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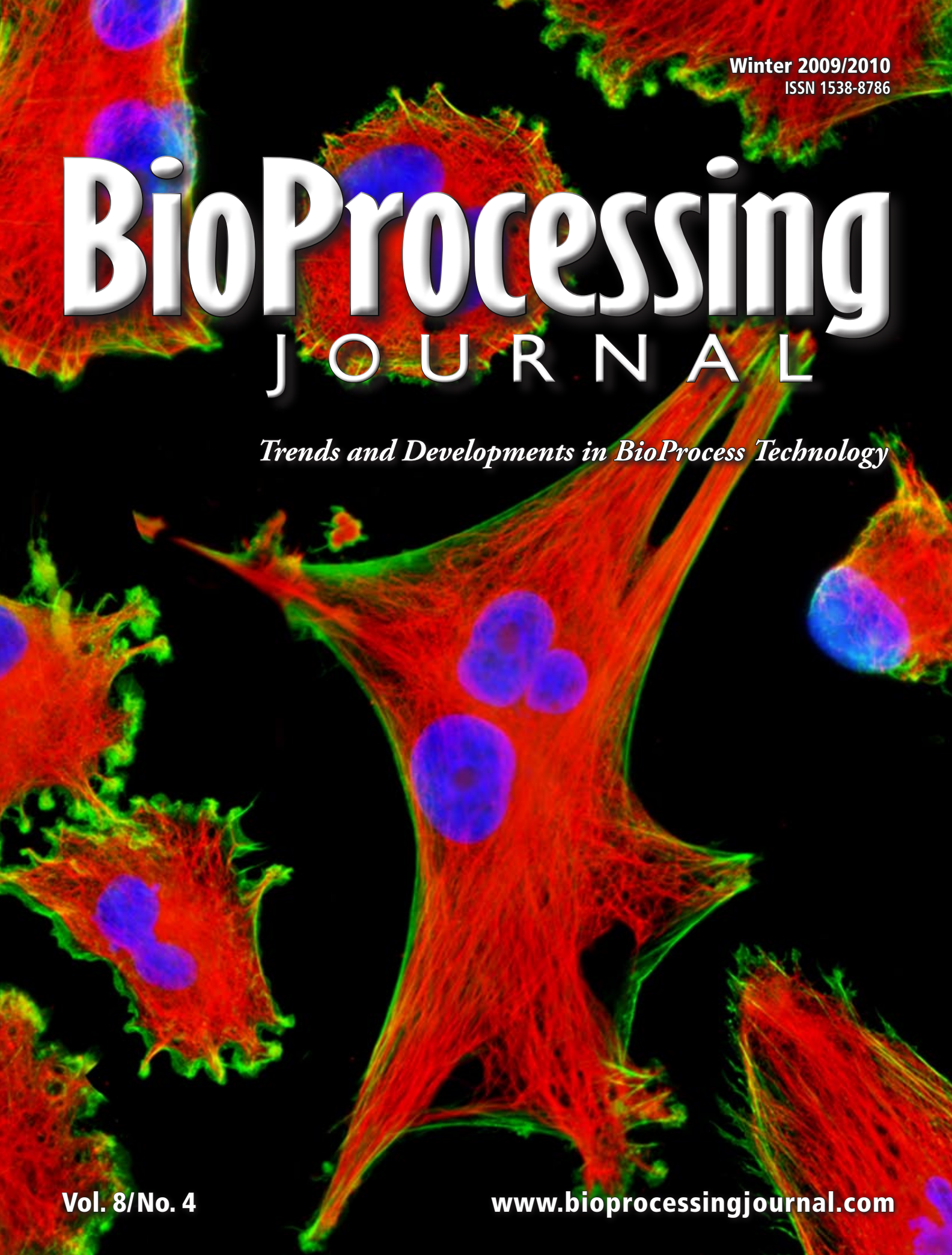

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Monitoring Viable Cell Density in Bioreactors Using Near-Infrared Spectroscopy

