Biotechnology Reports 1–2 (2014) 22–26

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Comparison of the behavior of CHO cells during cultivation in 24-square deep well microplates and conventional shake flask systems

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ARTICLE INFO

Article history:
Available online 6 May 2014

Keywords:
CHO cells
Duetz sandwich-cover system
Batch
Fed batch
Cell culture
Deep well plates

ABSTRACT

In biopharmaceutical production, the optimization of cell culture media and supplementation is a vital element of process development. Optimization is usually achieved through the screening of multiple media, feed and feeding strategies. However, most screening is performed in shake flasks, which makes the screening process very time consuming and inefficient. The use of small scale culture systems for the screening process can aid in the ability to screen multiple formulations during process development. In order to assess the suitability of 24 deep well (24DW) plates with the Duetz sandwich-covers as a small scale culture system for process development, we have tested growth and production performance of CHO cells in 24DW plates and conventional shake flask cultures. Multiple studies were performed to assess well-to-well and plate-to-plate variability in 24DW plates. Additional studies were performed to determine the applicability of 24DW plates for cell culture medium and supplement screening in batch and fed batch processes. Cultures in 24DW plates exhibited similar kinetics in growth, viability and protein production to those cultured in shake flasks, suggesting that 24DW plates with Duetz sandwich-covers can be effectively used for high throughput cell culture screening.

1. Introduction

The demand for antibodies and other glycoproteins has increased rapidly due to their importance as therapeutic agents. Production of these biologics is in large part reliant on mammalian cell culture systems. To meet the demand, various strategies are employed to increase production by enhancing cell performance, specifically improving bioproduction titers. Some of the most effective approaches to improve the cell culture process include media optimization and batch and fed batch supplementation. Development of these strategies involves intensive screening of components, mixtures and various feed schemes. Due to the difficulty of examining a large number of components at different concentrations or screening a large number of potential supplements, these types of studies cannot be efficiently performed using conventional bioreactor, shake flask, or spin tube cultures. Use of small scale cell culture systems, like multi-well plates can enable the exploration of a wider range of bioprocess operating conditions in a more efficient manner.

In general, multi-well plate cell cultures are performed in static or shaking formats in shallow well (SW) or deep well (DW) plates in a small culture volume for elongated time periods. Shaking plates are more suitable for suspension cell culture; however, the loss of media due to evaporation in outer wells (edge effects) poses a significant problem [1]. Edge effects result in well-to-well and plate-to-plate variability in cultures. Some laboratories have overcome the edge effects and evaporation issues by not using the outer wells of multi-well plates for cell-based assays. Omission of outer wells decreases analysis throughput by 38% and 66% in 96 well and 24 well plates, respectively. Other approaches to prevent evaporation include the use of self-adhesive plate seals, which are designed to maintain uniform gas exchange while keeping cultures sterile. A study of various commercially available adhesive plate seals showed multiple disadvantages and categorized them into two groups: (1) plate seals in which volume preservation is relatively low, but oxygen transfer is comparable to that of unsealed plates, and (2) plate seals in which volume preservation is high, but oxygen exchange is slower [2]. Therefore, adhesive plate seals may

Abbreviations: CD, chemically defined; 24DW, 24 deep well; VCD, viable cell density; CHO cells, Chinese Hamster Ovarian cells; %CV, percent coefficient of variation.

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http://dx.doi.org/10.1016/j.btre.2014.04.001

not adequately fulfill the requirements for cell culture screening in SW and DW multi-well plates. In addition to evaporation and oxygen transfer issues, culture volumes in most of the 24SW, 96SW, and 96DW plates ranges from 0.7 mL to 1 mL. These lower volumes are usually insufficient for multiple sampling and fed batch processes. Moreover, for suspension cultures, plates are being kept on shaking platform to keep cells in suspension. It is often challenging to keep plates secure on a regular platform while shaking.

