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# WHEATON® CELLine™ Overview • Application: Introduction

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The WHEATON® CELLine™ culture devices are based on a compartmentalization approach. Cells are cultured in a cell compartment separated from the nutrient medium by a semi-permeable membrane. Cells in the cell compartment are maintained in a small volume and supported by a larger volume of nutrient medium contained in the nutrient medium reservoir. Successful cultures in WHEATON® CELLine™ devices require manipulating both the cell compartment contents and the nutrient medium. Attributes such as high cell density, reduced serum use, and ease of handling are available to the user through this compartmentalization approach. The following overview highlights certain features and functions of the WHEATON® CELLine™ devices to assist the user in developing individual and varied applications.

**HIGH CELL DENSITY:** Cells can be cultured at high density within the cell compartment of the WHEATON® CELLine™. Compared to traditional static culture, the number of cells per milliliter, which can be maintained within the cell compartment of the WHEATON® CELLine™, is much higher. For example, hybridoma cells, lymphocytes, and leukemic cells can reach concentrations 20 to 30 fold greater when compared to growth in static vessels. This benefits the user by providing increased concentrations of secreted effector molecules, high antibody concentrations, and unique cell culture environments not available in traditional low-density cultures.

The separation of the cells from the nutrient medium by a semi-permeable membrane allows for trapping or retaining cell-secreted factors and the ability to conserve the use of exogenous culture factors. The 10,000 MWCO semi-permeable membrane allows the retention of many cytokines, which are approximately 10,000 MW or larger. For instance, IL-2 provided only to the cell compartment exerts full biological activity on the cells in culture. Conversely, secreted cytokines produced within the cell compartment can reach levels not normally associated with traditional static culture. Conditioned medium can be produced with significantly different levels of effector molecules in the WHEATON® CELLine™ than in traditional static culture.

For the production of monoclonal antibodies, the benefits of the compartmentalized strategy provide cost savings and labor savings. Hybridoma cells can be cultivated at high density within the cell compartment of the WHEATON® CELLine™, producing highly concentrated titers of antibody within the cell compartment volume. Antibody titers over 1 mg/ml are routinely achieved. Concentrated antibodies in small supernatant volumes can be diluted and used directly or concentrated supernatant can be applied directly to affinity purification columns.

Importantly, the consumption of serum can be significantly reduced by providing serum only to the cell culture compartment and eliminating serum from the nutrient medium. A 1-liter culture in the WHEATON® CELLine™ consumes only milliliters of serum in contrast to hundreds of milliliters of serum which would normally be consumed in static culture. These savings in serum cost quickly accumulate as the duration of culture increases. An added benefit is the removal of interference from serum proteins during antibody purification, as the product is obtained at mg/ml concentrations in a 10% serum supernatant. This is in marked contrast to concentrating serum components in conjunction with the desired antibody during processing leading to difficulties in purification and contamination of antibody preparations with nonspecific immunoglobulin molecules and other serum proteins. Using serum to generate highly concentrated supernatant of antibody molecules is not as problematic as downstream concentration steps are eliminated.

**CELL COMPARTMENT:** Understanding the concept of a viable cell capacity for the cell compartment is essential in the operation of the WHEATON® CELLine™ for maximum performance. The cell compartment in the WHEATON® CELLine™ has an upper limit to the number of viable cells that can be maintained. This is termed cell compartment viable cell capacity. As the number of viable cells within the cell compartment increases, the consumption of metabolic substrates and accumulation of metabolic byproducts also increases. Diffusion across the semi-permeable membrane begins to become limiting when the viable cell numbers reach the viable cell capacity in the cell compartment, even when a maximum diffusion gradient is provided across the semi-permeable membrane. Importantly, cell proliferation does not cease, as shown in ***Fig. 1***, when viable cell capacity has been reached. Cells continue to increase within the cell compartment after viable cell capacity is reached. Therefore, at viable cell capacity, the number of viable cells no longer increases, however, total cell numbers continue to increase. This can lead to an accumulation of very high numbers of total cells. Splitting back the cell compartment influences the ratio of total and viable cells. Splitting back the culture when the capacity of the cell compartment is reached will maintain high cell viability. Splitting back several days after capacity is reached does not lead to loss of viable cells but can lead to increased product concentration and increased total cell numbers.

The handling strategy for the WHEATON® CELLine™ should be based on the operator's needs. For high percent viability, it is crucial to split the cell compartment back when cells reach viable cell compartment capacity. This prevents the accumulation of nonviable cells. For higher total cell concentrations and increased product concentrations, less frequent splitting of the culture is acceptable. The ratio at which the cell compartment is split during harvest will determine the time required for cells to return to maximum viable cell capacity. Cultures splitiback twofold, will return to maximum viable cell numbers sooner than cultures split-back fourfold. To provide extended durations between handling, the cell compartment can be split further than 50%. Results obtained with the WHEATON® CELLine™ will depend on the strategy used in manipulating the cell compartment contents and the exchange of nutrient medium, as discussed below.

In summary, the cell compartment can be treated as a standard tissue culture flask except that cells are present at high concentrations. Splitting back cells in the cell compartment provides

control over cell numbers and percentages of viable cells. When cells have reached the maximum capacity of the cell compartment, proliferation continues, and accumulation of total cells will take place.

**NUTRIENT MEDIUM:** The rate of nutrient medium exchange also impacts culture results. Diffusion across the semi-permeable membrane is driven by concentration gradients established between the nutrient medium and the cell compartment medium. Glucose flux across the semi-permeable membrane is shown in ***Fig. 2***. As the nutrient medium is depleted, the driving force for solute diffusion across the semi-permeable membrane is also reduced. A decrease in viable cell mass is associated with a depleted nutrient medium and accumulation of metabolic byproducts.

The nutrient medium does not change color as significantly as it does in static culture flasks. The direct gas exchange across the bottom of the cell compartment reduces the accumulation of acid within the nutrient medium. Color change of nutrient medium can be used as an indicator of metabolic activity but is not as accurate for assessing when nutrient medium should be exchanged. Tracking cell numbers is the most accurate.

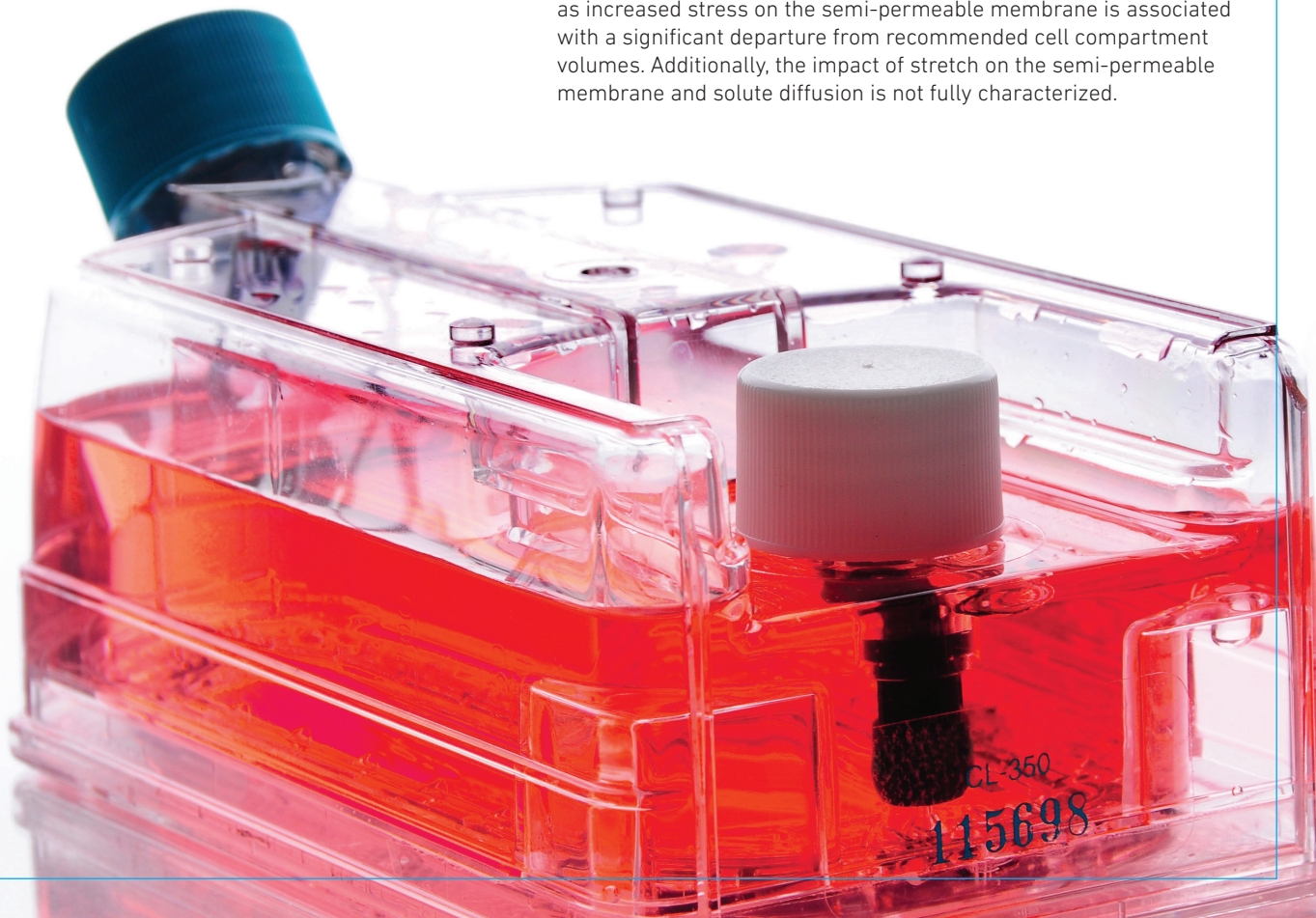
As shown in ***Fig. 3***, increased nutrient medium exchange does not significantly increase cell compartment capacity. Note the maintenance of the viable cell mass and the further accumulation of total cells after reaching viable cell capacity.