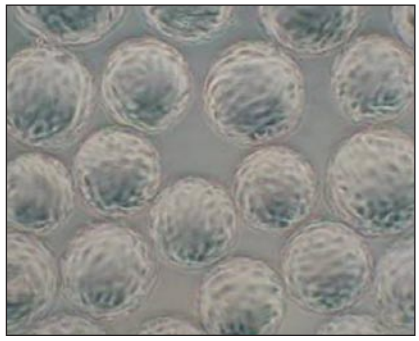
By ROBERT MATTES *et al.*

Viable cell density (VCD), the quantitative assessment of living cells, is commonly determined by laborious and inaccurate off-line cell counting methods. Single-function *in situ* probes have been developed using various technologies including optical density, radio frequency[1] and dielectric permittivity.[2] Optical density measurements predict total biomass but are sensitive to cell debris accumulation and inherent media turbidity.[3,4] Near-infrared (NIR) has the advantage of being able to measure many key analytes in the cell culture simultaneously. NIR has been used to acquire real-time measures of glucose, glutamine, glutamate, and media nutrients such as amino acids and metabolites (*e.g.*, lactate and ammonia).[5] NIR probes can be sterilized in place, and real-time measurements can be acquired throughout the mammalian cell culture processes.[6]

The fundamental absorption bands of functional groups occur in the mid-infrared region of the electromagnetic spectrum. These are very strong absorptions that require dilution to lower the absorbance within the linear range of a mid-infrared detector. The majority of the overtone absorptions and combination bands of these fundamental absorptions are detected in the near-infrared spectral region. Overtone absorptions occur at frequencies that are approximate integer multiples of the fundamental or first-excited vibrational state of the molecular bond.[7] NIR absorption in solutions of cell culture media is relatively weak, and direct measurement without sample preparation or dilution is possible. The NIR absorptions are due to the change of dipole moment in the covalent bonds, as found in organic compounds. The OH, CH, NH, and SH bonds, as well as C=O and C=C, have strong absorbance in the NIR region. NIR has been used to predict pH and osmolality in real-time in bioreactors and ionic species in aqueous solutions.[8,9]

The exact sources of the chemical cell characteristics that discriminate between viable and morbid cells in the NIR spectrum are not well understood. Differences in cellular and environmental pH might be the obvious phenomena triggering a specific NIR signature. Maintenance and regulation of intracellular pH is important for central biochemical pathways. At cell death, the cell membrane acting as a physical barrier will lose its integrity and let internal and external components reach equilibrium, subsequently leading to internal pH variation. Correlation between proliferation activity and intracellular pH has been demonstrated with fluorescent staining in various organisms.[10] Intracellular pH initiates multiple changes and has a direct impact at the mitochondrial level during cell death[11] by affecting the concentration of NA^+, K^+, and Ca^{++} accumulation.

More complex signals could provide NIR signatures that would correlate with cell viability. In a viable state, the cells maintain an equilibrium between cholesterol and sphingolipids in specialized regions called lipid rafts. These regions control membrane transport in viable cells and will differ in cholesterol content from morbid cells.[12,13] As soon as cell death occurs, these complex regions disappear, but the cholesterol remains present in the membrane and is no longer associated with the complex lipid raft structures.[14] The resulting fluidity/



SF Vero cells grown on Cytodex 1 microcarriers on day 4 post-seeding. Cells attach initially and spread over the microcarriers to reach confluence as they divide. (Photo: Misa Sugui)

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rigidity state of the cell membrane and its relation to cell viability might be a potential NIR spectral indicator. Further studies will be required to establish a relationship between biological markers and the NIR spectra.

One of the key goals for the FDA's Process Analytical Technology (PAT) Initiative[15] is to increase the manufacturing process understanding and control of a product. NIR can be used to collect a vast amount of information that allows efficient process monitoring and control in bioreactor. This information can also support process improvement by providing a critical content to multivariate data analysis. This study demonstrates that NIR fits well that description when applied to the monitoring of the VCD, as it can be used to produce feedback loop process signals directly from the bioreactor in order to optimize the cell performance and productivity.

Experimental

Bioreactor Settings

A cell line was adapted to serum-free growth conditions and inoculated into bioreactor at 2×10^5 cells/mL with 4g/L of microcarrier. The culture was grown at 37°C with pH controlled at 7.10 ± 0.05 with CO_2 and base for three days. Dissolved oxygen (DO) was allowed to float down from the initial 100% to the set point of 50% of air saturation by sparging pure oxygen. The agitation rate for cultures in the 3 L bioreactor (Biobundle™; Applikon, Foster City, California) was set to 125 rpm. Before infection, medium exchange was performed and cells were infected with a virus at a lower temperature. Agitation, pH, and DO were controlled as described above. The cells were adhered to Cytodex™ 1 microcarrier beads (GE Healthcare, Piscataway, New Jersey).