These problems can be adequately addressed using a closure system for multi well plates as described by Duetz et al. [3]. The Duetz sandwich-cover system is a shaking multi-well plate based system that consists of specialized multi-well plate sandwich covers and clamps to hold the closures in place. The sandwich cover is an autoclavable stainless steel lid containing layers of filters and silicon sealing that provides a positive seal on each well of the plate to promote efficient gas transfer. The headspace refreshment rate of each individual well is controlled by a small hole in the silicone layer above the center of each well and contamination is prevented using autoclavable gas permeable filters [3,4]. The system provides a headspace refreshment rate of 0.1–2 working volumes per minute in orbital shakers, permitting oxygen concentrations of at least 18% (v/v), even when oxygen uptake rates are as high as 40 mmol O_2/L/h. Evaporation at these conditions is kept at a minimum (as low as 2% per day), which permits longer culture times [5]. The clamps assure that the plates and sandwich covers are clamped together tightly and the individual wells are hermetically sealed. The cover clamp can be mounted onto a variety of regular orbital shaking platforms. The Duetz sandwich-cover system can be used with various multi well plates, including 24DW plates. A 24DW plate can hold up to four milliliter culture volume as compare to one milliliter in any other multi-well plates, which enables multiple samplings on various days of culture. This Duetz sandwich-cover system has been used for bacterial cell culture to maintain oxygen transfer and reduce evaporation [6]. The system has also been used for batch and fed batch culture studies with hybridoma and Chinese Hamster Ovary (CHO) cell lines in polystyrene 24 round well plates [7,8].

In this study, we have evaluated the Duetz sandwich-cover system for CHO cell screening studies. CHO cells are the most commonly used mammalian cells for production of biopharmaceuticals. We have tested monoclonal antibody (mAb) producing CHO cell lines in 24DW plates and compared performance to conventional shake flask cultures. Initially, a series of experiments were performed to assess well-to-well and plate-to-plate variability in the 24DW plate. Additional studies were performed to determine the application of the Duetz sandwich-cover system for cell culture medium and supplement screening in batch and fed batch processes. Multiple CHO cell lines were used to ensure that scalability to shake flask culture was not cell line specific. Overall, 24DW plates gave similar kinetics in growth, viability and protein production to those cultured in shake flasks, demonstrating a potential application of 24DW plates with the Duetz sandwich-cover system in high throughput screening for cell culture process development.

2. Materials and methods

2.1. Cell lines, cell culture media and supplements

Studies were carried out using five proprietary mAb producing CHO cell lines. Cell lines 1, 4 and 5 were derived from the CHO K1 host, while cell lines 2 and 3 were derived from CHO DHFR and GS CHO hosts, respectively. Each cell line, except CHO line 4, was cultured in its optimal basal chemically defined (CD) medium for maintenance and batch and fed batch studies. Cell line 4 was maintained in a peptone containing medium, while batch and fed batch studies were performed in CD medium. Cell culture media utilized were: BD Select CHO and BD Select CD1000 (BD Advanced Bioprocessing), CDM4CHO (Thermo Fisher Scientific), and EX CELL CD CHO3 (SAFC). Feeds and media supplements utilized were: TC Yeastolate (TCY) and Proteose Peptone 3 (PP3) (BD Advanced Bioprocessing) and CD Cell Boost 6 (Thermo Fisher). The Duetz sandwich-cover system and 24DW plates were obtained from Enzyscreen BV (Haarlem, Netherlands). The system includes 24DW plates (40 mm deep, pyramid bottom, volume 11 mL/well, transparent polystyrene plates), sandwich covers (CR1224a) and clamps (CR1700).

2.2. Bioassays

Bioassay cultures were seeded at identical seeding density in 24DW plates and shake flasks. Culture volume was 3 mL and 50 mL in 24DW plates and shake flasks, respectively. Plate and shake flask cultures were incubated on a shaking platform in 5% CO_2 at 37 °C. The shaking speed for plates was 300 rpm, while the shaking speed for flasks was 125 rpm. The orbital diameter of the shaking platform was 25 mm for both plates and shake flasks. For 24DW plates, 300 μl samples were collected from the same well on various days of culture. These samples were diluted 2–3 times with PBS (Cellgro®) for assessment of cell growth (Viable cell density; VCD), viability and protein production. VCD and viability were determined using a Vi-CELL® (Beckman Coulter) and protein production was measured using a ForteBio Octet® (Pall Life Sciences). For batch culture studies, peptones were added to basal media on Day 0. Fed batch cultures were fed with CD feeds on alternate days starting on Day 0 of the culture. Peptone titration studies were performed to test the effect of various concentrations of peptones on growth and production of CHO cells in a batch culture.

