ARTICLE

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Twenty-Four Well Plate Miniature Bioreactor System as a Scale-Down Model for Cell Culture Process Development

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ABSTRACT: Increasing the throughput and efficiency of cell culture process development has become increasingly important to rapidly screen and optimize cell culture media and process parameters. This study describes the application of a miniaturized bioreactor system as a scaled-down model for cell culture process development using a CHO cell line expressing a recombinant protein. The microbioreactor system (M24) provides non-invasive online monitoring and control capability for process parameters such as pH, dissolved oxygen (DO), and temperature at the individual well level. A systematic evaluation of the M24 for cell culture process applications was successfully completed. Several challenges were initially identified. These included uneven gas distribution in the wells due to system design and lot to lot variability, foaming issues caused by sparging required for active DO control, and pH control limitation under conditions of minimal dissolved CO_2. A high degree of variability was found which was addressed by changes in the system design. The foaming issue was resolved by addition of anti-foam, reduction of sparge rate, and elimination of DO control. The pH control limitation was overcome by a single manual liquid base addition. Intra-well reproducibility, as indicated by measurements of process parameters, cell growth, metabolite profiles, protein titer, protein quality, and scale-equivalency between the M24 and 2 L bioreactor cultures were very good. This evaluation has shown feasibility of utilizing the M24 as a scale-down tool for cell culture application development under industrially relevant process conditions.

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KEYWORDS: high throughput; miniaturized bioreactor; cell culture

Introduction

The success of several recombinant monoclonal antibodies as effective therapies for a wide range of clinical indications has made it the fastest growing sector of biologics. This has in part been fueled by the advances of our understanding of the biology of disease targets and has resulted in an unprecedented introduction of new molecular entities into early stage development. Many of these molecular entities are expressed in well-characterized host cell lines such as CHO or *E. coli*. Increasing throughput of bioprocess development has become progressively important to rapidly screen and optimize process parameters (Kensy et al., 2005; Maharbiz et al., 2004; Micheletti et al., 2006; Puskeiler et al., 2005). Traditionally small scale systems such as shake flasks and spinner flasks have been used to screen large number of clones and experimental conditions. However, due to the inability to monitor and control environmental parameters, the inability to conduct fed-batch cultivations on a routine basis, and the laborious nature of shake flask experimentation, makes these models less desirable for bioprocess optimization. Instrumented shake flasks such as those made by DASGIP (Julich, Germany) and TAP (UK) can provide the ability to control pH and perform fed-batch cultivations with the use of multi-channel pumps. In addition, it is also now possible, although not widely used, to monitor the respiration activity of shake flask cultures (Anderlei and Büchs, 2001). However the application of parallel instrumented shake flasks is still limited by challenges of non-disposable sensors, high parallelization and cost. Stirred tank bioreactors are able to provide online monitoring and control capability of process parameters; however, the low throughput and high operational costs does not permit the use of such systems for high-throughput applications. Due to the limitations of traditional small scale models, there is great interest in new high throughput processing technologies that mimic the performance of stirred tank bioreactors.

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In recent years, a number of high-throughput systems have been developed and reported in the literature. Several excellent reviews are available (Betts and Baganz, 2006; Kumar et al., 2004; Weuster-Botz, 2005) that describe the use of shake flasks, bubble columns, stirred tank bioreactors and shaken microtiter plates (MTPs) as parallel bioreactors for cell culture and fermentation applications.

MTPs are available in a variety of formats (6–1,536 wells), although for bioprocess applications, 24, 48, and 96 well formats appear to be most common. They were first introduced for analytical applications, and are now in widespread use in screening combinatorial chemistry, bioconversions, microbial fermentation and cell culture applications. Oxygen mass transfer rate (OTR) and mixing studies in MTPs have been extensively reported in the literature (Doig et al., 2002; Harms et al. 2006; Lye et al., 2003) and are generally more than sufficient to support suspension cell culture needs. OTRs have also been measured in different geometries of MTPs. The square-shaped vessel can provide high OTR; however, the turbulence caused by increased agitation may cause spillage of culture. The cylindrical vessel can prevent the spillage of culture at high agitation rate as well as maintaining good OTR (Kato and Tanaka, 1998). Some remaining challenges with the use of MTPs include minimization of fluid evaporation rates while maintaining suitable aeration and gas exchanges rates and parallel control of pH with feed strategies for fed-batch control. Integration of MTPs with liquid handling systems allows for predetermined intermittent step-wise feeding to simulate fed-batch operation and maintain pH via feedback control (Lye et al. 2003; Puskeiler et al., 2005).

In order to address the lack of online monitoring capability in MTPs, non-invasive fluorescent sensor technology is commonly used for monitoring dissolved oxygen (DO) and pH. In addition, this type of sensor technology often lowers the cost of MTPs and micro-bioreactors (MBRs) fabrication by keeping majority of the high-cost sensing infrastructure separate from the MTP/MBR, which allows the use of sensors as disposables (Zanzotto et al., 2004). Although a significant amount of research is being conducted on on-line sensors, only a small fraction are deemed suitable for applications in bioprocesses dues to constraints of sensor stability, accuracy and range, as well as issues related to leachables, heat sterilization and cost of fabrication (Harms et al., 2002). Commercially available sensors for DO are based on lifetime detection of fluorescence quenching and pH is generally based on ratiometric detection using pH sensitive fluorescent dyes. These sensors have readily been implemented in MTPs and MBRs (Harms et al., 2006; Isett et al., 2007; Kostov et al., 2001; Weiss et al., 2002).

Zanzotto et al. (2004) developed a 50 μL MBR capable of real time measurements in microbial cultivations and in a later study used the MBR for global gene expression analysis (Boccazzi et al., 2005; Zanzotto et al., 2006). De Jesus et al. (2004) developed a scaled-down system for suspension cell culture, coined the "TubeSpin," using 50 mL centrifugation tubes as culture vessels mounted on rotational shakers. The TubeSpin can be configured with vented caps, thus allowing the exchange of gases via the headspace. Although this system does not permit the in situ measurement and control of pH and DO, they are nevertheless suitable for large screening experiments where such information may not be crucial. It is also conceivable that the TubeSpin can be linked to a liquid handling system to allow an extension of its capabilities. Harms et al. (2006) reported on the design and performance of a 24-station MBR system equipped with non-invasive DO and pH sensors and individual impeller agitation. The authors demonstrated the feasibility of controlling DO in *E. coli* fermentations with a high degree of inter MBR reproducibility in DO, pH, and optical density (OD) profiles. Ge et al. (2006) tested a 12 MBR system equipped with disposable optical sensors and demonstrated a high degree of reproducibility in process parameters under non-growth conditions and using SP20/0 myeloma/mouse hybridoma cell line showed via transcriptional and HPLC analysis that the optical sensing system did not impact culture physiology.

A number of MBRs are also commercially available at scales of 50–300 mL (Fluorometrix, Baltimore, MD; AC Biotech, Julich, Germany; Infors AG, Bottmingen, Switzerland; DASGIP). The M24 microreactor (manufactured by MicroReactor Technologies, Inc., Mountain View, CA and marketed by Applikon Inc., Foster City, CA) utilizes the previously described non-invasive fluorescent sensor technology for DO and pH with gas sparging as well as the 24-well MTP format. In theory each of the 24 wells can be controlled similar to an individual bioreactor. The M24 combines the advantages of MBRs with lab-scale bioreactors by increasing throughput while maintaining data quantity and quality. Tang et al. (2006) first reported on the use of this system for microbial fermentations, although scalability of results to lab scale bioreactors was not addressed. The reproducibility of online pH and DO profiles in inter-wells and between MTP and bench scale bioreactor was found to be acceptable with little standard deviation (Isett et al., 2007). Isett et al. (2007) successfully demonstrated the reproducibility of pH, DO, and metabolite profiles between M24 and conventional 20-L stirred tank in microbial fermentations of *E. coli*, *S. cerevisiae*, and *P. pastoris*. In *P. pastoris* fed-batch fermentations, the M24 was capable of controlling process parameters at the desired set-points while supporting biomass levels of up to 278 g wet cell weight/L. Furthermore, OTR and mixing times were also measured under different conditions. Under non-sparged conditions, the k_La values for agitation speeds of 500, 600, 700, and 800 rpm were found to be around 33, 47, 52, and 56 h^{-1}, respectively. The mixing time, which was determined visually by the color change in the wells with bromothymol blue, for agitation of 500 rpm was less than 5 s.