In summary, nutrient medium can be exchanged to maintain the highest possible gradient for diffusion across the semi-permeable membrane, or it may be exchanged less frequently to maximize the efficiency of medium use. The user can determine which strategy is most suitable for a particular application.

**CELL INOCULATION:** For certain cells, a relationship between cell density at inoculation and the initial rate of cell growth in the cell compartment has been observed. The cell density dependent outgrowth of a murine hybridoma cell is shown in ***Fig. 4***. When cells were inoculated at lower acid accumulation they took longer to reach cell compartment capacity, in comparison to cells inoculated at higher density. The responses of certain cells to low density inoculation may be due to dilution of growth promoting or conditioning effects currently not well characterized. Certain cell lines may benefit from higher initial inoculation densities. For production purposes maximum performance is attained when the WHEATON® CELLine™ is operated at or near viable cell capacity and inoculating a higher number of cells at culture initiation can lead to more rapid attainment of viable cell capacity.

**OSMOTIC FLUX:** Water flux across the semi-permeable membrane is driven by differences in protein concentrations across the membrane. When serum is not provided in the nutrient medium, an oncotic gradient between the cell and nutrient medium compartments is established. This can lead to increases in the cell compartment volume during culture. The change in volume can be controlled by manipulating the oncotic gradient across the semi-permeable membrane through the addition of an inexpensive protein hydrolyzate (lactalbumin) to the basal medium. In most applications, the slight flux of water into the cell compartment is insignificant. It can be compensated by providing slightly higher serum concentrations or supplements to the cell compartment. As shown in ***Fig. 5***, changes in the cell compartment volume can be influenced by serum concentration differences across the semi-permeable membrane, the amount of cells in the cell compartment, and the duration between the handling of the cell compartment.

**CELL COMPARTMENT VOLUME CAPACITY:** The cell compartment volume is variable. As shown in ***Fig. 6***, the cell compartment can be varied significantly due to the compliance associated with the semi-permeable membrane. For the CL 1000 units, a slight change in the cell compartment volume can be induced with no significant increase in pressure. This allows the user to explore protocols with different cell compartment volumes. It is recommended that for routine use, the specified cell compartment volumes be adhered to as increased stress on the semi-permeable membrane is associated with a significant departure from recommended cell compartment volumes. Additionally, the impact of stretch on the semi-permeable membrane and solute diffusion is not fully characterized.



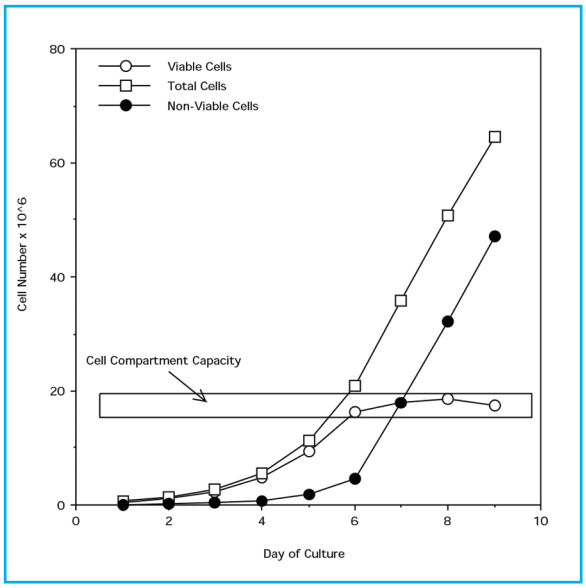



**Figure 1**: Growth curve example: Theoretical growth of cells within the cell compartment of the WHEATON® CELLine™ is depicted. The open circles represent viable cell numbers and plateau when the cell compartment capacity is reached. Total cells represented by open squares continue to increase throughout the culture period. Non-viable cells represented by filled circles begin to accumulate after cell compartment capacity is reached.

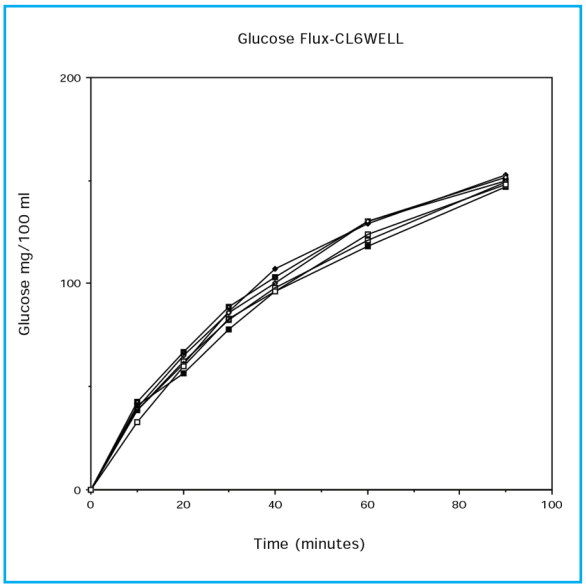



**Figure 2**: Glucose diffusion across semi-permeable membrane. Tests conducted at room temperature. RPMI-1640 was placed in nutrient reservoir of individual CL 6WELL compartments. The cell compartment filled with distilled water. Cell compartment harvested at indicated times and glucose concentration determined. The driving force for glucose flux decreased with time as the cell compartment glucose concentrations increased. Flux of nutrients across the semi-permeable membrane during culture will be influenced by depletion of nutrients during culture.

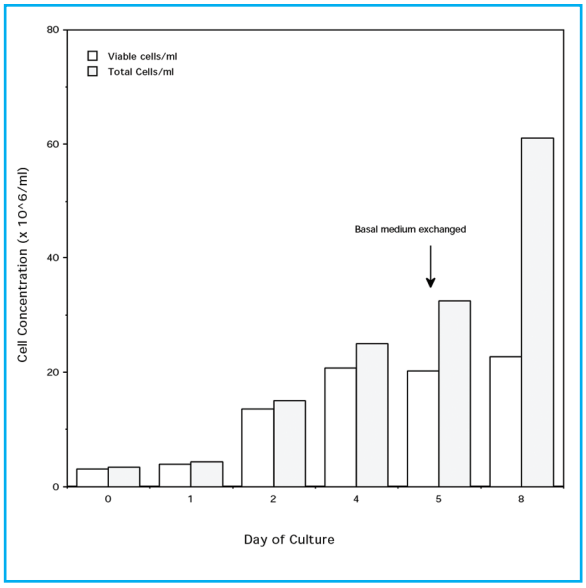



**Figure 3**: Growth curve of murine hybridoma cells in CL 1000. Cell compartment: 10% FBS, Nutrient compartment 0.8% FBS, .1% vitacyte. Concentrations of viable and total cells are plotted during culture. The viable cell concentration remained relatively constant after cell capacity had been reached. Total cell concentration continued to increase with culture. Note that exchange of basal medium did not significantly increase the viable cell numbers.

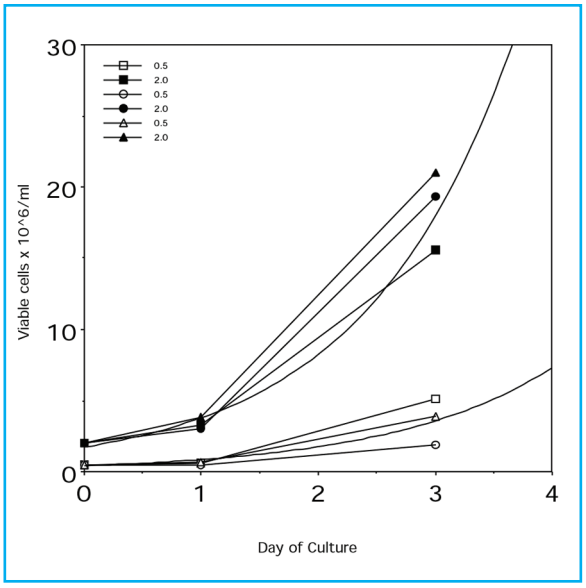



**Figure 4**: Cell outgrowth and intial cell concentration. AC.04 murine hbyridoma cells were inoculated at 0.5 x $10^6$ and 2.0 x $10^6$ cells/ml into individual wells of a CL 6WELL. Cell compartment 15% FBS, nutrient compartment 0% FBS, RPMI-1640. At days indicated cells were counted and viable cell concentrations determined by trypan blue exclusion.