NIR Settings

A ¾″ (~19 mm) diameter and 12″ long interactance immersion probe was installed in a 3 L Applikon bioreactor with a 1.5 L working volume. The adjustable path-length probe was set to a 1.0 mm gap (and 2.0 mm path-length) prior to being autoclaved. An XDS Process Analytics™ near-infrared spectrophotometer (FOSS NIRSystems, Laurel, Maryland) with a 3 m microbundle (40 illumination/40 collection) optical fiber was set up near the reactor. After the culture was prepared and the run was initiated, the optical fibers were inserted into the probe that was already in place and sterilized in the bioreactor.

NIR data acquisition and analysis were accomplished using Vision™ software supplied by FOSS. All sample spectra were collected over the range of 800–2200 nm in the interactance immersion mode. 32 spectra were averaged for each sample, resulting in a scan time of approximately 16 seconds per spectrum. The XDS Process instrument uses a standardized internal reference loop that compensated for lamp variations throughout the 12 day cell culture. Scans were collected every 15 minutes for the duration of 12 days. Laboratory samples were collected daily during the culture process.

Cell Viability Measurement

The off-line VCD values were determined by extracting samples at different time points from the bioreactor and analyzing them with the NucleoCounter™ (New Brunswick Scientific, Edison, New Jersey). Samples containing cells on microcarriers were mixed with a fluorescent dye, propidium iodide, in the NucleoCassette (New Brunswick Scientific) to stain the DNA and directly determine the nuclei count with the fluorescent reader. A dead cell count was obtained by directly mixing the cells with the dye to stain DNA in the permeable nuclei. A total cell count was measured by lysing the cell membrane and rendering the nuclei permeable to the dye. The VCD was calculated by subtracting the dead cell count from the total cell count.

Results and Discussion

Viable Cell Density NIR Model Development

During the initial spectral analysis, it was determined that the light scattering effect caused by the microcarriers could be removed by incorporating a second derivative mathematical treatment. This procedure was successful in minimizing the baseline variations due to the microcarrier light scattering effect and enhancing the peak definition for the analytes of interest. A first derivative was applied (segment of ten and a gap of zero) in place of the commonly used second derivative pretreatment in order to partially maintain the scattering effects. This approach ensured that the light scattering effect caused by the cells would still be detectable. First derivatives are frequently satisfactory for enhancing the absorption bands and normalize the spectra. It was found that the scattering effects correlated well with the total cell density. Moreover, the resulting normalized spectra permitted the identification of chemical variance that correlated to living cells as opposed to lysed or dead cells.

Because of the complexity of the growth media, a partial least squares (PLS) regression was used to develop the calibration model. PLS uses principal component analysis and is a variation of principal component regression (PCR). Principal component (PC) analysis is a linear algebraic method of data reduction where a great number of variables (or spectral absorbances at discrete wavelengths) can be reduced to a small number of factors or PCs. Each PC explains a successively decreasing amount of the variance in the data set. The PC is a vector with an angle between itself and the variable axis. The loadings are the cosine of that angle and therefore range between −1 and 1. If the loading is 0 (cosine of the angle is 0) then the PC and variable axis are perpendicular, indicating that none of the variation is contained in that PC. A loading that is 1 indicates that the PC points exactly in the same direction as an individual variable and the PC describes all of the variation in that variable axis.[16]

The strong absorption bands for water near 1400 nm and past 1800 nm were removed because the 2 mm path-length caused these regions to exceed the dynamic range of detection.

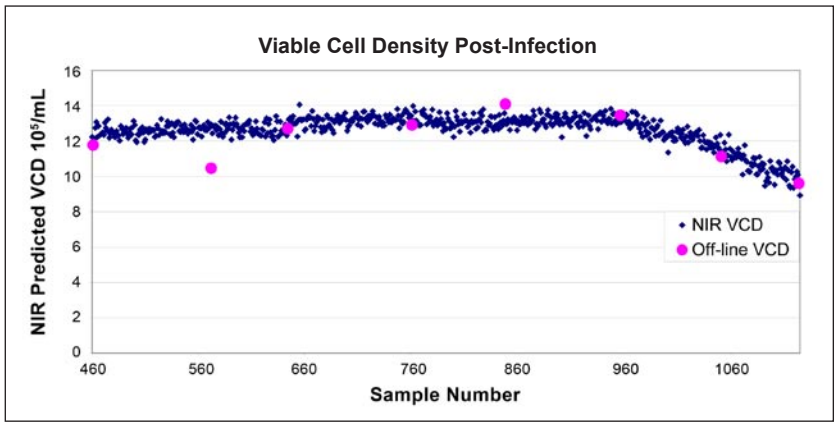

FIGURE 1. One-day interval off-line data for post-infection VCD and resulting NIR *in situ* model prediction, calculated at 15 minute intervals.