The present study reports on the evaluation of the M24 for mammalian cell culture process development. The performance of the M24 (5 mL working volume) was

compared to 3 L (2 L working volume) bench scale bioreactor with fed-batch cultivation of Chinese hamster ovary cells (CHO). One issue that has previously been reported is the uneven distribution of sparged gases in individual wells in MTPs. We developed a method to troubleshoot this issue and test improved MTPs designs and different manufacturing lot numbers. In addition, a critical evaluation of the accuracy of pH measurements and temperature control in individual wells was conducted. The inter-well and scale-up reproducibility was also determined by the comparisons of pH, DO, temperature, cell growth, production, and protein quality. This study also identified the challenges encountered when using the M24 for cell culture process development, and the appropriate measures taken for addressing these challenges. Currently no data has been published on the application of the M24 for suspension mammalian cell culture process development and as scale-down model to lab-scale bioreactors.

Materials and Methods

M24 Microtiter Plates and Sensors

The M24 utilizes a 24-deep-well plate (or cassette) as cylindrical baffleless vessels. Each well within the cassette is equipped with non-invasive pH, DO, and temperature sensors, thermal heat conductor, and a 0.2 μm sparge membrane for gas blending using air, oxygen, nitrogen, and carbon dioxide or ammonia (only three types of gases can be sparged at any given time). Vent caps were used to seal each well which also provided the sterile barrier. Schematics of the well plate and sensors have been shown in previous studies (Isett et al., 2007; Tang et al., 2006). The well plate was placed in an incubator and held in place by vacuum. Temperature control was provided by a Pelletier heating element and the incubator temperature was generally maintained at 2°C below the required temperature set-point. For mammalian cultures, the gasses used were typically clean air, CO_2, and N_2. pH, DO, and temperature were set and controlled at the individual well level; whereas, agitation was controlled at the plate level. The mechanism for pH and DO monitoring has been previously described (Isett et al., 2007). Briefly, the pH measurements employed dual referenced optical sensing (Presens precision Sensing GmbH). The pH measurement incorporated two fluorophores; one was a fast decay pH sensitive fluorophore and the other a slow decay reference fluorophore. The dyes were excited at 470 nm and emission intensity of the slow and fast decay fluorophores were measured at 530 and 600 nm, respectively. The emitted fluorescence of the reference fluorophore was entirely quenched at pH 4. The ratio of the peak intensity of the emitted spectra was proportional to pH in the linear range (typically 6–8). The DO sensor utilized an oxygen-sensitive fluorophore with a modulated excitation source. The concentration of the oxygen was inversely proportional to the phase shift, which can be described by the Stern–Volmer equation (Kostov et al., 2001). The excitation wavelength was at 505 nm and the emission was at 650 nm. This allows the non-invasive measurement of process parameters without interrupting shaking of the MTP. pH was typically measured in 40 s interval and DO was measured in 5 s interval. All data and parameter settings were acquired and controlled by the MicroReactor® software. The data logging rate was at 1 min interval.

Inter-Well Sparged Gas Consistency Evaluation

Each well was filled with 6 mL of proprietary industrial serum-free, non-selective cell culture medium with 300 ppm antifoam. The temperature was set at 37°C throughout the evaluation. Three plates from three different lots were tested. The sparging consistency of CO_2, clean air, and N_2 were evaluated by turning on each gas one-at-a-time and the consistency of gas flows across each well was reflected in the online DO, or pH profiles. The order of gas evaluation was CO_2, clean air, and N_2 (Table I). The CO_2 flows were determined by the DO and pH profiles from all wells, and the clean air and N_2 flows were determined by the DO profiles from all wells. Agitation was turned off during these tests to minimize gas exchange from surface aeration. In addition to testing of sparge gas reproducibility, the gas permeability of the cap used to provide a sterile barrier between the well and the atmosphere was also evaluated. This was done by turning off all gasses and turning on the agitation at 500 rpm and observing any increase in the steady state value of DO with time as an indicator of oxygen permeability from the atmosphere.

Accuracy of pH and Temperature Measurements

The accuracy of pH measurements was conducted by comparing pH readings between online and offline measurements using both pH 7.00 buffer (VW3447-2,

Table I. Systematic evaluation of CO_2, air, N_2 sparging consistency under static conditions in 5 mL media containing wells.

Test	rpm	N_2 (sccm)	pH	DO (%)	Temperature (°C)	Response
CO_2	0	OFF	5.00	OFF	37	pH/DO profile
Air	0	OFF	OFF	100	37	DO profile
N_2	0	0.40	OFF	OFF	37	DO profile
Cap D	500	OFF	OFF	OFF	37	DO profile

Four different plates from three different lots were used. All tests were conducted at 37°C. For CO_2, the initial pH was set at 5.0, N_2 flow switched off, the DO control loop was disengaged and the sparging consistency determined by the response of DO and pH profiles. Similar evaluations were conducted for air and N_2. Gas permeability of cap Type D from the incubator chamber was conducted at 500 rpm with pH and DO control loops disengaged. Prior to the tests, the wells were sparged with 0.4 sccm of N_2 to decrease the DO level and any increase in dissolved oxygen during the test was attributed to oxygen permeability of the caps.

VWR, West Chester, PA) and non-selective cell culture medium. In the latter case each 24 well plate was divided into quadrants, with each quadrant containing 6 wells (Fig. 3). Quadrant 1 had a pH set-point of 6.70; quadrants 2 and 4 had pH set-points of 7.00 and quadrant 3 had a pH set-point of 7.30. For both experiments, each well was filled with 6 mL of either buffer or media and was allowed to equilibrate overnight. Samples were taken using a syringe and the headspace in the syringe was minimized in order to prevent off-gassing of CO_2. The offline pH measurement was conducted using a calibrated NOVA BioProfile 400 (NOVA Biomedical, Waltham, MA). Three plates from three different lots were tested.

The ability of the M24 to control temperature accurately was conducted by comparing temperature measurements between the M24 online measurement and three calibrated 2 thermocouple device (FLUKE 52II, FLUKE, Everett, WA). Two types of tests were performed. Initial testing was conducted with the plate (all quadrants) at isotherm at temperature set-points of 33, 35, and 37°C. In addition, the system was challenged by introducing multiple temperature set-points to a single plate. Quadrants 1 and 3 had temperature set-points of 33°C, and quadrants 2 and 4 had temperature set-points of 37°C. The temperature set points were chosen based on typical ranges used in cell culture cultivations. The intention of this experiment was to explore whether the M24 could be used to cultivate cells under different temperature set-point conditions or introduce non-synchronized temperature shifts at the individual well or quadrant level. Each well was filled with 6 mL of PBS buffer and was allowed to equilibrate overnight with agitation set at 500 rpm. Six thermocouples were inserted into wells A1, B3, B6, C3, C6, and D6 (refer to Fig. 3). Three plates from three different lots were tested.