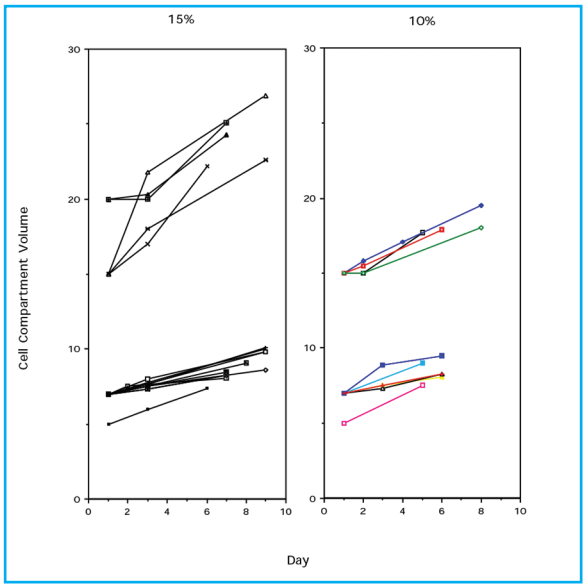



**Figure 5**: Osmotic water flux into cell compartment of CL 1000 and CL 350 units during culture. Cell compartment volumes are plotted at beginning and end of harvest intervals taken during culture. Cultures were conducted with cell compartment serum concentrations of 10% (0.8% nutrient compartment) and 15% (0% nutrient compartment).

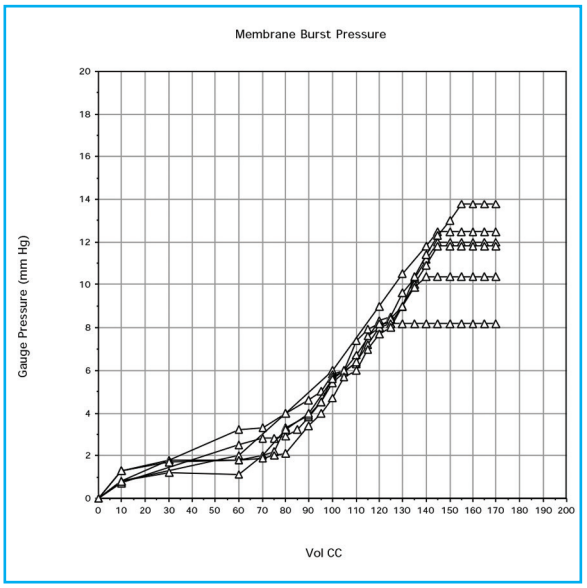



**Figure 6**: Cell compartment volume and pressure relationship. The volume of infused air into a wetted cell compartment of individual CL 1000 units is plotted versus gauge pressure. Units were tested with 250 ml of water placed atop of cell compartment. Pressure was incrementally increased in 20 sec intervals until membrane burst or developed leak.

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# Continuous Recombinant Protein Production in Baculovirus Infected SF9 Cells using WHEATON® CELLine™ classic 1000 Two-Compartment Bioreactors

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## INTRODUCTION

In recent years, baculovirus expression vectors (BEV) systems have become an efficient and popular method for the production of recombinant proteins in insect cell lines. The system allows to achieve relatively high levels of heterologous gene expression and the posttranslational modifications of the gene products are closely parallel to the modifications within mammalian cells. Recombinant proteins expressed in the cytoplasm of an insect host cell often reach concentrations of up to 1mg/mL. In contrast, secreted proteins are usually only found to be present at concentrations below 0.01mg/mL. Therefore milligram-amounts of a secreted protein can only be obtained by handling several litres of cell culture volume using either a small scale bioreactor or handling large numbers of standard cell culture disposables. As a consequence such experiments can become time consuming and cost intensive.

The WHEATON® CELLine™ Two-Compartment Bioreactors are designed to ensure an optimal nutrient and oxygen supply of the cells and thereby allow the cultivation of eukaryotic cells to densities which are 50 times higher than compared to standard homogenous cell culture vessels. This protocol describes the cultivation of SF9 cells in a WHEATON® CELLine™ bioreactor to a density of 5–10 x $10^7$ cells/mL. The culture can be continuously maintained over several weeks and cells can be harvested every sixth day. Upon transfection of the SF9 cells with a BEV, this high cell densities drive the accumulation of recombinant protein titers which are between 1 to 2 magnitudes higher than in a standard homogenous cell culture vessel. The method was designed to minimize the necessary handling time and to allow significant cost savings, mainly resulting from a 95% reduction of the required serum supplementation. In addition, the condensed harvesting volumes prevent a laborious concentration of culture supernatants and the high specific product concentrations facilitate the protein purification from the contaminating serum background.

i Best Results were obtained with SF900 II medium (Invitrogen), as alternative standard Grace's Insect Medium can be used.

ii In order to obtain optimal performance, the semi- permeable membrane of the CELLine bio reactor needs to be equilibrated for 5 minutes prior to inoculation by the addition of 25 ml of basal medium to medium compartment.

iii In order to prevent protein precipitations to damage the semi-permeable membrane between the cell and the medium compartment, we recommend to store the CELLine bioreactor in 1x PBS in between two BEV infection cycles.

## OPERATING PROCEDURES

### A. Preculture

- Cultivate SF9 cells in 225cm$^2$ Tissue Culture Flask to a density of 1×$10^6$ cells/mL in 50mL SF900 II[i] supplemented with 10% FCS.

### B. Continuous Cultivation of SF9 cells

- Resuspend the 5×$10^7$ cells obtained from the preculture in 15mL of fresh medium supplemented with 10% FCS and inoculate the cell compartment of a WHEATON® CELLine™ 1000 bioreactor[ii] (cultivation reactor) with the suspension. Add 1000mL of fresh basal medium (not supplemented with FCS) to the medium compartment
- Incubate the WHEATON® CELLine™ Reactor for 6 to 7 days at 28°C
- Harvest the cells from the cell compartment at a density of around 5 x $10^7$ cells/mL and remove
- 90% (about 17 to 18mL) for baculovirus transfection
- Spin down the remaining 10% (2 to 3mL) of the cells, resuspend them in 15mL fresh medium with 10% FCS and continuously cultivate the cells as described above
- Perform subsequent harvests every 4 to 6 days. SF9 cultures can be continuously maintained over 2 to 3 months

### C. Baculovirus Transfection and Recombinant Protein Production

- Spin down the cells harvested from the cultivation reactor and resupend them in 15mL of fresh medium containing 10% FCS
- Add Baculovirus with an MOI 2 (around 15 x $10^8$ virus particles) into the cell compartment of a second WHEATON® CELLine™ 1000 bioreactor (production reactor) and subsequently add the prepared cell suspension
- Add 1000mL of fresh medium without FCS to medium compartment of the production reactor
- Incubate the infected cells for 4 to 5 days at 28°C
- Harvest the culture supernatant and/or cells from cell compartment containing your protein of interest
- The production reactor can be used continuously for several transfections of SF9 cells with the same BEV[iii]





DWKWP_002a 01/2022

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# Comparison of batch vs. WHEATON® CELLine™ culture for production of monoclonal antibody in vitro as alternatives to ascites Application: Murine Hybridoma

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As an alternative to ascites, in vitro production of monoclonal antibody can be accomplished with a variety of methods (1-5). Comparison of in vitro methods indicates not all methods are equally cost effective or user friendly. The ascites method has a number of perceived advantages over most in vitro methods, primarily a low cost of p roduction based on mg of antibody produced and a concentrated product which eases down stream processing requirements.

Many systems and instruments (Bioreactors) designed specifically to produce mAb in vitro are burdened by capital instrument expense and additional culture ware costs. These systems also require a learning curve for successful use. For small scale production, many of these systems are not cost effective. These systems become more cost effective as production increases, due to amortization of costs over larger amounts of produced antibody. In contrast, the ascites method does not become less expensive as the amount of antibody to be produced increases. Ascites production costs are generally linearly related to the amount of antibody required (number of mice).

For small production runs, ascites has been considered more cost effective than most other methods, as it can be readily scaled to small production needs. A single mouse can produce milligram amounts of antibody and provide it in a concentrated form. To effectively compete with ascites for production of modest amounts of monoclonal antibody, in vitro methods must have no capital costs and provide additional cost saving benefits. For example, production of concentrated antibody, reduced serum use, and reduced labor are benefits which make in vitro methods competitive to ascites for production of modest amounts of antibody. These benefits are unfortunately available only with more costly and complicated systems and are not cost effective for producing smaller amounts of antibody. The WHEATON® CELLine™ culture devices have no capital equipment costs, a low purchase cost, provide concentrated product, reduce serum use, and reduce handling. Importantly all of these attributes are available in a device as simple to use as a standard tissue culture flask. The WHEATON® CELLine™ 1000 was used to produce modest amounts of monoclonal antibody in a manufacturing laboratory. The WHEATON® CELLine™ 1000 is a member of a product family of high density cell culture devices available from DWK Life Sciences. The WHEATON® CELLine™ 1000 has a reservoir volume of 1 liter with a cell compartment capacity of 2030 ml. The results obtained with the WHEATON® CELLine™ 1000 are presented to provide a demonstration of the cost savings and additional benefits obtained by producing antibody in these devices.

