugation time, the pellet continued to expand after 5 h at room temperature. The results also showed that

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most of the pellet expansion occurred within 10 min after the centrifugation (Fig. 2C). During this time, the increase of the PCV due to pellet expansion was approximately 2%. For this reason the measurement of the PCV was typically performed immediately after centrifugation, thus increasing the reproducibility of the method. We assumed that the expansion of the cell pellet after centrifugation was due, at least in part, to the recovery of a more spherical cell shape which may have been deformed during the process.

The accuracy of the PCV measurement was evaluated by measuring 10 individual samples from the same CHO DG44 suspension culture. The PCV measurement was then repeated 1 h later with the same culture. First, the statistical analysis of the results showed that the standard deviation of the 10 independent PCV measurements was typically less than 1% of the average value, which indicated a high degree of reliability. Second, the increase of biomass within a period as short as 1 h was statistically relevant with the average PCV values of 0.484% and 0.492% for the two time points. The high degree of precision illustrated in this example opens new perspectives in cell culture growth monitoring. Previously, the measurement of cell growth within very short time periods was limited, mainly because of the low resolution of conventional methods of biomass analysis. Manual counting using a hemocytometer typically produces errors between 5% and 20%. For the PCV method described here, the most probable source for error was in sampling and the transfer of the material into the mini PCV tube. However, the error was very low since only a single pipetting was required and no dilution step was necessary.

To verify the resolution of the PCV method the biomass increase of suspension growing cells was monitored within very short time intervals. Exponentially growing CHO DG44 and HEK 293 cells were cultivated in 500 mL square-shaped bottles and samples were taken every 30 min for 4 h. Results showed a measurable increase in biomass from a starting PCV value of 1.17% for both cell lines up to 1.49% and 1.36% for CHO DG44 and HEK 293 cells, respectively. By fitting the data with an exponential curve, the specific growth rates (m) were determined to be 8.6×10^{-4} and 6.1×10^{-4}/min for CHO DG44 and HEK 293 cells, respectively (Fig. 2D). These values corresponded to doubling times of approximately 13 and 19 h, respectively. This example showed that differences in growth kinetics between cell lines or due to different process conditions can be easily and reliably assessed with the PCV method even within brief time intervals. The same procedure is not feasible with manual cell counting due to the level of error associated with this method.

To determine the correlation between manually obtained cell density values and PCV in exponentially growing cultures, CHO DG44 and NS0 cells were maintained in agitated 50 mL TubeSpin® bioreactors with subcultivation every 3–4 days. The PCV and cell density were assessed in duplicate twice every day for 30 days giving a total of 120 samples for each cell line. Each average PCV value was plotted as a function of the corresponding average cell density (Fig. 3). It is obvious from the results that the two cell lines have different individual cellular volumes. However, for both cell lines, up to a density of 4×10^6 cells/mL, the relationship between PCV and cell density was approximately linear (Fig. 3). At higher cell densities, however, the average cell volume was slightly reduced and the correlation was not linear (data not shown). This suggested that the cell volume changed when the batch cultures approached stationary phase. This may be explained by the fact that an increase in cell volume is not necessarily correlated with the progression through the cell cycle. In fact, while the cell density increases, the availability of growth factors may be reduced, resulting in a lower maximal cell volume (Conlon and Raff, 2003).