M24 Fed-Batch Cultivations of CHO

An IgG1 recombinant protein was expressed in an industrial suspension Chinese hamster ovary (CHO) cell line of Genentech, Inc. The cell line was derived from a dihydrofolate minus (dhfr-) CHO host (Urlaub and Chasin, 1980). Cells were genetically engineered to secrete the recombinant protein using a dhfr/methotrexate selection method similar to that used by Kaufman and Sharp (1982). Cells were adapted and maintained in a proprietary industrial chemically-defined selective medium based on an adaptation of DMEM and Ham's F-12 media. Selective medium was used in the seed train stage, and non-selective medium was used in the inoculum and production stages. All media solutions were sterilized using a 0.2 μm filter. Cells were thawed from a vial and were maintained in selective medium for more than 200 days. Typically, cells were maintained in either 500 mL or 1 L (working volume 200 or 400 mL, respectively) baffled Erlenmeyer shake flasks (Corning, Corning, NY). The cell culture process conditions for this stage were 37°C and 150 rpm for 500 mL shaker flask and 120 rpm for 1 L shake flask in an incubator with 5% CO_2 and no humidity control. The orbital diameter of shaker platform was 19 mm. Cultures from the seed train were transferred to non-selective medium approximately 3–4 days before the production stage by transferring 300 mL of cell culture fluid (CCF) to 900 mL of non-selective medium to a 3-L baffled shake flask. Cells were allowed to expand and adapt to the medium. Cell culture process conditions in the inoculation stage were similar to those in seed train stage except that the agitation rate was reduced to 75 rpm (with the same throw).

When the viable cell density (VCD) reached 4×10^6 cells/mL, the appropriate volumes of CCF and non-selective medium were transferred to a 500-mL baffled shake flask to result in a cell density of 1.4×10^6 viable cells/mL. The culture from the shake flask was transferred to the M24 wells. Anti-clumping agent (01-0057AE, Invitrogen, Carlsbad, CA) and anti-foam (7-9245, Dow Corning, Midland, MI) was added to the flask at concentrations of 0.003 mL/mL and 300 ppm, respectively. The CCF, non-selective medium, and additives were allowed to mix in the incubator at 150 rpm for at least 30 min. Six milliliter of cell culture was aseptically transferred to each well in the M24 and each well was sealed with vent caps to maintain asepsis, thus allowing the operation of the M24 outside of a biosafety cabinet. The plate was transferred to the M24 platform and was clamped down by vacuum.

The cell culture process was operated at 35°C, 500 rpm, initial pH set-point of 7.00 and no active DO control. On day 3 of the cultivation, 25% (v/v) of a proprietary feed medium, which contained 900 ppm of anti-foam, was added to the culture, and the pH set-point was shifted from 7.0 to 6.80. Anti-foam was not added to shake flasks and bioreactor cultures. For pH control, 50% CO_2 gas blend (with clean air) was added on demand for decreasing pH, and 0.1 M of sodium carbonate was added (if needed) for increasing pH. Glucose was added on a need basis to maintain $\geq$3 g/L concentration.

Samples were taken on days 0, 3, 6, 8, 10, and 12. On day 0, 1 mL sample was taken from each of the 24 wells so that the final culture volume on day 0 was 5 mL per well. Among the 24×1 mL samples, twelve samples were used for viable cell counts and the remaining twelve samples were used for metabolite measurements. On culture day 3, 1 mL sample was taken from each well and 1 mL feed medium was added back to each well so that the final volume on day 3 was still about 5 mL per well. Day 3 samples were processed in a similar manner to the day 0 samples. The wells were further divided into quadrants for sampling purposes on days 6, 8, 10, and 12. On days 6, 8, and 10, a 2 mL sample was taken from each well in quadrant 1, 2, and 3 (Fig. 3), respectively and split between viable cell counts and metabolite measurements. On culture day 12, the entire volume from each well in quadrant 4 was harvested for cell counting, metabolite, titer measurements, and assays for protein quality.

Two-Liter Stirred Tank Cultivations of CHO

The two liter bioreactors (total volume 3-L) were controlled by a DeltaV based BioNet system (Broadley-James, Irvine, CA). The cell source (CCF), and medium without anti-foam and anti-clumping agent, used in 2 L bioreactor cultures were the same as in the M24 experiment. The bioreactors (Applikon Inc.) were equipped with calibrated DO, pH and temperature probes. Temperature control was achieved via a heating blanket. DO was controlled on-line through sparging with air and/or oxygen, and pH was controlled through additions of CO_2 or 1 M Na_2CO_3. Cells were inoculated at a VCD of 1.4×10^6 cells/mL target density. The process conditions were 35°C and 275 rpm with one pitched-blade impeller commonly used in cell culture technology. The DO set-point was 30% and the initial pH set-point was 7.00 and shifted to 6.80 on culture day 3. On day 3, 25% (v/v) of the proprietary feed medium containing 60 g/L glucose was added to the culture. Samples were taken and similar assays were performed, on the same culture days as in the M24 cultivation.

Sample Analysis

One milliliter samples were used for measurements of viable cell counts and percent viability by the Trypan Blue exclusion method using an automated VI-CELL AS cell counting system (Beckman Coulter, Fullerton, CA). Metabolites were analyzed by using a NOVA BioProfile 400 (Nova Biomedical). Six hundred microliters of harvested sample was analyzed for glucose, lactate, glutamine, glutamate, and ammonia concentrations. The analysis was done by using the tray rather than the syringe mode due to the large number of samples. Offline pH readings are not included due to pH drift caused by off-gassing of CO_2. Supernatant of samples was used for titer and protein quality assays. Titer was analyzed by HPLC using a protein A column (1200 Series, Agilent Technologies, Santa Clara, CA). The samples for protein quality assays were first purified by protein A plate (plate: 7700–2804, Whatman, Florham Park, NY, resin: 17-5438-02, GE Healthcare Bio-Sciences, Piscataway, NJ). The samples were tested for protein molecular size distribution by using size-exclusion chromatography (SEC) (1200 Series, Agilent Technologies), protein charge heterogeneity by using imaged capillary isoelectric electrophoresis focusing (iCIEF) (iCE280, Convergent Bioscience, Toronto, Canada) (Wu et al., 1998), and n-linked oligosaccharide analysis by capillary electrophoresis (CE) (PA800, Beckman Coulter, Fullerton, CA) (Ma and Nashabeh, 1999).

Results and Discussion

Evaluation of Gas Distribution in Wells

Our initial assessment of the M24 focused on the evaluation of gas sparging consistency amongst the wells in the M24 which is a pre-requisite to conducting reproducible cultivations. This problem has also been reported in the literature (Lamping et al., 2003) and is generally due to uneven gas pressure distribution across all levels as well as manufacturing inconsistency of the spargers. Figure 1 demonstrates the worst case of inconsistent profiles of DO measured in response to the tests described in Table I. The DO response constant was calculated by taking the slope of $\ln(DO_t/DO^*)$ versus time. DO_t was the DO reading at time t, and DO^* was equivalent to 100% air saturation. The DO response constant for the worst case scenario during the CO_2 test was approximately -0.10/h, and was -0.02/h during the N_2 test. The initial test revealed a gas distribution problem in about 33% of the wells, which was clearly unacceptable. Most of the problematic wells were located at the edge of the MTP. The problem was mainly caused by leaky vacuum gaskets, which in turn caused incomplete vacuum seal of the plate. The majority of the vacuum suction points were located toward the center of the plate and therefore, with inadequate vacuum seal at the edge, a portion of gas sparged would leak through the gasket. This agreed with the observation that the wells at the edge tended to fail more often than those at the center. This problem was exacerbated by the slightly convex nature of the bottom of the well plate. The uneven gas distribution problem was addressed by the vendor by installing new generation of vacuum gaskets and redesign of the MTP to ensure a flat bottom and therefore, a superior vacuum seal. These changes led to a dramatic increase in the reliability of the of inter-well gas distribution as shown in Figure 2A (DO profile) and 2B (pH profile). The improved DO response constants were approximately -0.35 ± 0.09/h during the CO_2 test and -0.21 ± 0.08/h during the N_2 test. The large standard deviation of the constants suggested that the difference in gas sparge rates among wells might still be approximately twofold. However, the dynamics of cell culture metabolism