The results from 7 different murine hybridoma cultures carried out in the WHEATON® CELLine™ 1000 are presented. The cultures were carried out by a commercial manufacturing laboratory which produces and sells antibody reagents to the research community. A minimal handling protocol (continuous batch process) was employed by the manufacturing laboratory to reduce the handling of the cultures. The hybridoma cells were maintained in the cell compartment of the WHEATON® CELLine™ 1000 and harvested at approximately 7 day intervals. During harvest, cells and supernatant were collected from the cell compartment, a fraction of the harvest was reinoculated into the cell compartment with fresh medium. Nutrient medium was removed and replaced with fresh medium on day of harvest. Results obtained from cultures in the WHEATON® CELLine™ 1000 are compared to previous results obtained with the same cell lines cultured in a batch method in traditional tissue culture flasks at the facility.

**Methods:** Murine hybridoma cell lines (Table 1) were thawed from frozen stocks and expanded in static culture (RPMI-1640, 10-15% FBS, 2X L-Glutamine, Pen-Strep) . After demonstration of consistent cell doubling in static culture, cells were inoculated into the WHEATON® CELLine™ 1000 devices.

**TABLE 1**

| Hybridoma | Isotype | Fusion Partner | Inoculation Concentration Viable cell/ml |
|---|---|---|---|
| AC.1 | IgG1 | Ns-1 | $7.5 \times 10^6$ |
| AC.2 | IgG2a | 653 | $6.8 \times 10^6$ |
| AC.3 | IgG1 | Ns-1 | $3.7 \times 10^6$ |
| AC.4 | IgG2a | Sp20 | $5.6 \times 10^6$ |
| AC.5 | IgG1 | Sp20 | $7.2 \times 10^6$ |
| AC.6 | IgG1 | not known | $7.2 \times 10^6$ |
| AC.7 | IgG1 | not known | $5.5 \times 10^6$ |

**Cell compartment medium:** RPMI-1640; 2X L-glutamine (5 mM), penicillin G (66 mg/L), streptomycin sulfate (144 mg/l). Basal medium was supplemented with 10% FBS (Hyclone, Logan Utah). Additional supplementation of medium with a hybridoma growth supplement (0.1% Vitacyte, J. Brooks Irvine, CA) was done to remain consistent with prior batch production runs of the same cell lines in traditional flasks.

**Nutrient medium:** RPMI-1640; 2X L-glutamine (5 mM ) , penicillin G (66 mg/L) streptomycin sulfate (144 mg/l) with 0.8% FBS, 0.1% Vitacyte.

**Inoculation:** Cells were inoculated from static culture at Day 0 in a 20 ml volume into the cell compartment of the WHEATON® CELLine™ 1000 devices. Inoculation density was maintained above $3.0 \times 10^6$ cells/ml. Cells were removed from frozen stock initiated cultures and resuspended in fresh cell compartment medium prior to inoculation. Nutrient medium (1000 ml) was supplied to the nutrient medium compartment and the devices placed into a 5% CO2, 37°C humidified tissue culture incubator.

**Harvest:** At harvest, the total cell compartment volume was removed from the WHEATON® CELLine™ 1000 units by pipette. Cell numbers were determined by diluting and counting samples using a standard hemacytometer. Viable cells were discriminated from non-viable cells by trypan blue staining and phase contrast microscopy. Cell compartment contents were split back between 3-5 fold determined by cell numbers in the cell compartment at time of harvest. Fresh cell compartment medium (17-15 ml) was added to the cell fraction (35 ml) to achieve a 20 ml volume and the cell suspension returned to the cell compartment. The harvested cell containing supernatant fraction was kept sterile and stored at 4°C until purification by affinity chromatography. Nutrient medium (1000 ml) was removed and replaced with fresh medium at the time the cell compartment was harvested . Devices were returned to incubator until next harvest. Devices were stacked atop of each other in the incubator.

**Antibody purification:** Culture supernatant was processed by eluting antibody from protein A affinity chromatography columns following manufacturers protocol. Eluted antibody fractions were collected, pooled and antibody quantified by spectrophotometer and ELISA. Sandwich ELISA was performed with polyclonal goat anti-mouse IgG or IgM capture antibody and polyclonal anti-mouse IgG or IgM antibody labeled with peroxidase. Color was developed with ABTS. Antibody purity was assessed by SDSPAGE and Coomassie blue staining. Purified antibody was subjected to further labeling reactions to generate Fluorescein and Phycoerythrin conjugates. Purified antibody was subjected to internal quality testing and released.

**Results:** A representative cell growth curve is shown in Fig. 1. The total cells and total viable cells are plotted against day of culture. The cell compartments were split back at harvest and growth of the reinoculated cells resumed. The semi continuous batch process generated over $1x10^9$ total cells within the cell compartment at harvest. The total viable cell numbers obtained prior to or at day of harvest indicate the maximum capacity for viable cells in the cell compartment. The maximum cell numbers counted from a single harvest during the entire culture period for the individual cultures are shown in Table 2.

**TABLE 2**

| Hybridoma | Maximum Viable Cells (cell compartment) | Maximum Total Cells (cell compartment) |
|---|---|---|
| AC.1 | $561 \times 10^6$ | $1701 \times 10^6$ |
| AC.2 | $660 \times 10^6$ | $3304 \times 10^6$ |
| AC.3 | $520 \times 10^6$ | $2140 \times 10^6$ |
| AC.4 | $731 \times 10^6$ | $2080 \times 10^6$ |
| AC.5 | $682 \times 10^6$ | $1957 \times 10^6$ |
| AC.6 | $570 \times 10^6$ | $1476 \times 10^6$ |
| AC.7 | $504 \times 10^6$ | $1120 \times 10^6$ |

The production results for the individual cultures are shown in Table 3. The mean number of harvests for the 7 cultures was 5. A mean harvest volume of 114 ml was processed for antibody purification. Clone AC.5 had an unusually low yield of antibody and analysis indicated that the majority of the antibody present was not intact (SDSPAGE; size analysis). Supernatant from batch culture of this clone in traditional flasks also contained partial antibody fragments. Results from this clone were not included in the following mean values as it was considered to be a non-productive clone. It should be noted that a modest amount of recoverable antibody was obtained from the WHEATON® CELLine™ culture while supernatant from batch cultures were unable to be used.

A mean of 118 mg of purified antibody per culture was obtained. The mean duration for the 6 cultures was 39 days. The mean antibody concentration recovered from harvested supernatant was 1.02 mg/ml. This concentration was calculated by dividing the volume of processed supernatant (116 ml) by the amount of antibody which was recovered following purification (118 mg). The mg of antibody produced per liter of nutrient medium consumed was 23.7 mg/l. The mean antibody concentration obtained from the standard batch culture method used previously in production for the 6 different clones was 31 mg/l. The batch cultures were maintained until viable cell numbers were nearly exhausted.

The WHEATON® CELLine™ 1000 devices consumed significantly less serum to produce antibody when compared to results from the traditional batch cultures. Nutrient medium in the WHEATON® CELLine™ 1000 was supplemented with only 0.8% FBS and the cell compartment was supplemented with 10% FBS. About 50-60 ml of FBS was consumed for an individual culture. In contrast a batch culture consumed approximately 380 ml of FBS to produce equivalent amounts of antibody. Supplementation of the nutrient medium in the WHEATON® CELLine™ 1000 with FBS has been demonstrated not to be necessary for many hybridoma clones.

Purification of the culture supernatant from the WHEATON® CELLine™ 1000 resulted in both time and labor savings when compared to traditional batch culture. Instead of concentrating more than 3 liters of medium to recover comparable amounts of antibody, the WHEATON® CELLine™ supernatant (mean:116 ml) was centrifuged and applied directly to the affinity purification columns. Consequently, there was no simultaneous concentration of serum protein in the supernatant which can lead to purification difficulties associated with concentrating traditional culture supernatant.

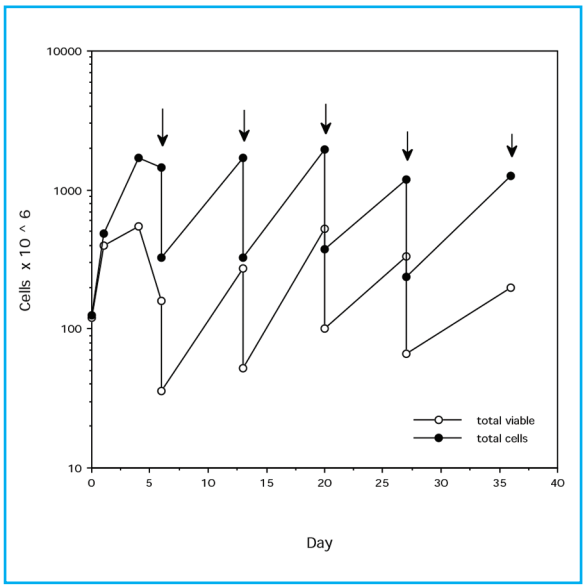


**Figure 1:** Growth Curve (AC.5). The number of total cells and viable cells which were recovered from the cell compartment of the CL 1000 during culture are shown. The cells were counted using a standard hemacytometer and non viable cells determined by trypan blue staining. The culture was harvested at times indicated by the arrows. The cell numbers were split back as indicated and cell growth between harvests is shown. The semi continuous batch process; harvests followed by resumed cell expansion of the inoculum is readily seen. The viable cell capacity of the WHEATON® CELLine™ CL 1000 was demonstrated during the initial 5 days of culture. Note, that the number of viable cells did not continue to increase, but declined as nutrient medium was consumed. Addition of fresh nutrient medium does not lead to increased viable cell numbers within the cell compartment beyond its capacity. The viable cells do continue to proliferate at maximum capacity and lead to the very high total cell numbers seen at harvest. Splitting back of the cell compartment allowed for continued cell proliferation and removal of excess cells. The growth of all the hybridoma clones was similar to the growth shown for AC.5.