2.3. Statistical analyses

Minitab 16 software (Minitab Inc) was used to generate multivariate charts and to perform other statistical analyses. Correlation analysis was performed to determine growth and production performance relationship between shake flask and 24DW plates. The Pearson correlation coefficient is a measure of linear association between two variables. Values of the correlation coefficient are always between −1 and +1. A correlation coefficient of +1 indicates that two variables are perfectly related in a positive linear sense. Two-Way ANOVA was used to assess effect of plates and peptone titration in plate-to-plate variation study.

3. Results

3.1. Comparison of 24DW plate with Duetz sandwich-cover system and conventional shake flask system

Four CHO lines were grown concurrently in their respective optimal base media in 24DW plates with Duetz sandwich-covers and in conventional shake flasks. Samples were collected on various days of culture and operational parameters for each culture system were as described in Material and Method. Regardless of medium and cell line, growth kinetics and mAb production of CHO cell lines in 24DW plates was comparable to those grown in shake flasks (Fig. 1). As shown in Fig. 2, cell viabilities were maintained above 80% on Day 7 of culture for all cell lines in both shake flask and 24DW plates. These results indicate that 24DW plates may simulate the performance and dynamics of shake flask and can be used for cell culture process development studies.

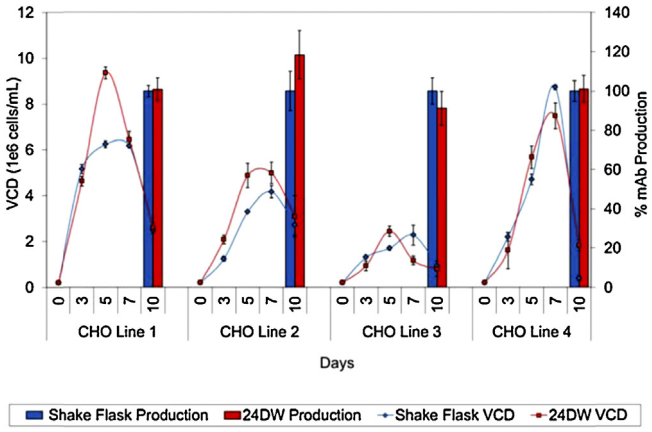

Fig. 1. Comparison of cell growth and mAb production of four CHO lines (CHO line 1–4) in 24DW plate with Duetz sandwich-cover system and conventional shake flask system. Lines represent VCD, while bars represent % of mAb production on Day 10. Each data point represents the mean ± SD of six replicates for plates and duplicates for shake flasks.

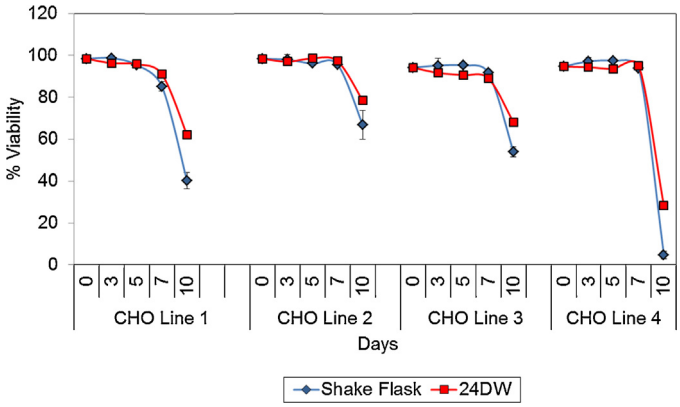

Fig. 2. Comparison of viability of four CHO cell lines (CHO line 1–4) in 24DW plate with Duetz sandwich-cover system and conventional shake flask system. Each data point represents the mean ± SD of six replicates for plates and duplicates for shake flasks.

Table 1
Assessment of well-to-well variation in growth of CHO line 3 in 24 DW plate.