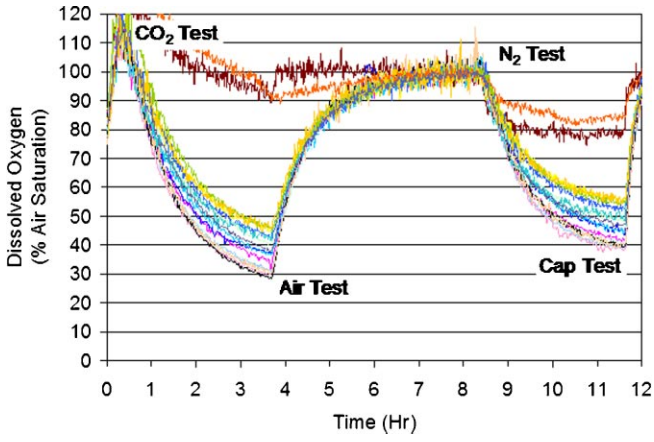

Figure 1. Inconsistency of sparged gas distribution amongst wells is reflected in the dissolved oxygen profiles. Each colored line represents the online profile of a well. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

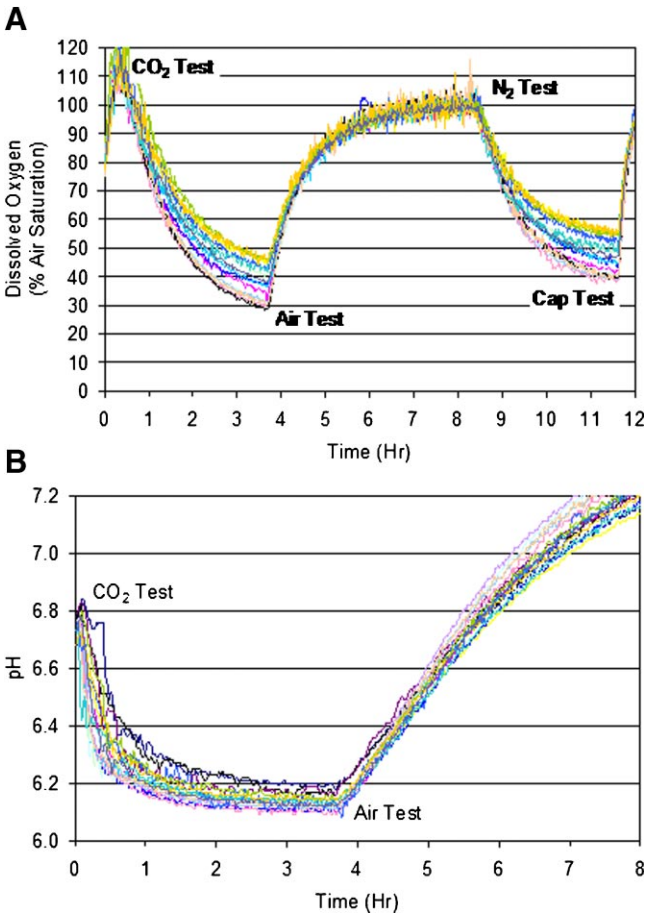

Figure 2. Dissolved oxygen (**A**) and pH (**B**) profile consistency subsequent to improved MTP design and system modifications. Each colored line represents the online profile of a well. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

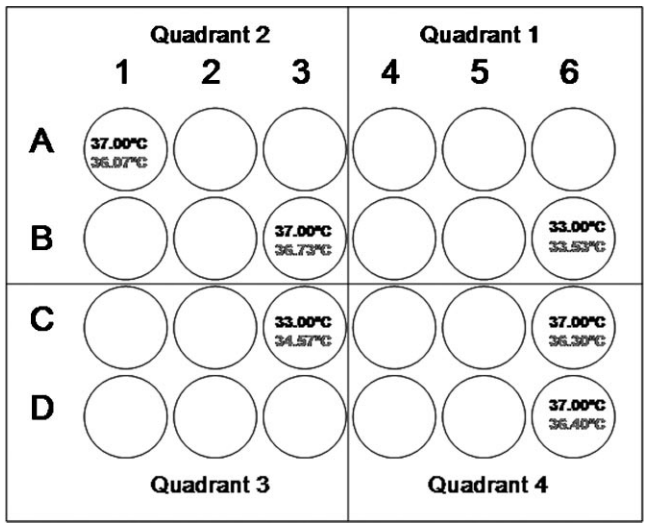

Figure 3. Schematic representing the 4 quadrants of the M24 MTP for inter-well temperature measurements. The temperature set-points in quadrants 1 and 3 were maintained at 33°C, whereas in quadrants 2 and 4, the set-point was 37°C. The set-points are shown in black text whereas on-line thermocouple readings are shown in white.

are slow enough to tolerate these differences. The gas sparging test could be used effectively as QC checks for the reliability of the MTPs.

Accuracy of pH and Temperature Measurements

The online M24 and offline pH readings, determined by a calibrated bench pH probe, consistently differed by about 0.25 pH unit. With pH 7.0 buffer, the offline reading was 7.01 ± 0.01 and the online reading was 6.77 ± 0.02, whereas at 37°C; the actual value of pH 7.0 buffer should be 6.98. This problem was solved easily by implementing a 0.25 pH offset in the M24 control software. After implementation, the pH difference between online and offline readings was reduced to less than 0.05 pH unit.

The temperature control of the M24 was good when all 24 wells were at the same temperature set-point. When the temperature of the M24 was set at isotherm at 33, 35, and 37°C, the system was able to control the temperature within ± 0.10°C of the set-point. In addition, the averaged standard deviation of the temperature readings between the online M24 sensor and calibrated thermometer was 0.02°C. However, when challenged with different temperature settings, it was difficult to maintain the temperature at the desired set-points. According to the manufacturer, the incubator temperature is required to be at least 2°C below the set-point of the MTP. In these tests the incubator temperature was set at 31°C. With temperature set-points of 33°C (quadrants 1 and 3) and 37°C (quadrants 2 and 4), the standard deviations became larger. For the 37°C set-point the difference between online M24 and thermocouple readings ranged from 0.27°C to 0.93°C (wells A1, B3, C6, and D6 in Fig. 3). For 33°C set-point, both readings were higher than the set-point, although this difference was greater up to 1.6°C in the inner well location (well C3, in Fig. 3) whereas it was up to 0.5°C higher in the outer well (well B6 in Fig. 3). This was in part due to the adjacency of 37°C wells, which acted as a heat source. The inability of controlling two temperature set-points with large temperature differentials in the inner well locations seemed to be an inherent system limitation which cannot be addressed easily.

Preliminary Cell Culture Experiments in the M24

Initial experiments focused on the performance of the M24 relative to shake flasks. The M24 was intentionally operated as a shake flask with no active sparging, pH, or DO control. The working volume of the 500 mL shake flask was 150 mL

(agitation 150 rpm), whereas the M24 wells were operated at an initial volume of 6 mL (agitation 500 rpm). The shake flasks were cultured using 5% CO_2. Aeration in the M24 was provided via the headspace using air. The scale and intra-well equivalencies between M24 and shake flask were assessed as shown in Figure 4. The acceptance criterion for each parameter was determined based on typical cell culture process variations of each parameter (Table II). The scale comparison results were generally good with 75% of the VCD data points meeting the criterion of ±20% of shake flask data and 63% of the titer values meeting the criterion of ±20% of shake flask data by culture day 14 (Table II). Furthermore, the absolute difference between the shake flask and mean M24 titer values were about 0.10 g/L, which were well within the normal variation in cell culture processes. The comparisons of lactate and glutamine profiles between two scales were poor. For both metabolites, 0% of the data points fell within the acceptance criteria. The absolute difference of lactate between two scales ranged from 7 to 11 mM. The poor correlation of lactate could be explained by the initial pH difference between the M24 and shake flask due to the presence of 5% CO_2 in shake flask culture. The initial pH readings were 7.1 and 7.4 for the shake flask and M24, respectively. The culture in shake flask was grown under a 5% CO_2 environment; whereas, the culture in the M24 was grown under an ambient air environment. The

Table II. Summary of comparisons between M24 and shake flask cultures.