TABLE 3

| Hybridoma | Number of Harvests | Total harvest volume (ml) | Mg Ab Total mg | Culture Duration days | mAb concentration mg/ml | mAb mg/liter nutrient medium |
|---|---|---|---|---|---|---|
| AC.1 | 5 | 128 | 121 | 42 | 1.06 | 24.2 |
| AC.2 | 6 | 130 | 158 | 42 | 1.21 | 26.3 |
| AC.3 | 4 | 100 | 80 | 30 | 0.8 | 19.9 |
| AC.4 | 6 | 120 | 141 | 50 | 1.17 | 23.5 |
| AC.5 | 5 | 104 | 23 | 38 | 0.22 | 4.6 |
| AC.6 | 4 | 110 | 121 | 37 | 1.1 | 30.4 |
| AC.7 | 5 | 110 | 87 | 36 | 0.8 | 17.6 |
| Mean n=6 | 5.0 | 116.3 | 118.1 | 39.5 | 1.02 | 23.7 |

**Summary:** The results obtained from hybridoma cultures in WHEATON® CELLine™ 1000 culture flasks indicate the following:

1. Cell growth was obtained in all of the 7 cultures (One clone AC.5 was not considered a productive clone and excluded from the mean values derived for the cultures).
2. The mean production of the remaining 6 clones was 118 mg of purified antibody over a mean culture duration of 39 days.
3. Significant reductions in serum consumption was obtained compared to traditional batch cultures of the same cell lines.
4. Nutrient medium consumed per mg of antibody produced in the WHEATON® CELLine™ 1000 was slightly greater than that obtained in batch culture, 23.7 mg/l compared to 31 mg/l. The nutrient medium in the WHEATON® CELLine™ 1000 was less expensive when compared to that used in batch culture as it contained only 0.8% FBS. The lower efficiency in nutrient medium consumption was more than offset by reduced nutrient medium costs by significant reduction in consumption of FBS.
5. A concentrated supernatant (1 mg/ml; mean) was obtained from the WHEATON® CELLine™ 1000 cultures when compared to prior batch cultures, reducing downstream processing requirements and eliminating the need for a concentrating step prior to antibody purification.

The results indicate that a single WHEATON® CELLine™ 1000 can produce greater than 100 mg of purified antibody in approximately 39 days while requiring only 5 harvests. The semi-continuous batch method allowed for sequential harvests and reduced handling. The WHEATON® CELLine™ 1000 units were no more difficult to handle than traditional tissue culture flasks and did not require syringes to access cells or medium. The WHEATON® CELLine™ 1000 units were stacked in the incubator and did not take up extra incubator space or require any support systems such as a roller apparatus or perfusion pump. A concentrated product was obtained from all of the cultures which simplified volume handling and processing downstream of the culture, eliminating any concentration steps normally associated with processing traditional culture superntant.

In conclusion; the WHEATON® CELLine™ 1000 units are considered cost competitive to ascites as an in vitro method for producing antibody. The operating costs (medium and serum) were significantly less than those associated with traditional in vitro batch methods using standard tissue culture flasks or gas permeable bags. Additional benefits such as concentrated product, reduced handling, and simplified downstream processing were also obtained with the WHEATON® CELLine™ 1000 devices. Most importantly, the ease of use and reduced handling needed to achieve the above mentioned benefits make the WHEATON® CELLine™ culture flasks a viable alternative to ascites for producing monoclonal antibody.


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# Antibody Manufacture in the CELLine CL1000 Application: Murine Hybridoma

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The successful production of antibodies in vitro is dependent upon numerous factors. A variety of methods and devices for producing monoclonal antibodies are available. One of the most important variables impacting production rates and costs is the clone itself from which antibody is made (1-3). Not only do the same clones perform differently under varied culture conditions, but different clones produce markedly different amounts of antibody when cultured under the same conditions (4, 5).

For many manufacturers of antibody, a large number of different clones are routinely cultured to produce monoclonal antibodies. The production amounts of antibodies required may be only 100-200 mg. For these applications, a simple to use culture method that provides benefits associated with more expensive and complicated systems for larger production amounts is beneficial. The ability of the WHEATON® CELLine™ culture flasks to meet the needs of the small-scale manufacturing laboratory is provided below.

To assess the performance of the WHEATON® CELLine™ culture devices in the production of antibody in vitro under manufacturing conditions; a large number of hybridoma clones (32 individual clones) were cultured in WHEATON® CELLine™ 1000 units by an independent manufacturing concern. The clones were selected at random and cultured by the independent commercial laboratory producing monoclonal antibodies for use in research. The antibody produced was purified from culture supernatant by affinity chromatography. All produced antibody passed quality standards (purity, activity, specificity) except for one clone (see below) and was released as a product.

The results indicate a range of production achieved using the WHEATON® CELLine™ 1000 in routine in vitro monoclonal antibody production under manufacturing conditions. The manufacturing laboratory chose a reduced handling protocol to minimize labor and handling costs. Changes in FBS supplementation and medium were evaluated during the process by the manufacturing laboratory. The protocol was not optimized for the individual clones.

Methods: Murine hybridoma cell lines were thawed from frozen stocks and expanded in static culture (RPMI-1640, 10-15% FBS, 2X L-Glutamine, Pen-Strep). After the demonstration of consistent cell doubling in static culture, cells were inoculated into the WHEATON® CELLine™ 1000 devices. The hybridoma clones were obtained from sources worldwide and included both clones obtained under license and clones generated by the manufacturer. The clones (fusion partners, isotypes) were randomly selected based on production needs. The cell lines cultured were derived from fusion partners, including 653, NS-1, and SP20. Clones are coded by the manufacturer for confidential reasons.

Cell compartment medium: RPMI-1640 or DMEM; 2X L-glutamine (5 mM), penicillin G (66 mg/L), streptomycin sulfate (144 mg/l). Basal medium was supplemented with 10%, 15%, or 20% FBS (Hyclone, Logan Utah). Additional supplementation of medium with an additional hybridoma growth supplement (0.1% Vitacyte, J. Brooks Ivrine, CA) was done for some cultures and not others.

Nutrient medium:RPMI-1640 or DMEM; 2X L-glutamine (5 mM ), penicillin G (66 mg/ L), streptomycin sulfate (144 mg/l) with 0%, 0.8%, or 5% FBS. Additional supplementation of medium with an additional hybridoma growth supplement (0.1% Vitacyte) was used for some cultures.

Inoculation: Cells were inoculated from the static culture at Day 0 in a 20 ml volume into the cell compartment of the WHEATON® CELLine™ 1000 devices. Inoculation density was maintained above 2.0 x $10^6$ cells/ml. Cells were removed from frozen stock initiated cultures and resuspended in a fresh cell compartment medium prior to inoculation. A nutrient medium (1000 ml) was supplied to the nutrient medium compartment, and the devices were placed into a 5% $CO_2$, 37°C humidified tissue culture incubator.

Harvest: At harvest, the total cell compartment volume was removed from the WHEATON® CELLine™ 1000 units by pipette. Cell numbers were determined by diluting and counting samples using a standard hemacytometer. Viable cells were discriminated from non-viable cells by trypan blue staining and phase-contrast microscopy. Cell compartment contents were split back between 3-4 fold determined by cell numbers. A fresh cell compartment medium was added to the cell fraction (4-5 ml) to achieve a 20 ml volume returned to the cell compartment. The harvested cell containing supernatant fraction was kept sterile and stored at 4°C until purification by affinity chromatography. Nutrient medium (1000 ml) was removed and replaced by pouring during cell compartment harvest. Devices were returned to the incubator until the next harvest. Devices were stacked atop of each other in the incubator.

Antibody purification: Culture supernatant was processed by eluting antibody from protein affinity chromatography columns following affinity resin manufacturers protocol. Eluted antibody fractions were collected, pooled, and quantified by spectrophotometer and ELISA. Sandwich ELISA was performed with polyclonal goat anti-mouse IgG or IgM capture antibody and polyclonal anti-mouse IgG or IgM antibody labeled with peroxidase. Color was developed with ABTS. Antibody purity was assessed by SDS Elector phoresis and Coomassie blue staining. Purified antibody was subjected to further labeling reactions to generate Fluorescein and Phycoeyrthrin conjugates. Labeled antibody was subjected to internal quality testing and released.

Results: As shown in Table 1, a total of 33 individual murine hybridoma cultures were completed in the CELLine devices. Antibody isotypes included IgG1, IgG2a, IgG2b and Ig-M. A myeloma (AC 19 MOPC) producing IgG1 was also cultured. Cultures are presented in the sequence in which they were run. Changes in serum supplementation and medium were implemented by the laboratory as indicated. If cell growth was not established following inoculation, increased serum was provided to either the cell compartment or the nutrient medium as indicated.