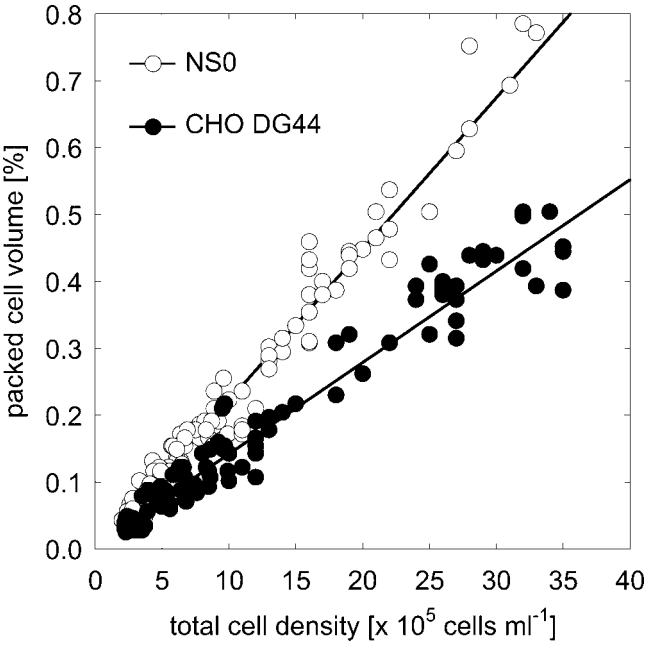

Figure 3. PCV of CHO DG44 and NS0 myeloma cells as a function of total cell density assessed by manual cell counting. The cells were subcultivated every 3–4 days and samples were taken twice per day. The linear regression gives the correlation between the two biomass assessment methods for each cell line. Average PCV values and manual cell counts are reported ($n = 2$).

Usually, cell growth includes the increase in both cell volume and number. However, the comparison of PCV and manual cell counts indicated that the two biomass assessment methods provide information which is intrinsically different. This aspect of biomass analysis was considered in an experiment in which the variation in cell volume due to osmolarity changes was assessed. An aliquot (200 mL) of a suspension of either CHO DG44 or HEK 293 cells was mixed with the same volume of phosphate-buffered saline (PBS) of various concentrations so that the osmolarity was varied from hypotonic (150 mOsm/kg) to strong hypertonic conditions (1,500 mOsm/kg). The resulting PCV values were assessed for each different osmotic condition and normalized to that obtained at 150 mOsm/kg (Fig. 4A). Surprisingly, a threefold difference in cell volume was observed between 150 and 800 mOsm/kg for both cell lines. However, little difference in

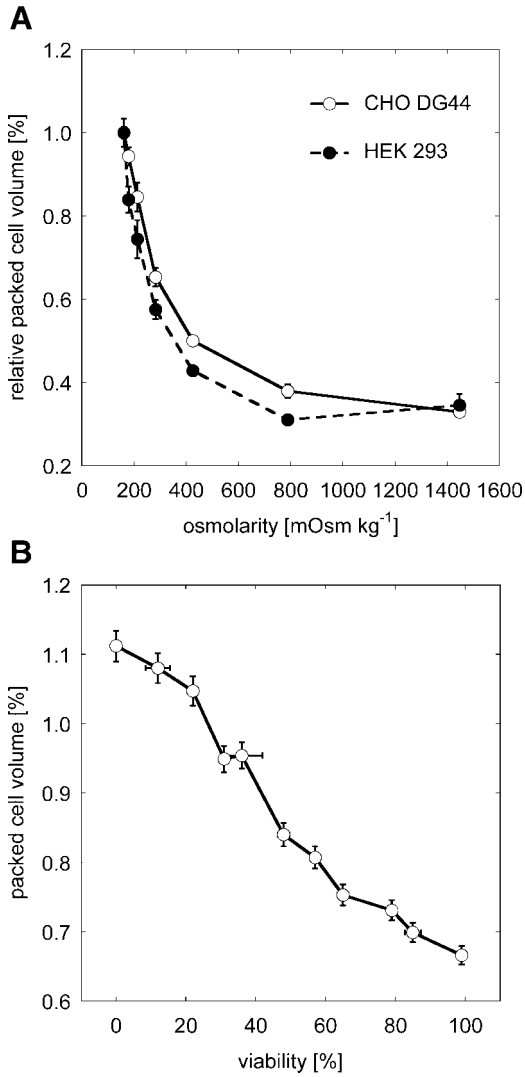

Figure 4. **A**: Relative PCV of CHO DG44 and HEK 293 cells under different osmotic conditions. Sample (200 µL) were mixed with 200 µL of PBS at different concentrations and transferred to a mini PCV tube for measurement. The samples were taken from the same initial cultures of CHO DG44 and HEK 293 cells, respectively. Average relative PCV values are reported ($n = 3$). **B**: Packed cell volume of CHO DG44 cells under hyperosmotic conditions (1,300 mOsm/kg) as a function of cell culture viability. The viability was assessed in duplicates by Trypan blue exclusion. Average PCV values are reported ($n = 2$).

PCV was observed from 800 to 1,500 mOsm/kg (Fig. 4A). The osmolarity of most commercially available cell culture media is between 260 and 310 mOsm/kg. The data shown here indicated that small osmolarity shifts in this range affect the cellular volume quite dramatically.