Parameters	Acceptance criteria (%)	% Data within criteria
VCD	±20	75
Viability	±20	88
Titer	±20	63
Glucose	±20	85
Lactate	±20	0
Glutamine	±20	0
Glutamate	±20	100
Ammonia	±20	100

The acceptance criteria were chosen based on typical variations observed in the cell culture process. The percentage of data points were calculated based on the number of data points falling within the acceptance criteria. Figure 4 shows the trends for comparison.

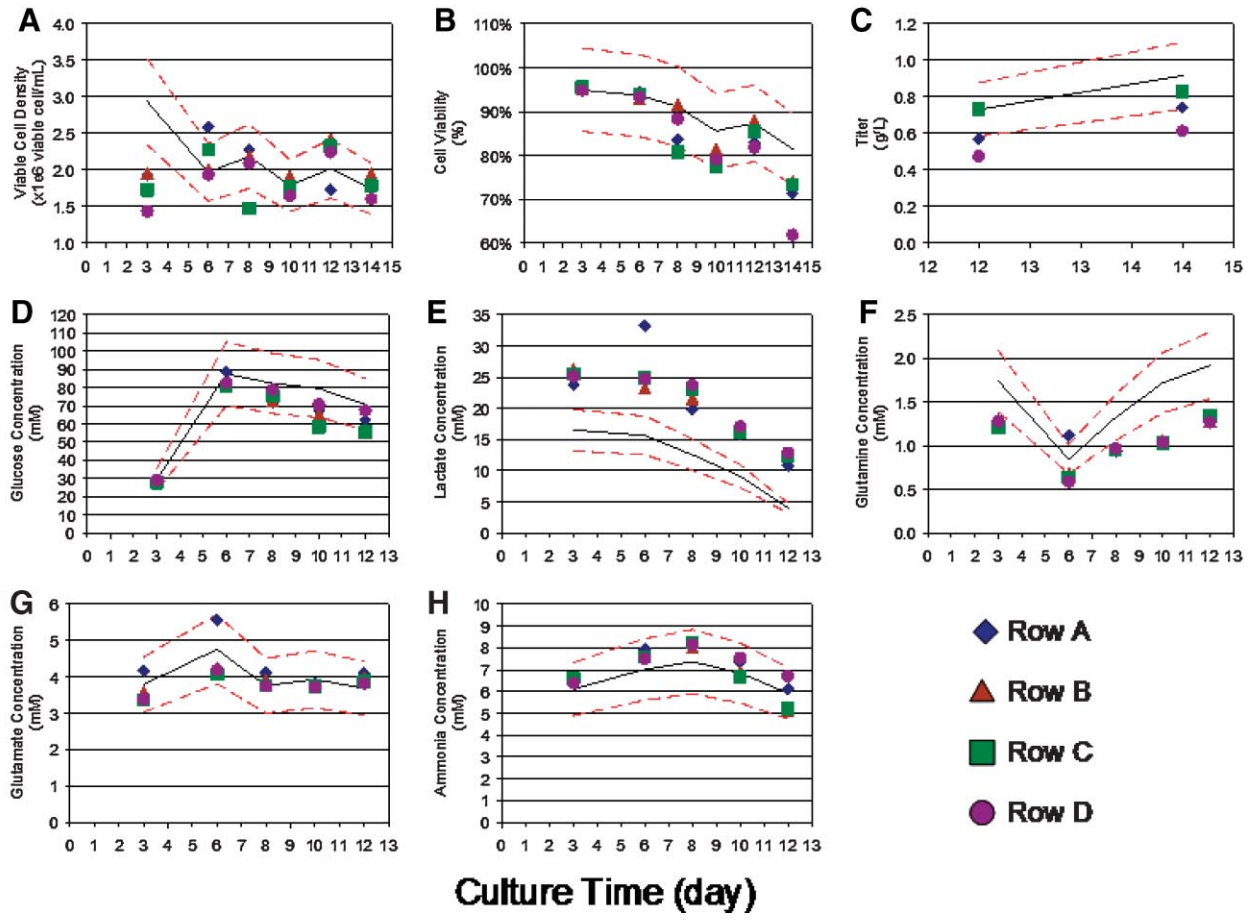

Figure 4. Scale-up and intra-well reproducibility of M24 versus shake flask cultures. The black line represents the shake flask data, and the red lines represent the acceptance criteria. The acceptance criteria were determined based on the typical cell culture process variations of each parameter. The symbols are the data from M24. Intra-well reproducibility can be assessed by the spread of data points on a given culture day. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

influence of pH on lactate production is a well-known phenomenon (Wagner 1997; Wu et al., 1993; Xie et al., 2002). Although glutamine had a poor correlation between two scales, it is important to point out that the largest absolute difference was less than 0.70 mM and this can be within the normal variation in cell culture processes. The intra-row reproducibility can be assessed by the spread of data points in a given culture day. The intra-row reproducibility was also reasonably good when using the M24 to simulate a shake flask process.

Problems arose initially when simulating the 2L bioreactor process. The first problem was severe foaming due to gas sparge into culture to maintain DO and pH set-points, which clogged the filter cap and eventually caused the cap to pop due to pressurization of wells compromising the sterility of the culture. In addition, the foaming was not consistent across all wells, where typically the wells located in the inner rows (B and C) experienced greater foaming due to the higher sparge rates caused by uneven gas distribution. The foaming issue was addressed by several measures. Firstly, the inconsistency of foaming across wells was resolved by the measures described earlier (new vacuum gaskets and redesigned well plate) to ensure even gas distribution. Secondly, addition up to 900 ppm of anti-foam in the culture was tested (300 ppm was used in shake flasks) reduced the foaming, but did not eliminate it. Finally a reduction in the gas sparge rate from 0.4 to 0.2 sccm, and elimination of DO control (to minimize gas sparging) resulted in the prevention of foaming while using an antifoam concentration of 300 ppm.

Another problem that we encountered was the limitation of pH control, especially under conditions of high lactate production. Unlike microbial fermentations where ammonia can be used in the M24 to control pH (Isett et al., 2007) and unlike traditional bioreactor based cell culture processes where liquid base is used, the M24 uses N_2 gas to strip off CO_2 for increasing pH for cell culture processes. Ammonia was not used due to concerns of toxicity (McQueen and Bailey, 1990). However, when the dissolved CO_2 is completely stripped off using N_2, the pH could no longer be increased to maintain the set-point. This limitation in pH control was addressed by manual addition of liquid base (0.1 M sodium carbonate). The amount of sodium carbonate required to adjust pH was calculated based on Equation (1)

$$V_{\text{add}} = V_{\text{total}} \frac{P_{CO_2}}{k_H 2[HCO_3^-]_{\text{add}}} \left[10^{(pH_f - pK_a)} - 10^{(pH_i - pK_a)}\right] \quad (1)$$

where V_{add} is the volume of sodium carbonate to be added, V_{total} is the total volume of culture, P_{CO_2} is the partial pressure of CO_2 in the culture, k_H is the Henry's Law constant for CO_2, $[HCO_3^-]_{\text{add}}$ is the concentration of carbonate in the liquid base, pH_f is the final post-adjustment pH, pH_i is the pH value prior to adjustment, and pK_a is the first dissociation constant of sodium carbonate base. Typically only one liquid base addition was required throughout the entire 12-day culture duration. The Henry's Law constant at 37°C and the pK_a used in V_{add} calculation were 36 (L atm)/mol and 6.30, respectively.