	Viability (%)			VCD (1e6 cells/mL)		
	Mean	SD	%CV	Mean	SD	%CV
Day 4	96.71	0.47	0.48	2.91	0.26	8.95
Day 7	62.28	2.16	3.46	5.00	0.22	4.47

3.2. Plate uniformity assessment

3.2.1. Well-to-well variation

In order to assess well-to-well variation, CHO line 3 was cultured in all wells of a 24DW plate in a basal medium supplemented with 3 g/L of PP3 peptone. Samples were collected on Day 4 and 7 for assessment of growth. As shown in Table 1, the percent coefficient of variation (%CV) for VCD and viability was <10%, which was consistent with the shake flask culture system (<15%) as observed in our laboratory (data not shown). As shown in Table 2, growth data was uniform across the plate on various days of culture and edge effect was not observed. Protein production was determined on the last day of culture and %CV for protein production was less than 5% for entire plate (Table 3). Together, these results show well-to-well consistency and lack of edge effect in 24DW cultures with Duetz sandwich-covers.

3.2.2. Plate-to-plate variation

To assess plate-to-plate variation, a peptone titration study was performed in three 24DW plates with CHO line 5 as described in Materials and Methods. Each plate contained six different concentrations of TCY peptone in duplicate wells. Sample locations were identical across three plates as shown in the plate map (Table 4). Samples were collected and analyzed for growth (Day 5) and production (Day 7). In Fig. 3, growth and production data is presented in a multivariate charts, where each panel represents a plate. Peptone showed a dose dependent effect on growth and protein production in all plates. All three plates did not show significant differences in mean VCD or production, indicating that the average response was similar across plates, regardless of titration point. Two way ANOVA

Table 2
Assessment of uniformity of VCD (1e6 cells/mL) of CHO line 3 in different wells of 24DW plate.

VCD-Day 4	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
Row A	2.9	3.2	3.7	3.1	3.2	3.1
Row B	3.2	2.9	2.9	3.1	2.8	2.6
Row C	2.8	2.5	2.8	2.9	3.0	2.6
Row D	2.7	2.7	2.9	3.0	2.5	2.9
VCD-Day 7	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
Row A	5.0	5.3	5.1	4.8	4.9	5.0
Row B	5.5	5.1	5.2	5.3	4.7	5.2
Row C	4.8	4.9	5.1	4.9	4.7	4.9
Row D	5.1	5.1	4.9	4.8	4.5	5.2

Note: Table shows locations of rows and columns in a 24DW plate.

Table 3
Assessment of uniformity of protein production of CHO Line 3 in different wells of 24DW plate.

Production (ug/mL)	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
Row A	535.55	546.40	524.55	559.20	556.55	549.20
Row B	559.75	593.05	540.75	581.65	568.50	542.6
Row C	522.00	518.90	508.80	544.40	566.75	575.20
Row D	575.45	538.70	574.55	556.80	557.00	579.20

Note: Table shows locations of rows and columns in a 24DW plate.

Table 4
Assessment of plate-to-plate variation – plate map.

	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
Row A	TCY (0 g/L)	TCY (3 g/L)	TCY (6 g/L)	TCY (9 g/L)	TCY (12 g/L)	TCY (15 g/L)
Row B	TCY (0 g/L)	TCY (3 g/L)	TCY (6 g/L)	TCY (9 g/L)	TCY (12 g/L)	TCY (15 g/L)
Row C	NT	NT	NT	NT	NT	NT
Row D	NT	NT	NT	NT	NT	NT

NT – not tested (empty wells).

analyses were performed to determine the effect of plates and titration on growth. There was a significant difference among titration points ($P = 0.00$) while plate effect was insignificant ($P < 0.081$). These results demonstrate 24DW plate-to-plate consistency.

3.3. Process development in 24DW plates

Common strategies for enhancing cell performance for biologics production include batch or fed batch supplementation with peptone and/or CD supplements to provide sufficient nutrition. Studies were performed to assess the applicability of 24DW plates for batch and fed batch processes and to determine the correlation between 24DW plate and shake flask culture systems.

3.3.1. Batch culture study – peptone supplementation in 24DW plate and shake flask

To compare the performance of 24DW plates and shake flasks in a batch culture process, CHO line 4 was grown in both culture systems in the presence of various concentrations of PP3 peptone. Samples were collected on various days of culture and data is shown (Fig. 4) for Day 7 and 9 for growth and production, respectively. PP3 enhanced growth of CHO line 4 in shake flask cultures and 24DW plates in a dose dependent manner. Production was enhanced in presence of 1 g/L of PP3 peptone compared to no peptone in both shake flasks and 24DW plates. Higher concentrations of PP3 did not show further enhancement in protein production in either culture system. Correlation analysis of data from both systems gave a Pearson coefficient value of 0.986 for growth and 0.900 for production with a P value <0.05. This indicates that there is a positive linear relationship between the data sets obtained from the two culture systems and they are highly correlated.