Figure 1 shows a process trend plot of the VCD over the course of a culture run after infection. The off-line VCD data are superimposed over the NIR predicted values acquired at 15 minute intervals. The NIR prediction VCD trend is smoother than the off-line sampling VCD trend which contains inherent manual sampling process error.

The partial least squares model loadings indicating where correlated variance is modeled between the off-line data and the spectral absorbances are shown in Figure 2. The first factor loadings are comparatively strong and are likely correlated to total cell density as determined by the light scattering effects. The first factor loadings are very strong compared to subsequent factors characterized by more subtle correlations to cell viability. The PLS loading for factors 2–5 are possibly related to the chemical absorbances used to discriminate viable cells from morbid cells, and enabling the prediction for VCD (Figure 3). Further in-depth studies need to be set up in order to establish the specific chemical absorbance.

The optimal number of factors used to develop the model was chosen where the predicted residual error sum of squares (PRESS) reaches a minimum (Figure 4). The PRESS is calculated from the leave-one-out cross validation predictions. Five factors achieved minimum PRESS value. A calibration curve was built using the five factors determined by the PRESS plot and shows the calibration set between the NIR VCD predictions plotted against the off-line data from the NucleoCounter (Figure 5). The PLS VCD model using five factors (or PCs) has an R^2 value of 0.9923 with a standard error of 0.4021×10^5/mL and a standard error of cross validation of 0.902×10^5/mL. These values indicate a high correlation and smaller variation of the NIR prediction value to the off-line data.

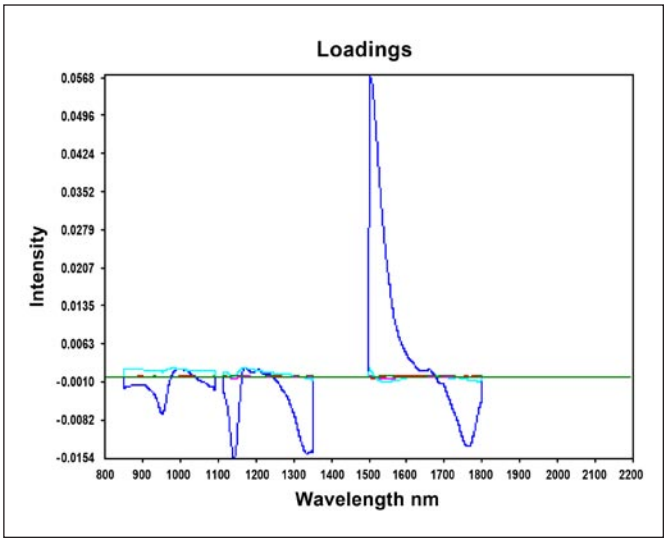

FIGURE 2. PLS loadings from the VCD model. The first principal component (dark blue) shows where the strongest correlated variance occurs in the NIR spectrum. The first factor loadings are strong and are likely correlated to total cell density determined by physical property obtained by light scattering effects.

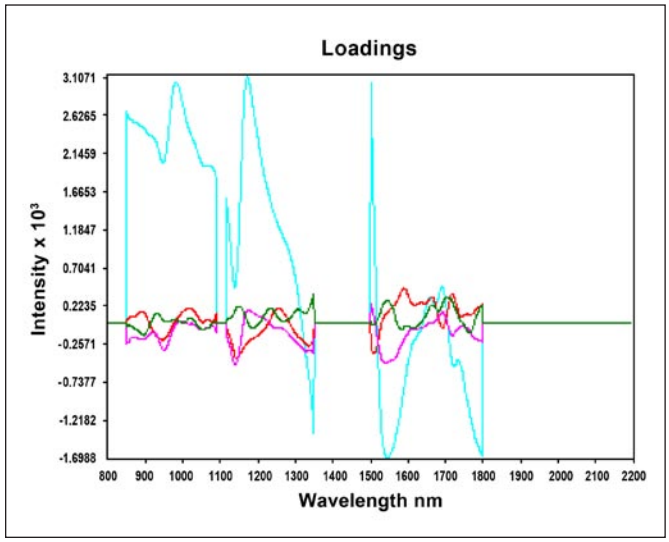

FIGURE 3. PLS loadings from factors 2–5 from the VCD model. These factors, 3–5 in particular (red, pink and green), are believed to be related to chemical absorbences helping to discriminate viable cells from morbid cells. Prediction of VCD is enabled by these factors.