Fed-Batch Cultivation of CHO Cells in the M24

After addressing all of the problems identified in a systematic manner, cell culture experiments were designed to evaluate the reproducibility among all 24 wells and the performance of the M24 as a scaled-down system for the 2 L bioreactor culture process. Environmental parameters such as online pH, DO, and temperature as well as cell growth, metabolic, production, and protein quality were used for assessing the reproducibility among wells and scalability between M24 and 2 L bioreactor.

Dissolved Oxygen, pH and Temperature Profiles

DO control was not activated throughout the 12-day culture in order to minimize foaming due to gas sparging. Instead oxygen was provided through surface aeration. The oxygen mass transfer of the M24 at the operating speed of 500 rpm and non-sparged conditions resulted in a $k_L a$ value $>30\ h^{-1}$ (Isett et al., 2007), which is more than sufficient for the oxygen demand under the present conditions of culture growth. Although DO control was not activated, reviewing DO profiles can provide indirect comparison of similarity in cell growth performance among wells. The profiles shown in Figure 5A were taken from quadrant 4 since the other quadrants were sampled before the final culture day. Overall, the DO did not drop below 30% and the DO profiles were similar among wells with the exception of well 6C, for the entire culture duration, and 4C, after culture day 9. The addition of anti-foam and elimination of DO control addressed the severe foaming issue. However, it did not completely eliminate foaming. The low DO profiles of these two wells were due to minor to moderate foaming. The foam acted as a mass transfer barrier and affected the oxygen exchange between the culture and head space, leading to lower DO in the culture broth. The DO spikes on culture days 3, 6, 8, and 10 were due to sampling. Based on our experience and published literature, cell culture performance is generally insensitive to DO levels in the range 10–100% (Oh et al., 1992), although in some cases it can impact the product quality.

In general, when there was active pH control, the pH profile fluctuated within ±0.2 pH unit. Only profiles from quadrant four are presented here since those six wells were harvested at the end of 12-day culture (Fig. 5B). The pH profiles were extremely reproducible among wells. However, pH profiles from days 3, 6, and 9 prior to sampling also indicated a high degree of reproducibility. The standard deviation of pH readings from all 24 wells in the first 2 culture days was 0.06 pH unit, and the standard deviation of pH readings from quadrant 4 was also 0.05 pH unit. The pH set-point was decreased to 6.8 as desired and remained at

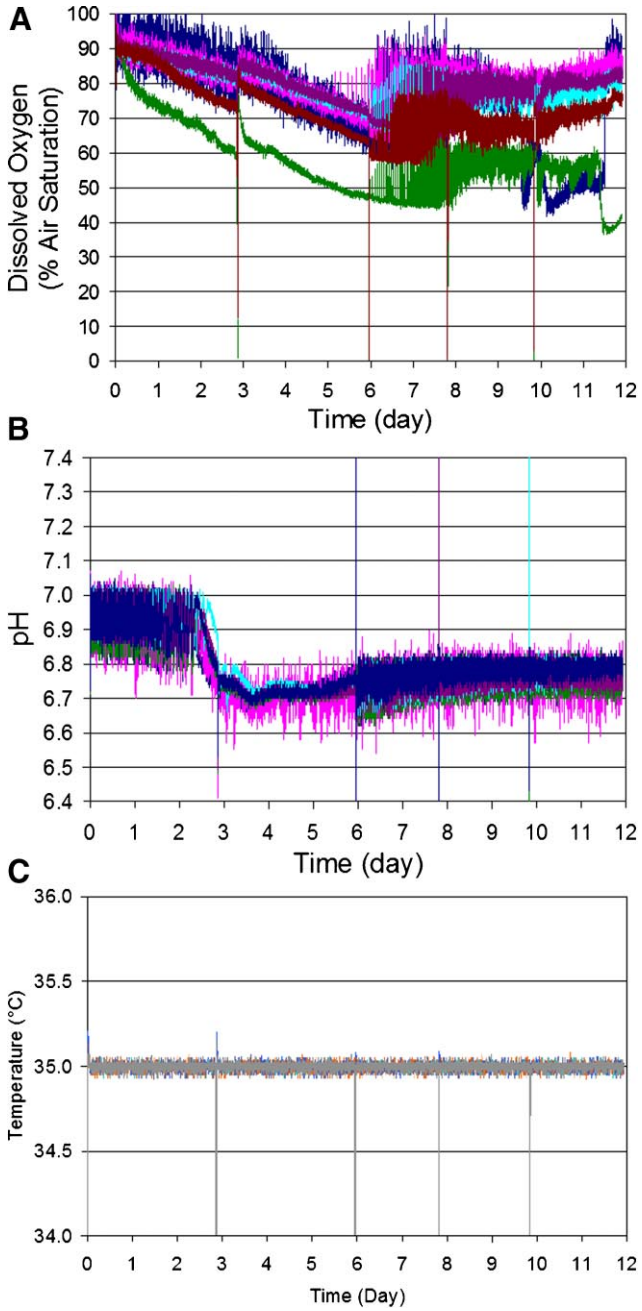

Figure 5. M24 quadrant 4 on-line measurements of (**A**) dissolved oxygen, (**B**) pH, and (**C**) temperature. Each colored line represents the online profile of a well. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

6.8 ± 0.1 through culture day 5. On culture day 6, 17.8 μL of 0.1 M of sodium carbonate was added to the culture according to Equation (1) in order to maintain pH around 6.8. Only one liquid base addition was required throughout the entire culture duration. This was likely due to the consumption of lactate after culture day 6. Multiple base additions might be needed for more lactogenic cultures. The pH spikes around culture day 3, 6, 8, and 10 were due to sampling. The offline pH measurements are not available due to CO_2 off-gassing during analysis. However, the difference between online and offline pH readings was less than 0.05 pH unit in the range of pH 6.80–7.30. Temperature profiles were similar among M24 wells and between scales (Fig. 5C). The standard deviation of all temperature readings from all wells was 0.20°C. The cells were cultivated at a constant 35°C set-point. The spikes on culture day 3, 6, 8, and 10 were due to sampling.

Although the manufacturer claims the M24 is capable of controlling pH and DO, we were not able to demonstrate simultaneous control of both parameters across the entire 12-day fed-batch culture under our operating conditions. In separate experiments, we were able to simultaneously control the pH and DO for a few days before either CO_2 was stripped completely or foaming occurred. Once all of the dissolved CO_2 was stripped off from the culture, the pH could no longer be increased by the M24 system unless base was added manually. On the other hand, the challenge of DO control was due to the severe foaming issue with culture duration. The N_2 purge and clean air sparge were turned off in order to address this issue. In the absence of foaming it was possible to control DO at better than ±10%. It is still a challenge to fully control the pH and DO using the M24 system. However, there are ways to accomplish this goal. One of the options might be to develop a medium and cell culture process which will limit the pH fluctuation by increasing the buffer capacity or minimizing lactate production. In terms of DO control, more work can be done on identifying the optimal anti-foam concentration as well as the sparge rate/aeration strategies of various gasses. Operator independent base addition for pH control can be automated (e.g., the AutoReact™ from Hudson Control).

Viable Cell Concentration and Viability

A similar method (as described in Preliminary Cell Culture Experiments in the M24 Section) for comparing both the M24 and 2 L bioreactor cultures was employed (Fig. 6). For both VCD and percent cell viability, the percentage of the data within the acceptance criteria were 85% and 97%, respectively. The culture doubling time for both M24 and 2 L bioreactor was 1.51 and 1.70 days, respectively. Both VCD and percent cell viability were similar between the M24 and 2 L bioreactor cultures (Fig. 7A). The integrated viable cell counts were 31×10^6 and 32×10^6 viable cells/mL/day for the M24 and 2 L bioreactor cultures, respectively.