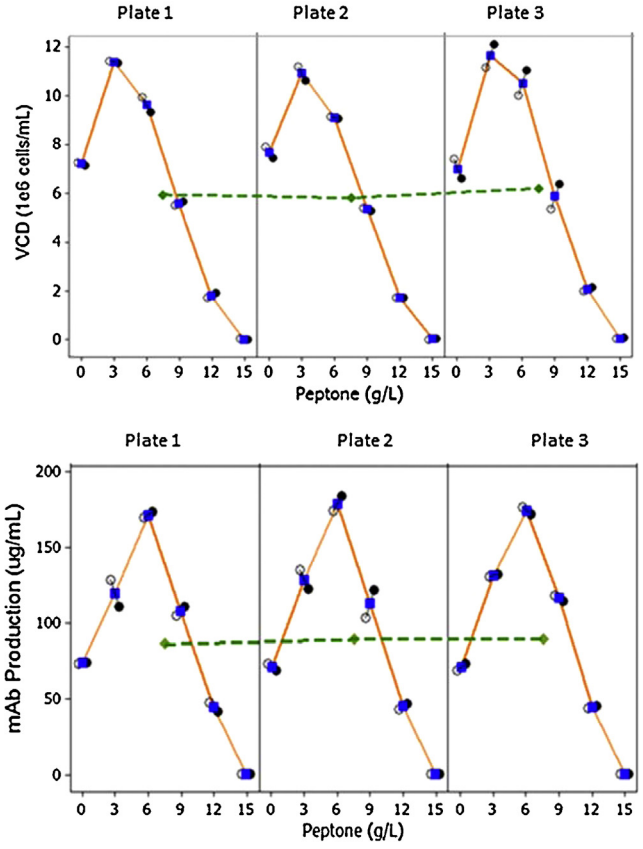

Fig. 3. Multivariate charts showing plate-to-plate variation in a peptone titration study. VCD (upper panel) and mAb production (lower panel) responses of CHO line 5 in TCY peptone titration study. Open and closed circles represent duplicate samples at each TCY peptone concentration and blue squares represent the means of duplicates. The orange solid line connects the titration level means and green diamonds represent overall mean for each plate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.2. Fed batch culture study – CD supplementation in 24DW plate and shake flask

To compare the performance of 24DW plates and shake flasks in a fed batch culture process, CHO line 1 was grown in a basal medium in both culture systems, fed with a CD supplement (5%, v/v) on days 0, 2, 4, and 6, and sampled on various days of culture. As shown in Fig. 5, the CD supplement enhanced the growth of cells in both 24DW plates and shake flasks, however somewhat higher growth was observed in shake flask cultures. Despite lower growth in 24DW plates, both systems showed equivalent protein production. In a separate study (data not shown), six different feeds were tested in fed batch process on CHO line 1 in both culture systems and protein production was determined on various days of culture. A high and significant correlation was obtained between 24DW plates and shake flask for protein production on three different days of culture (Pearson correlation coefficient 0.94 with $P = 0.00$). Results obtained from these fed batch studies indicate that while the overall cell growth patterns show some differences, the production response is highly correlated between two systems.

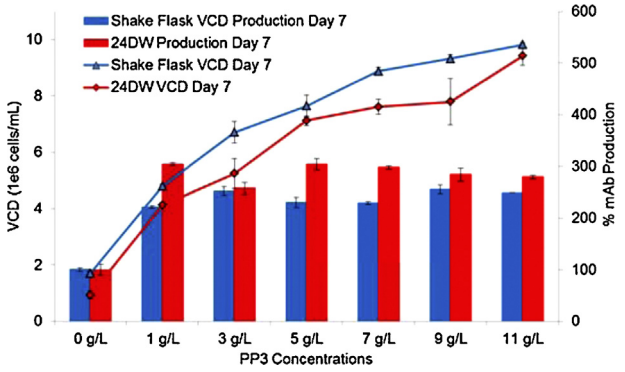

Fig. 4. Representative batch culture study in 24DW plate with Duetz sandwich-cover system and conventional shake flask system. A concurrent batch study was performed in 24DW plates and shake flasks. CHO line 4 was exposed to various concentrations of PP3 on Day 0 of culture and samples were collected on various days of culture for assessment of growth and mAb production. Lines represent VCD, while columns represent % mAb production. Each data point represents mean of duplicate samples.