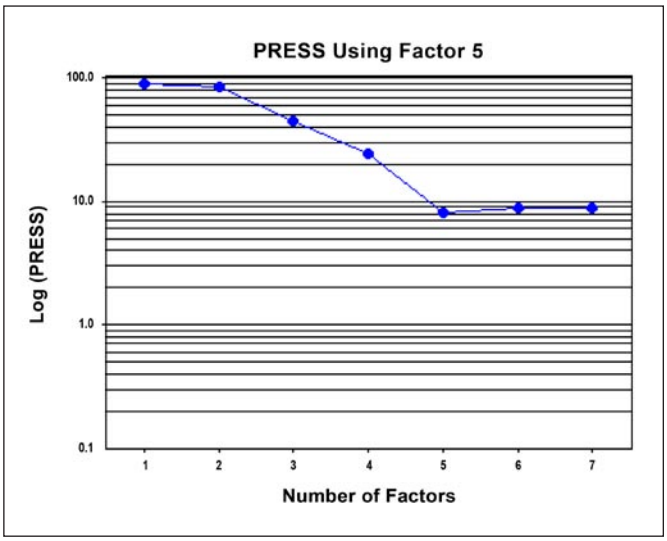

FIGURE 4. PRESS plot of PLS factors used to predict VCD. The PRESS is calculated from the residual variance of the standard error of calibration (SEC) and the standard error of cross-validation.

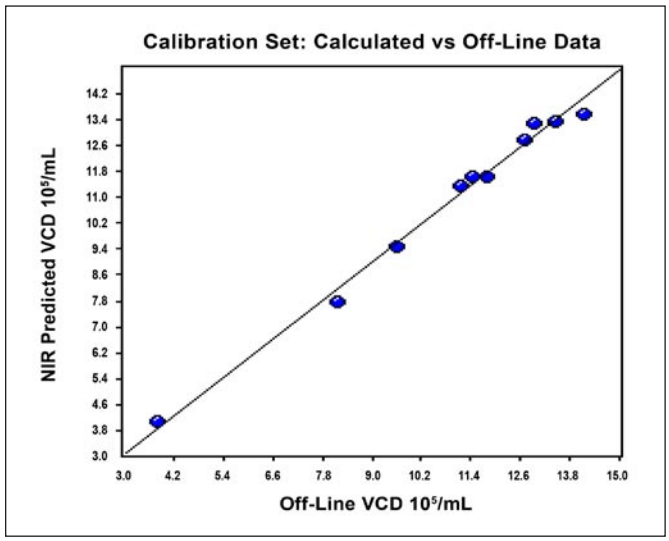

FIGURE 5. Calibration set obtained by plotting NIR model against the off-line data. VCD model is using five factors, with $R^2 = 0.9923$, SEC = 0.4021 x 10^5/mL, and cross-validation = 0.902 x 10^5/mL.

NIR is an empirical modeling technique requiring the spectra to be correlated to primary reference methods. The accuracy of the primary method and the primary data will greatly determine the accuracy of the resulting NIR model. It will be important when developing a robust model to include all common sources of variation that may affect the absorbance of the analyte being modeled. To develop a more robust model that can better predict analyte concentration, a higher number of samples is required. On the other hand, spectra from subsequent runs and other multiplexed probes can be added to increase both the variance and robustness.

Conclusion

A real-time *in situ* near-infrared probe can predict viable cell density of mammalian cells attached to microcarriers in culture media. Building a robust model from multiple runs is necessary to incorporate the run to run variability. NIR spectroscopy can monitor bioreactor phenomena *in situ* and in real-time, and is also capable of replacing off-line VCD. Near-infrared spectroscopy may be used to improve cell viability and hence, productivity as a PAT tool. Further studies will be needed to evaluate the correlation with the NIR signal and off-line signals such as intracellular pH or lipid cell membrane state.

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NOTES

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