Metabolites

Overall, the trending of metabolite profiles was also similar between the M24 and 2 L bioreactor cultures. Cells consumed glucose throughout the entire culture (Fig. 7B). The cell specific glucose consumption rates were 2.55 and 2.92 pmol/(cell-day) for the M24 and 2 L bioreactor cultures, respectively, during the first three culture days, and were 2.24 and 2.43 pmol/(cell-day) thereafter. Glucose concentration increased from culture day 3 due to the feeding event. The amount of

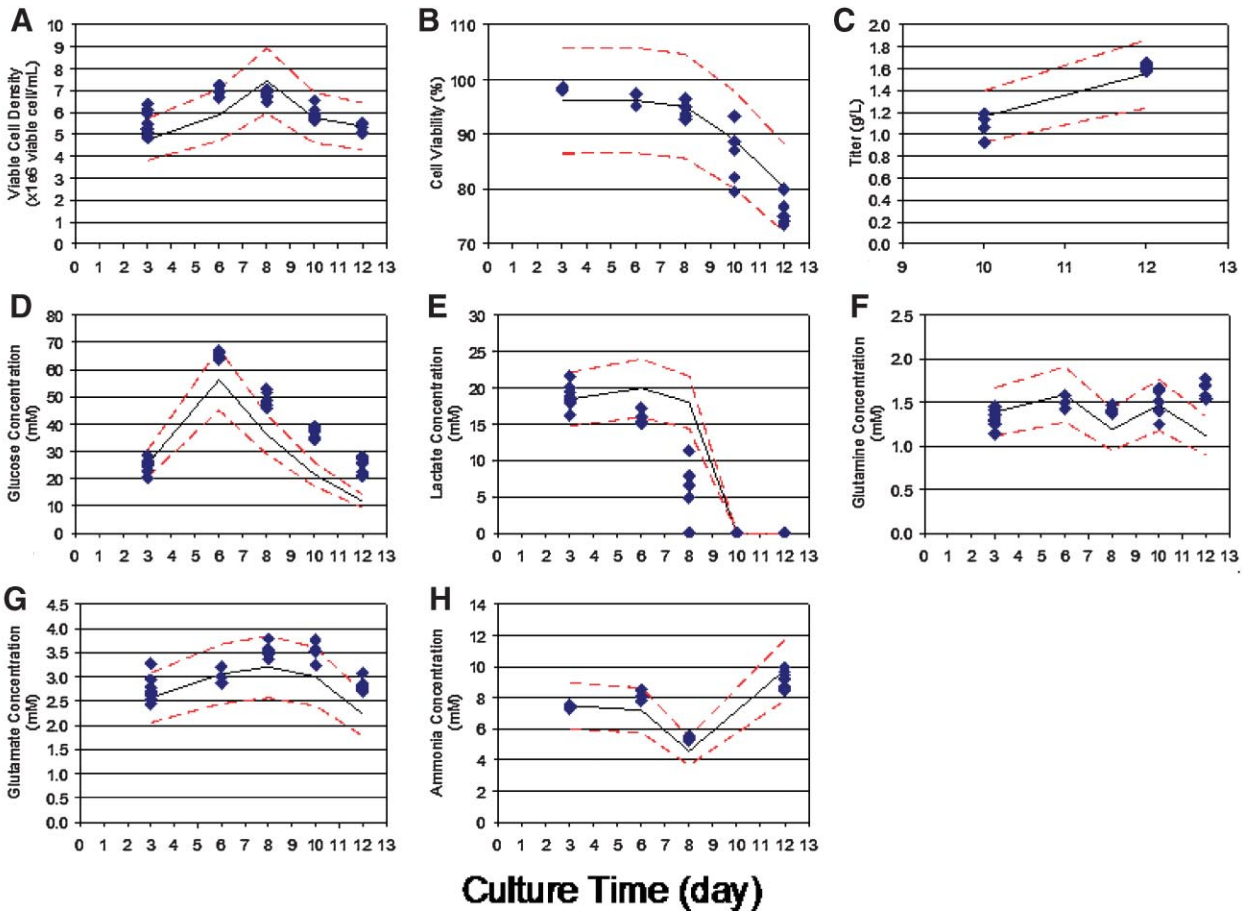

Figure 6. Scale-up and intra-well reproducibility of M24 versus 2 L bioreactor cultures. The black line represents the data from the 2L bioreactor, and the red lines represent the acceptance criteria. The acceptance criteria were determined based on the typical cell culture process variations of each parameter. The symbols are the data from M24. Intra-well reproducibility can be assessed by the spread of data points on a given culture day. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

glucose supplied to the culture was enough for the entire culture duration. The mean standard deviation of glucose in the M24 was 2.26 mM throughout the entire culture. The highest standard deviation was 2.81 mM on culture day 8. For glucose, some of the differences between two scales could be due to feeding error. The method we used for feeding the 2 L bioreactor could have a ± 5 mM of glucose error, whereas, feeding in the M24 was much more accurate by using calibrated pipettes. Before considering this possible feeding error, the difference in glucose values between scales ranged from 10 to 15 mM. This range is reduced down to 5–10 mM (0.9–1.8 g/L) with the feeding error considered. This difference of 5–10 mM is within the normal variation of our cell culture process.

Lactate was produced on the first 3 days of culture and slowly got consumed after that (Fig. 7B). The cell specific lactate production rates were 2.52 and 2.86 pmol/(cell-day) for the M24 and 2 L bioreactor cultures, respectively, during the first three culture days. The cell specific lactate consumption rates were 1.25 and 3.12 pmol/(cell-day) for the M24 and 2 L bioreactor cultures, respectively, during the late culture days. The maximum lactate concentration was around 4 g/L for both scales and was completely consumed by culture day 10. The mean standard deviation of lactate in M24 was 1.72 mM throughout the entire culture. The highest standard deviation was 2.73 mM on culture day 8. Although 76% of the lactate data were within the criteria, the reasons for the day 8 variability is not clear, although this was also the culture duration when glucose concentration deviations were also measured.

Cells consumed glutamine on the first 3 days of culture and the concentration of glutamine stayed constant around 1.5 mM for the rest of culture duration (Fig. 7C). The cell specific glutamine consumption rates were 0.44 and 0.49 pmol/(cell-day) for the M24 and 2 L bioreactor cultures, respectively, during the first 3 days of culture. The mean standard deviation of glutamine in the M24 was 0.09 mM throughout the entire culture. The highest standard deviation was 0.15 mM on culture day 10. Glutamate concentration increased from culture day 0 to day 8 and