The hybridoma clones were obtained from sources around the world and included clones obtained under license (majority) and clones generated by the manufacturer. The clones were randomly selected based on manufacturers production needs and represented a random sampling of isotypes and fusion partners. Many of the clones were newly received by the manufacturing laboratory and did not have extensive production records indicating expected production levels.

Clones AC 11, 12, 13, and 16 were difficult to establish initially but were successfully propagated after increasing inoculation densities and or providing increased serum concentrations in the nutrient medium. One clone AC 9 grew well in the devices but did not produce sufficient amounts of antibody. Sub-cloning (AC 17) of clone AC 9 was not satisfactory in restoring antibody production to acceptable levels. The clone was unable to produce suitable product amounts when cultured in static culture flasks. Several clones (AC 18, 31) were treated with an anti-mycoplasma agent (enrofloxacin, Baytril) to treat suspected mycoplasma contamination. These clones were difficult to establish initially but grew and produced antibody at satisfactory levels following treatment. Cells were treated outside of WHEATON® CELLine™ 1000 flasks.

The total cell compartment supernatant volumes collected from each culture is shown in Table 1 (Harvest Vol). AC 15 was run simultaneously in two units to increase production. The number of harvests are also shown, indicating the total number of harvests/ handling operations for each culture. Clone AC 2 was cultured in 3 WHEATON® CELLine™ 350 flasks. The harvest volume was determined by pooling the three flasks. Cell numbers are those counted from a single flask. The WHEATON® CELLine™ 350 has 1/3 the surface area as the WHEATON® CELLine™ 1000 and has 1/3 the capacity.

Maximum viable and maximum total cell numbers that were counted in supernatant from the cell compartment during harvest are also shown for cultures which were tracked by counting cells. The maximum cell counts reflect the highest numbers obtained during a harvest during the culture period and reflect the maximum cell capacities attained in the cell compartments during culture for the particular hybridoma. Representative growth curves from individual cultures are shown in Fig. 1.

Cells proliferated throughout the interval between harvests, as indicated by the accumulation of total cells. Maximum viable cell numbers ranged between 318 and 800 x $10^6$ cells per cell compartment for the different clones. The maximum number of total cells counted in a harvest from the cell compartment ranged between 648 and 3304 x $10^6$ cells for the different clones. The continued proliferation of cells after viable cell capacity was reached accounted for the large numbers of total cells recovered at harvest and contributed to overall antibody production.

Nutrient medium was exchanged during harvest and was usually depleted, as evidenced by a decline in the total viable cell numbers in the cell compartment at harvest. Nutrient medium contained 0.8% FBS in the initial experiments, supplemented with 0.1% Vitacyte. Supplementation with 0.1% Vitacyte was done to duplicate culture conditions used in prior production runs in batch culture. Cell compartment medium was initially supplemented at 10% FBS. Subsequent experiments were run with 0% FBS in the nutrient medium and 20% FBS in the cell compartment, and no additional supplementation. The manufacturer selected the protocols. The manufacturing concern reported no significant difference in antibody production. The final protocol conditions were left at 20% FBS in the cell compartment and 0% FBS in the nutrient medium. The manufacturer chose the 20% FBS to allow prolonged intervals between handling to compensate for water flux into the cell compartment. Water flux into the cell compartment was observed in all cultures resulting in increased cell compartment volumes compared to inoculation volumes at harvest. The volume increase was influenced by the duration between harvests and the present cell numbers.

A mean of 136 mg of purified antibody was recovered following affinity column purification of supernatant from the 30 individual cultures (Table 2).

The average culture duration was 36 days. The mean harvest volume was 144 ml of supernatant. The harvest supernatant's mean (purified) antibody concentration was nearly 1 mg/ml. This was determined by dividing the harvest supernatant volume by the amount of antibody recovered following purification of the supernatant. The range of (purified) antibody concentration was between 2.21 mg/ml and 0.36 mg/ml for the individual cultures. The longest culture was 54 days (AC 18) which included a lack of initial growth, subsequently established. Cell lines AC 29, AC 9 and 17 were excluded from the determination of the mean values for the following reasons: AC 9 and AC 17 produced a partial antibody molecule that did not pass manufacturing criteria, AC 17 was sub-cloned from AC 9 and was also incapable of producing satisfactory antibody. AC 29 was excluded due to poor growth. The manufacturer has reported that this cell does not appear to be producing in static culture and cannot be considered a productive clone.

Summary: The WHEATON® CELLine™ 1000 proved to be a suitable production vessel for manufacturing limited amounts of monoclonal antibody. It provided cost savings by reducing serum use, handling, and processing. A concentrated product was obtained in a small volume of culture supernatant when compared to traditional batch cultures. The applicability of the WHEATON® CELLine™ 1000 was established in over 30 different hybridoma cell lines, which comprised various immunoglobulin isotypes, and which were derived from various fusion partners.

The protocol was based on a continuous batch production method which reduced handling and was tailored by the manufacturing concern to its needs. The positive attributes of reduced overall costs, concentrated product, and most significantly reduced labor were confirmed in this study. The data provides a range of production results from the culture of a large number of distinct murine hybridoma lines. It should be pointed out that multiple units can be employed for the same cell lines if increased production is desired. Scale-up can be accomplished by operation of multiple flasks simultaneously. In conclusion, the WHEATON® CELLine™ 1000 flasks proved well suited for the small manufacturing laboratory and provided cost savings, reduced handling, and ease of use when compared to prior batch methods used.


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**TABLE 1: Cumulative Production Record WHEATON® CELLine™ 1000**

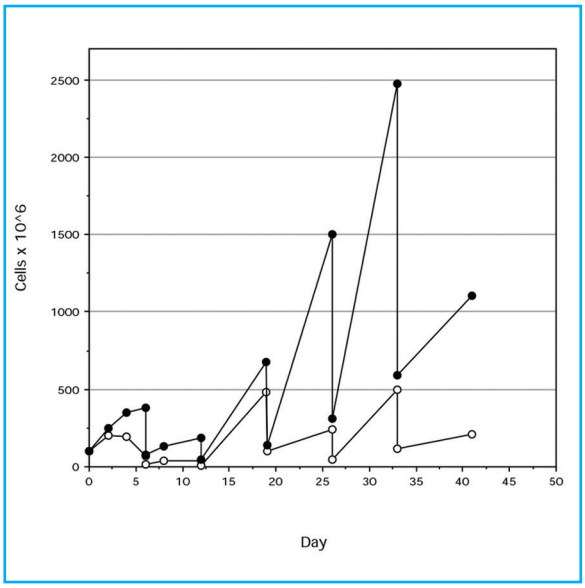
| Clone | Ig | Harvest Volume (ml) | mAB total (mg) | mAB conc. (mg/ml) | Duration (days) | Harvests | Nutrient Medium (%FBS) | Cell Compartment | 0.1% Vitacyte | Medium | Maximum Viable cells (x $10^6$) | Maximum Total cells (x $10^6$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AC.1 | G1 | 120 | 89 | 0.74 | 47 | 5 | 0.8 | 10 | Y | RPMI | 616 | 1664 |
| AC.2 | G1 | 65 | 101 | 1.55 | 25 | 3 | 0.8 | 10 | Y | RPMI | 220 | 576 |
| AC.3 | G1 | 128 | 121 | 0.94 | 42 | 5 | 0.8 | 10 | Y | RPMI | 561 | 1701 |
| AC.4 | G1 | 45 | 33 | 0.73 | 22 | 3 | 0.8 | 10 | Y | RPMI | 543 | 1940 |
| AC.5 | G1 | 110 | 122 | 1.10 | 37 | 4 | 0.8 | 10 | Y | RPMI | 570 | 1476 |
| AC.6 | G1 | 100 | 80 | 0.80 | 30 | 4 | 0.8 | 10 | Y | RPMI | N/A. | N/A. |
| AC.7 | G2a | 120 | 141 | 1.17 | 50 | 6 | 0.8 | 10 | Y | RPMI | 731 | 2080 |
| AC.8 | G2a | 130 | 158 | 1.22 | 42 | 6 | 0.8 | 10 | Y | RPMI | 660 | 3304 |
| AC.9 | G1 | 104 | 23 | 0.22 | 38 | 5 | 0.8 | 10 | Y | RPMI | 682 | 1957 |
| AC.10 | G1 | 110 | 88 | 0.80 | 36 | 5 | 0.8 | 10 | Y | RPMI | 504 | 1120 |
| AC.11 | G1 | 108 | 95 | 0.88 | 35 | 4 | 10-5 | 15-20 | Y | RPMI | 800 | 1540 |
| AC.12 | G1 | 140 | 109 | 0.78 | 48 | 5 | 5-0.8 | 10-20 | Y | RPMI/ DMEM | 557 | 1425 |
| AC.13 | G1 | 144 | 71 | 0.49 | 28 | 4 | 2-0.8 | 20 | N | RPMI | 312 | 1172 |
| AC.14 | G2a | 123 | 44 | 0.36 | 39 | 5 | 3 | 20 | Y | RPMI/ DMEM | 456 | 1707 |
| AC.15 | G1 | 190 | 165 | 0.87 | 39 | 6 | 0.8 | 20 | N | RPMI | 615 | 1660 |
| AC.16 | M | 160 | 124 | 0.78 | 32 | 4 | 2-0.8 | 20 | N | RPMI | 300 | 992 |
| AC.17 | G1 | n.d | n.d. | n.d. | 38 | 4 | 5 | 15 | N | DMEM | 682 | 3000 |
| AC.18 | G1 | 130 | 138 | 1.10 | 54 | 5 | 5 | 10-20 | Y | RPMI | 535 | 1435 |
| AC.19 | G1 | 110 | 243 | 2.20 | 32 | 4 | 0.8 | 20 | N | RPMI | 644 | 1092 |
| AC.20 | M | 135 | 95 | 0.70 | 30 | 4 | 0.8 | 20 | N | RPMI | 394 | 1160 |
| AC.21 | G1 | 180 | 120 | 0.70 | 29 | 5 | 0.8 | 20 | N | RPMI | 402 | 1608 |
| AC.22 | G2b | 190 | 105 | 0.60 | 38 | 6 | 0.8 | 20 | N | RPMI | 444 | 1110 |
| AC.23 | G2b | 180 | 123 | 0.70 | 29 | 5 | 0.8 | 20 | N | RPMI | 504 | 2102 |
| AC.24 | G1 | 150 | 179 | 1.20 | 31 | 5 | 0.8 | 20 | N | RPMI | 318 | 648 |
| AC.25 | G1 | 150 | 209 | 1.40 | 32 | 5 | 0 | 20 | N | RPMI | N/A | N/A |
| AC.26 | G1 | 170 | 163 | 1.00 | 38 | 6 | 0 | 20 | N | RPMI | N/A | N/A |
| AC.27 | G1 | 170 | 163 | 1.00 | 36 | 6 | 0 | 20 | N | RPMI | N/A | N/A |
| AC.28 | G1 | 200 | 152 | 0.80 | 42 | 6 | 0 | 20 | N | RPMI | N/A | N/A |
| AC.29 | G2a | 168 | 24 | 0.14 | 31 | 5 | 0 | 20 | N | RPMI | N/A | N/A |
| AC.30 | M | 185 | 169 | 0.91 | 37 | 6 | 0 | 20 | N | RPMI | N/A | N/A |
| AC.31 | G1 | 198 | 135 | 0.68 | 39 | 6 | 0 | 20 | N | RPMI | N/A | N/A |
| AC.32 | G2b | 144 | 197 | 1.40 | 27 | 5 | 0 | 20 | N | RPMI | N/A | N/A |
| AC.33 | G1 | 220 | 176 | 0.80 | 38 | 7 | 0 | 20 | N | RPMI | N/A | N/A |