Similar experiments with osmolarity changes have been carried out previously with red blood cells and chondrocytes where it was shown that the Boyle-van't Hoff relationship predicts the cell volume under various osmotic conditions (Guilak et al., 2002; Yang and Kamino, 1995). This "ideal" osmotic behavior of eukaryotic cells was exploited to distinguish between osmotically active (viable) and inactive (dead) cells. During batch cultivation of cells beyond 3 or 4 days the viability of a culture usually decreases, probably due to nutrient depletion and the accumulation of metabolic waste products. We assumed that dead cells lose their ability to adapt their volume to the osmotic environment. Aliquots of CHO DG44 cells with identical cell densities but ranging in viability from 0 to 99% were prepared and mixed with an equal volume of a concentrated PBS solution giving a final osmolarity of 1,300 mOsm/kg. The PCV values of the various cultures were then plotted as a function of the cell culture viability (Fig. 4B). Whereas cells at 99% viability had a PCV value of 0.7% under these conditions, the same number of non-viable cells had a PCV value of more than 1.1% (Fig. 4B). Thus, as assumed, non-viable cells (as monitored by Trypan blue exclusion) did not respond to the increase in osmolarity. These findings showed that the PCV method may also be used as a tool for the rapid assessment of cell condition (i.e., viability) during bioprocesses.

Lastly, the PCV method was applied to the monitoring of recombinant protein production with an NS0 cell line at the 20-L scale in a stirred tank bioreactor. The manual cell counts and the PCV values were used to determine the growth profile of the culture (Fig. 5). During the entire exponential growth phase, an excellent correlation between both measurement methods was observed. However, the average of the errors was lower with the PCV method (1.7%) as compared to manual cell counting (5%). After reaching the maximal cell density, the PCV remained relatively constant, whereas the total cell density decreased (data not shown). This probably reflected the accumulation over time of dead cells and cell fragments that were included in the PCV measurements but not in the manual cell counts. In the stationary and death phases of the culture, the manual cell counting was extremely

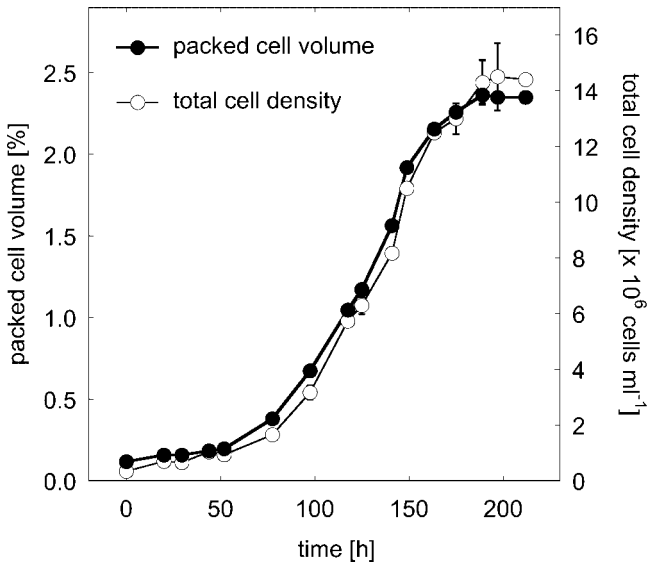

Figure 5. PCV and total cell density of a recombinant NS0 myeloma cell line. The cells were cultivated in a 20-L fed batch bioreactor for the production of a monoclonal antibody. Average PCV and cell counts are reported ($n = 2$). The average errors for all the PCV measurements and the manual cell counts were $1 \times 7\%$ and 5%, respectively.

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inaccurate due to the presence of numerous particles, cell fragments, and aggregates. In contrast, because all centrifugable material was packed in the pellet, the error for the PCV measurements remained very low even at the late phases of the batch cultivation.

The biomass assessment method described here was found to provide a unique combination of speed and precision at low cost. The validation of the method allowed the identification of the optimal conditions for centrifugation. The study also demonstrated the potential use of the method under different conditions, with different cell lines, media, and culture techniques. Unlike manual cell counting, the PCV method allows statistically reliable measurements of the total biomass including cell fragments and aggregates present in late phases of batch cultures. Due to the high sensitivity of the method, even slight variations in metabolic activity were monitored with a high degree of precision. The new method is another step in the introduction of low-cost, disposable technologies in modern bioprocessing.

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