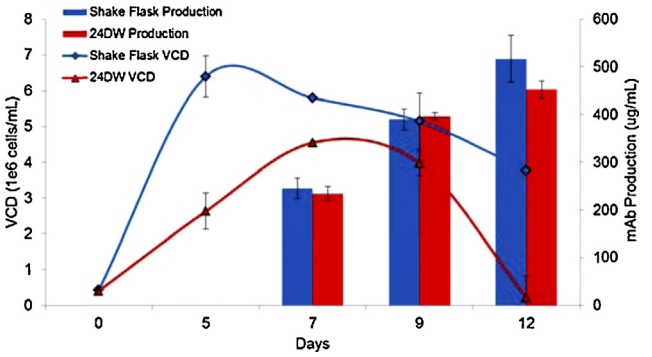

Fig. 5. Representative fed batch culture study in 24DW plate with Duetz sandwich-cover system and conventional shake flask system. A concurrent fed batch study was performed in 24DW plates and shake flasks. CHO line 1 was fed with a CD supplement on day 0, 2, 4 and 6 of culture. Lines represent VCD, while columns represent mAb production. Each data point represents mean of duplicate samples.

4. Discussion

The premise of our approach was that the miniaturized cell culture system (shaking 24DW plates) can be used for cell culture process development, if the system shows significant correlation with conventional shake flask system. To assess this approach, concurrent studies were performed in 24DW plates with the Duetz sandwich-covers and conventional shake flask systems. Feasibility studies included screening of multiple CHO cell lines in 24DW plates concurrently with shake flasks to understand cell line dependent variability. Other studies included assessment of well-to-well and plate-to-plate variation for CHO cell growth and mAb production. Regardless of the medium and cell line, growth kinetics of the cells grown in 24DW plates showed similar patterns to cells grown in shake flask. Moreover, the production levels in 24DW plates were equivalent to shake flasks. Determination of inter- and intra-plate variability is important for data consistency and accuracy in any plate based assay. Edge effect is a very common phenomenon observed in a multi well plate assays caused by differential evaporation across the plate. To confirm that Duetz sandwich-cover system would resolve these issues, well-to-well variability was assessed by culturing the same samples in all 24 wells of a 24DW plate. Data for well-to-well variation study depicted that cell growth was uniform across the plate and % CV for growth was <10% across the plate on various days of culture. plate-to-plate variation was assessed by culturing the same set of samples in duplicate on multiple plates. Two way ANOVA analysis data from three plates displayed no significant differences in growth and production responses among three plates ($P = 0.775$).

Biopharmaceutical production of recombinant proteins often uses batch and fed batch culture systems. During process development shake flasks are used to evaluate various supplements and feed strategies to finalize manufacturing process. Use of multi well plates in place of shake flasks can help increase efficiency and reduce time lines for process development projects. We have performed several studies to determine the correlation between shake flasks and 24DW plates, when used for batch and fed batch processes. Here, we have shown data from a representative batch culture study where strong correlation was found between the performances of shake flasks and 24DW plates (Pearson coefficient for growth = 0.98, production = 0.90). In the fed batch process, a significant correlation was observed between 24DW plate and shake flasks for protein production (Pearson Coefficient = 0.94, $P = 0$) however growth patterns in 24DW plate and shake flask did not show a high correlation (Pearson Coefficient = 0.40; $P = 0.096$) in the cell lines tested in this study. The data from fed batch studies suggests that 24DW plates will be indicative of titer levels and can be used for screening of feeds and fed batch strategies.

5. Conclusions

The biopharmaceutical industry has a substantial interest in scale-down and high-throughput cell culture platforms that can facilitate scalable media and process development with significant cost and time savings. We have shown with a series studies that CHO cell cultivation in 24 DW gives well-reproducible results that are comparable to those in Erlenmeyer shake flasks, provided that the exchange-of-headspace air of each individual well is controlled by a high-quality cover system. The procedures used were found to be well applicable for the screening of media and supplement formulations.

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