N/A - Not Available

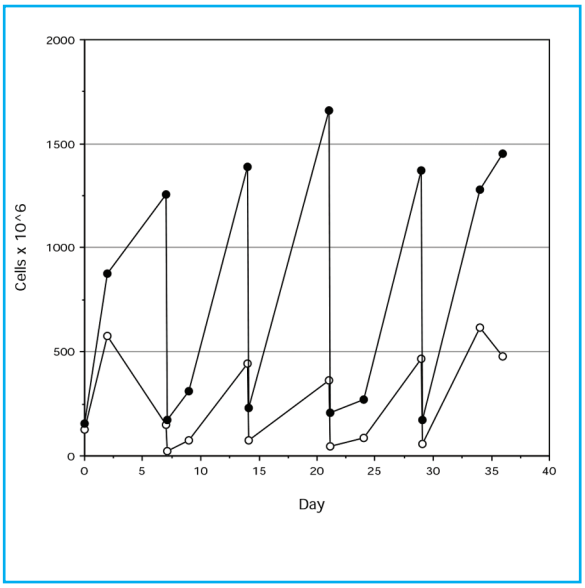
**TABLE 2**

| Mean (n=30) Harvest Volume (ml) | Mean (n=30) mAb Total (mg) | Mean (n=30) mAb Concentration (mg/ ml) | Mean (n=30) Duration (days) | Mean (n=30) Harvests |
|---|---|---|---|---|
| 144 ml (s=40) | 136.3 (s=47.5) | 0.94 (s=.26) | 36 (s=7.5) | 5 (s=1.0) |

**Figure 1:** Growth curves of representative cultures: The number of viable cells and total cells present in the cell compartment of the WHEATON® CELLine™ 1000 during culture are shown. Viable cells are plotted with open circles, total cells are plotted with filled circles. The cell compartment was sampled on days indicated (usually at harvest) and the cells counted. Cultures were split back as indicated and cell growth can be seen by increased numbers at next havrest. Cultures were split back by mixing cells with fresh cell compartment medium and reinoculating into the cell compartment.




**CELL LINE 22A5**

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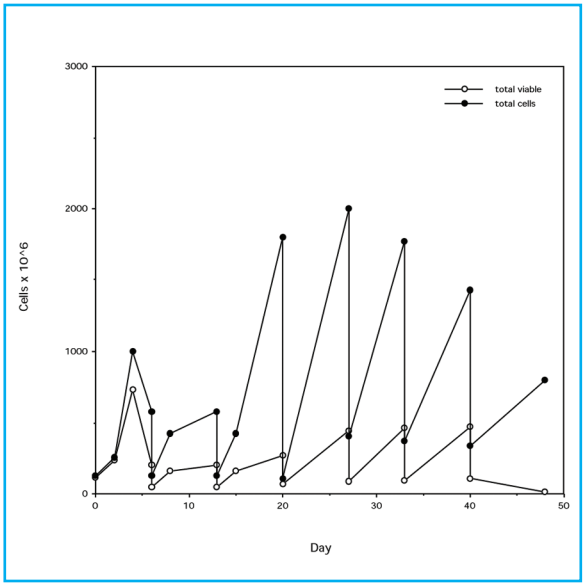
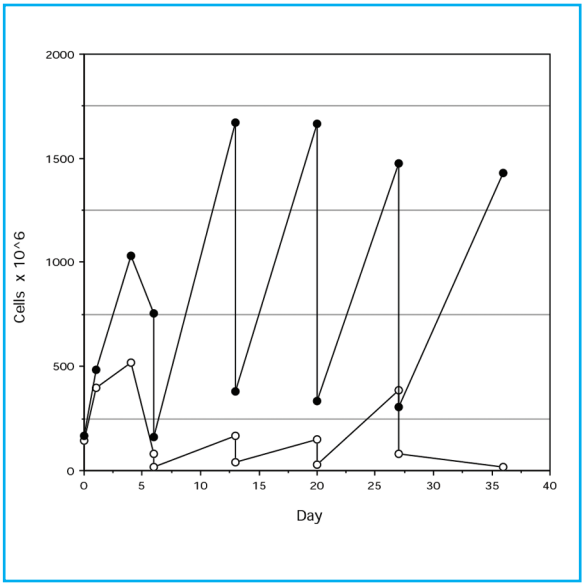



**CELL LINE HA5-6**




**CELL LINE P2W7R**

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# High-Density Suspension Culture for Recombinant Protein Production from CHO cells in WHEATON® CELLine™

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**Abstract:** Culture of CHO cells at high density as suspension cells in a protein-free medium is described. The production of a recombinant protein from the cells was compared during the early adaption phase in the presence of several different commercially available serum and protein-free mediums. Following adaption and medium evaluation, long-term continuous culture was maintained by a regular schedule of harvesting and feeding from multiple WHEATON® CELLine™ 1000 bioreactor flasks. Soluble recombinant protein at high concentration (greater than 40X) was obtained from multiple WHEATON® CELLine™ cultures compared to traditional monolayer cultures.

**Introduction:** We evaluated commercial culture medium to support CHO cell proliferation and protein production in high-density suspension culture. Screening and medium selection were made in small-scale CL 6- well units. After selecting a suspension medium, the cultures were scaled up into the larger WHEATON® CELLine™ 1000 units (1000 ml) for production.

CHO cells are routinely cultured as a monolayer but have also been cultured as suspension cells, and specialized media are available for this purpose (1-6). Other monolayer-forming cell types have also been shown to be adaptable to suspension culture (7-10). A CHO cell line secreting a recombinant fusion protein construct (rec protein) was evaluated. The CHO cells were previously engineered and selected in traditional monolayer culture for protein production. The protein construct is proprietary (B cell activation molecule which binds to cell surface ligand) and is functional when tested in cell-based assays and flow cytometry. A sandwich ELISA was developed to measure this protein in culture supernatant and during purification. Affinity purified rec protein was used to generate the standard curve in the ELISA.

The following mediums were evaluated: CHO -S-SFM II (Life Technologies, Rockville, MD), CD CHO (Life Technologies, Rockville, MD), SFX-CHO (Hyclone, Logan, UT), and HyQ PF CHO (Hyclone, Logan, UT). The control monolayer culture medium was RPMI-1640 supplemented with EXCYTE VLE (1:300) (Bayer, Kankakee, IL), ITS (1 ml/liter) (Collaborative Biomedical Products, Bedford, MA) and 0.5% FBS (Hyclone, Logan, UT). All medium was further supplemented with antibiotics and L-Glutamine (4 mM).