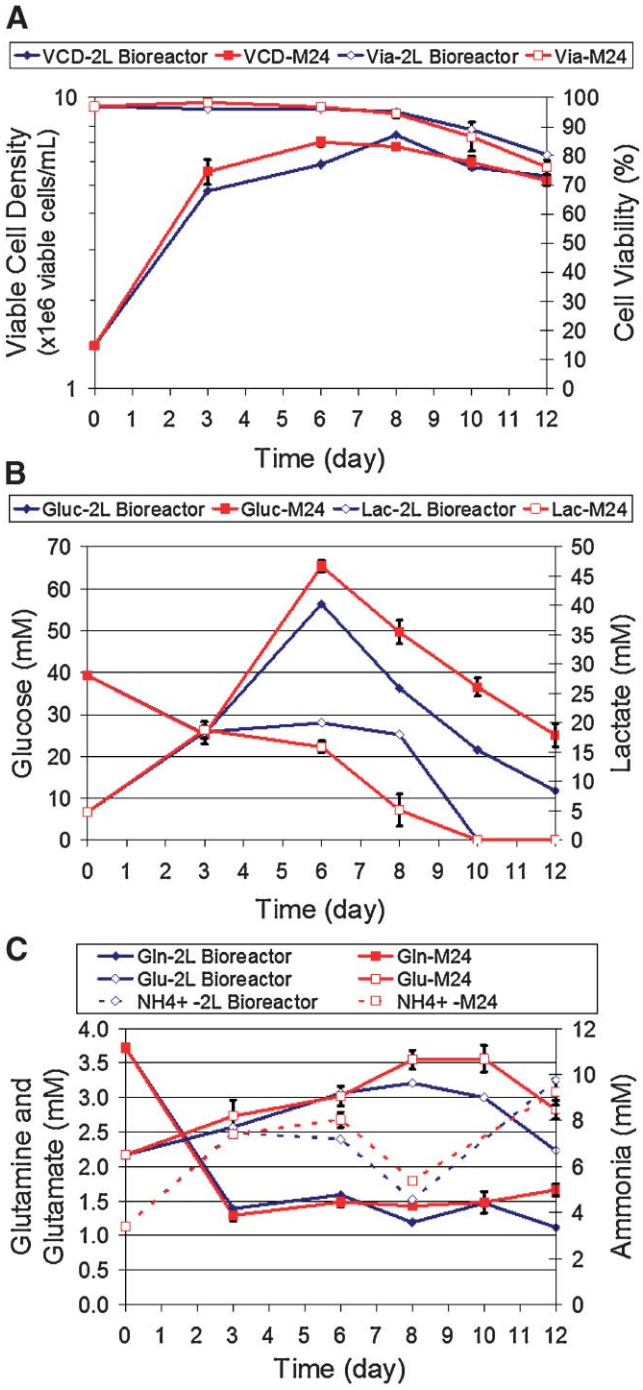

Figure 7. Cell culture performance comparison between the M24 and 2 L bioreactor: (**A**) cell growth and viability curves, (**B**) glucose and lactate profiles, and (**C**) glutamine, glutamate, and ammonia profiles. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

slowly decreased to around 2.5 mM at the end of culture (Fig. 7C). The mean standard deviation of glutamate in the M24 cultures was 0.17 mM throughout the entire culture duration. The highest standard deviation was 0.23 mM on culture day 3. Ammonia was produced during culture; the final ammonia concentration was around 9.30 mM for both M24 and 2 L bioreactor cultures (Fig. 7C). The mean standard deviation of ammonia in M24 was 0.33 mM throughout the entire culture. The highest standard deviation was 0.57 mM on culture day 12. The metabolite comparisons between the M24 and 2 L bioreactor are summarized in Table III. All metabolites exhibited reasonable similarity between scales. The minor difference exhibited in some metabolites could be due to the normal variation in cell culture process.

Table III. Summary of comparisons between the M24 and 2 L bioreactor cultures.

Parameters	Acceptance criteria (%)	% Data within criteria
VCD	±20	85
Viability	±20	97
Titer	±20	92
Offline pH	±0.10 pH	50
Glucose	±20	47
Lactate	±20	76
Glutamine	±20	82
Glutamate	±20	79
Ammonia	±20	100

The acceptance criteria were chosen based on typical variations observed in the cell culture process. The percentages were calculated based on the number of data points falling within the acceptance criteria. Figure 6 shows The trends for comparison.

Protein Titer and Quality

The protein concentrations on the final culture day were 1.55 and 1.60 ± 0.03 g/L for the 2 L bioreactor and M24 cultures, respectively. The results of size exclusion chromatography to determine protein aggregation and charge variant analysis are shown in Table IVa. The percentage monomer of the protein produced at each scale was similar at approximately 99% and the low and high molecular species were also similar. The protein charge variants were also very similar with % main peak at 66%, the % acidic species between 29.2% and 31.6% and the basic species between 3.6% and 4.7%. Glycoform analysis also revealed similarity, with G0 values at 77.5% and 77.7 ± 0.67% for the protein expressed in cultures from the 2 L bioreactor and

Table IVa. Protein quality data: (a) protein charge heterogeneity by using imaged capillary isoelectric electrophoresis focusing and protein size distribution by size-exclusion chromatography.

	% Acidic	% Main peak	% Basic
2 L bioreactor	30.61	65.83	3.57
M24 ($n = 6$)	29.19 ± 0.51	66.14 ± 1.70	4.67 ± 1.61
	HMWS	Monomer	LMWS
2 L bioreactor	0.50	98.80	0.75
M24 ($n = 6$)	0.45 ± 0.08	98.67 ± 0.29	0.92 ± 0.29

Table IVb. Protein quality data: (b) N-linked oligosaccharide analysis by capillary electrophoresis.

	G0-F	G0-1	Man5
2 L bioreactor	1.16	5.17	0.69
M24 ($n=6$)	1.20 ± 0.05	5.67 ± 0.65	0.87 ± 0.25
	G0	Peak 1	G1-1
2 L bioreactor	77.53	0.16	0.13
M24 ($n=6$)	77.71 ± 0.67	0.20 ± 0.03	0.11 ± 0.02
	Man6	G1*-1	Peak 2
2 L bioreactor	0.08	0.15	0.57
M24 ($n=6$)	0.08 ± 0.09	0.16 ± 0.02	51 ± 0.05
	G1	G1'	G2
2 L bioreactor	10.31	3.29	0.78
M24 ($n=6$)	9.80 ± 0.99	2.99 ± 0.41	0.73 ± 0.13

the M24, respectively (additional glycoform distribution data is shown in Table IVb). Both protein concentration and glycoforms were measured over six replicates for M24. The small standard deviations (± 0.03 g/L for protein concentration and $\pm 0.67\%$ for G0 glycoform) for these measurements also showed consistency among well replicates. Overall there was no difference in protein quality between the M24 and 2 L bioreactor cultures (Tables IVa and IVb).

Automation

The M24 has been shown to be a versatile high-throughput platform and scale-down model for both cell culture and microbial applications (Isett et al., 2007). In terms for future enhancements to the M24 platform, it is important to integrate automation technologies for inoculation, feeding, sampling and analytics similar to the use of liquid handling systems with MTPs. This concept has recently been realized by Hudson Control Group (Springfield, NJ). Using their proprietary robotics technologies, the system automatically loads, samples and feeds (substrate and base) cells growing under 24 independent reaction conditions simultaneously. The entire unit is placed in a standard biosafety cabinet to allow aseptic feeding and sampling. The samples can be transferred to chilled MTPs for further analysis and users can input triggered automated feeding using multiple feeds and/or sampling of the M24 based on measured events and/or time.

CONCLUSIONS

A systematic evaluation of the M24 for cell culture process applications was successfully completed. Several challenges were initially identified. These included uneven gas distribution in the MTP due to system design and lot to lot variability, foaming issues caused by sparging required for active DO control and pH control limitation under conditions of minimal dissolved CO_2. The uneven gas distribution was evaluated by implementing step changes in gas flow rates and measuring the consistency of the DO and pH sensor response. A high degree of variability was found which was addressed by changes in the systems design. Foaming issues were resolved in general by addition of anti-foam, reduction of sparge rate, and elimination of DO control. The pH control limitation was overcome by a single manual liquid base addition. Our results indicate that although it was possible to achieve parallel control of both pH and DO under conditions where CO_2 was not completely stripped and/or foaming did not occur, it was clear that control of both parameters for the entire 14-day fed-batch culture in the M24 remains a challenge (under our operating conditions). However, this can be achieved by intervention of liquid base addition as well as by strategies to eliminate foaming (optimal anti-foam concentration as well as the sparge rate/aeration strategies). Intra-well reproducibility of the M24 MTP and scale-equivalency between M24 and 2 L bioreactor cultures, as indicated by measurements of process parameters, cell growth, metabolite profiles, protein titer and protein quality, were very good. This evaluation has shown feasibility of utilizing the M24 as a scale-down tool for cell culture application development under industrially relevant process conditions. With increased high throughput, online monitoring and control capability, and potential automation, the M24 system is an attractive option to broaden the experimental design space while potentially lowering the cost of process development.

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