**Results:** To adapt cells to the various suspension medium, established monolayer cultures (6.0 x $10^6$ cells) in T-25 flasks (10 ml) in the control monolayer culture medium was weaned into the various medium formulations by stepwise dilution into the suspension medium. Starting at a 50% suspension medium concentration, cells were switched to a 75% suspension medium after three days. Following an additional two days of culture, cells were harvested by trypsin from the flasks and reseeded in 100% suspension medium into T-25 flasks and into the CL 6-well cell compartments. At harvest in 75% suspension medium, the following cell numbers were harvested from the flasks and the corresponding concentration of recombinant protein was assayed by ELISA present in the culture supernatant.

| Medium | Cells/Flask | Rec Protein (µg/ml) |
|---|---|---|
| CHO-S-SFM II | 10.3 | 0.920 |
| CD CHO | 9.1 | 0.657 |
| SFX-CHO | 6.5 | 0.574 |
| HyQ PF CHO | 4.8 | 0.822 |
| Control | 6.0 | 0.969 |

Following transfer to 100% suspension medium and new culture flasks (1.0 x $10^6$/flask), the cells no longer demonstrated monolayer growth on the surfaces of the T-25 culture flasks. Although a few cells could be seen attached to the flask surfaces, most cells grew in loose aggregates of viable cells. The control medium cells were in a traditional confluent monolayer. In the control medium, cells still formed a confluent monolayer. All suspension medium tested resulted in suspension cultures and the absence of monolayer formation.

Cells harvested from the various (75% suspension medium) flasks were inoculated (2.0 x $10^6$/750µl) into the cell compartment of CL 6-well units in duplicate. The nutrient medium was matched to the cell compartment medium for each suspension medium. Following six days of culture in a 100% suspension medium, the supernatant from the reseeded T-25 flasks and the cell compartments of the CL 6-well were assayed for recombinant protein concentration.

**Rec Protein Concentration (µg/ml)**

| Medium | T-25 | CL 6-well |
|---|---|---|
| CHO-S-SFM II | 0.43 | 9.32 |
| CD CHO | 0.38 | 2.91 |
| SFX-CHO | 0.30 | 3.11 |
| HyQ PF CHO | 0.43 | >10 |
| Control | 1.67 | 3.19 |

On day 9 of culture, the cell compartment medium was removed and replaced with fresh medium leaving the cells in the cell compartment. Cells were left in the cell compartment to maximize cell numbers, and the nutrient medium was replaced. On day 13, the cell compartments were harvested by mixing and removing 400 µl for cell counting and ELISA assay of supernatant. The nutrient medium was replaced.

| Medium | Viable Cells x $10^6$/ml | Rec Protein (µg/ml) |
|---|---|---|
| CHO-S-SFM II | 4.49 | 17.33 |
| CD CHO | 0.14 | 1.01 |
| SFX-CHO | 1.08 | 10.58 |
| HyQ PF CHO | 10.44 | 22.65 |
| Control | 1.58 | 7.10 |

On day 15 of culture, the cell compartment contents were removed from the CL 6-well and again analyzed for cell numbers and protein concentrations. At this time, two of the suspension medium were supporting more cell growth as determined by cell counts and visible appearance of the cell mass in the cell compartment and the size of cell pellet following centrifugation of the cell compartment suspension (CHO-S-SFM II and HyQ PF CHO).

| Medium | Viable Cells x $10^6$/ml | Rec Protein (µg/ml) |
|---|---|---|
| CHO-S-SFM II | 4.39 | >50 |
| CD CHO | 0.14 | 1.53 |
| SFX-CHO | 2.50 | 8.05 |
| HyQ PF CHO | 6.82 | 17.06 |
| Control | 1.21 | 4.22 |

Control cultures (monolayer medium) in the CL 6-well contained viable cells; however, no increase in cell numbers could be seen. Cells did not form monolayers in the control medium in the cell compartment or any of the other mediums. The contents of the cell compartment from the CHO-S-SFM II and HyQ PF CHO cultures were reseeded (1.1 and 1.51 x $10^6$/ml, respectively) into CL 6-well units in replicates of 6 cell compartments to further assess cell production with the two different medium types.

Following four days of culture, a 50 µl sample was taken from each replicate well and cell counts and protein concentration were determined. At this time, considerable cell mass was evident in both medium types. Cells were present as aggregates within the cell compartment. The aggregates contained viable cells as determined by trypan blue staining. Although most cell aggregates could be dissociated by pipetting with a P200 pipette tip, significant numbers of cells were still present as multicellular aggregates containing predominantly viable cells. This made an accurate assessment of cell numbers difficult due to the presence of aggregates remaining. The mean and standard error for the replicate wells is shown below.

| Medium | Viable Cells x $10^6$/ml | Rec Protein (µg/ml) |
|---|---|---|
| CHO-S-SFM II | 5.17 ± 1.09 | 32.17 ± 2.47 |
| HyQ PF CHO | 3.46 ±1.16 | 56.29 ± 11.17 |

After an additional three days in culture, the cell compartment contents were removed entirely, and cell counts and protein concentrations were determined. The entire cell compartment volume was removed by pipetting to suspend cells, followed by an additional rinse of 500 µl medium to recover as many cells as possible from the cell compartment. Cell counts were determined for each cell compartment, and the individual supernatants were pooled for ELISA determination of protein levels.

| Medium | Viable Cells x $10^6$/ml | Rec Protein (µg/ml) |
|---|---|---|
| CHO-S-SFM II | 11.95 ± 1.08 | 44.38 |
| HyQ PF CHO | 12.47 ± 0.67 | 80.86 |

At this time, cell growth in both mediums was marked in the cell compartments of the CL 6-well. Although greater concentrations of rec protein were obtained from the HyQ PF CHO cultures, both mediums appeared to support cell growth and production.

Assessment of a nutrient medium substitute with an alternative less expensive medium was done in cultures continued in the presence of alternative nutrient medium choices as well as the control medium (cell compartment medium). The alternative nutrient medium choices were based on the basal medium from which the suspension medium was derived. For the CHO-S-SFM II medium, an S- MEM (Life Technologies, Rockville, MD) was selected, and for the HyQ PF CHO medium, RPMI-1640 (Hyclone, Logan, UT). The nutrient medium was used without any supplementation or in the presence of 2% FBS. The control monolayer medium was also evaluated as a nutrient medium as it maintained cell viability, although in the absence of cell proliferation.

Cells were reinoculated into the cell compartments of the CL 6-well at 2.4 x $10^6$ cells/ml in duplicate with the various nutrient medium choices and the control medium. By day 4, visual inspection revealed differences between the various nutrient medium cultures. Cell mass was most significant in the control medium cultures (cell compartment = nutrient compartment). The next best condition appeared to be the control monolayer medium. The cultures were harvested after seven days, and the cell counts and protein concentrations were determined for the various culture conditions in duplicate.

**Cell Compartment-HyQ PF CHO**

| Medium | Viable Cells x $10^6$/ml | Rec Protein (µg/ml) |
|---|---|---|
| RPMI-1640 | 4.08 | 19.31 |
| RPMI-1640 2% FBS | 7.42 | 22.32 |
| Monolayer Medium | 9.94 | 26.89 |
| S-MEM | 1.50 | 20.92 |
| S-MEM 2% FBS | 0.44 | 26.24 |
| HyQ PF CHO | 19.14 | 46.21 |

**Cell Compartment-CHO-S-SFM II**

| Medium | Viable Cells x $10^6$/ml | Rec Protein (µg/ml) |
|---|---|---|
| RPMI-1640 | 1.96 | 15.41 |
| RPMI-1640 2% FBS | 2.32 | 24.13 |
| Monolayer Medium | 3.10 | 15.13 |
| S-MEM | 0.76 | 11.42 |
| S-MEM 2% FBS | 0.82 | 17.13 |
| CHO-S-SFM II | 8.18 | 19.65 |

At this time, the substitution of an alternative nutrient medium to replace the more expensive cell compartment medium was not shown to be as effective as using the same medium in both the cell compartment and the nutrient medium reservoir. Although continued evaluation of small molecular weight supplements to a basal medium appears promising in that the presence of ITS and Excyte present in the monolayer medium appeared to be beneficial when compared to RPMI-1640 with or without 2% FBS. The poor performance of the S-MEM as an alternative nutrient medium is not understood at this time. HyQ PF CHO medium was chosen as the medium for production due to its protein-free composition and increased production levels compared to CHO-S-SFM II.

In parallel to the evaluation of alternative nutrient medium, large cultures were established in WHEATON® CELLine™ 1000 units for production purposes. The cultures were maintained on a twice-weekly handling protocol in which the cell compartment was harvested and the nutrient reservoir medium exchanged. At harvest, the nutrient medium was poured from the bioreactors, and the cell compartment contents were mixed by gentle sliding of the bioreactors in a swirling motion on the work surface. This allowed near-complete removal of the cell compartment contents by pipetting. It was difficult to remove the cell contents entirely by pipetting with visible cell mass remaining in the cell compartment if mixing was not done. After removing the cell compartment contents, cells were centrifuged, and the supernatant was removed and saved for harvest. Roughly 1/2 of the cell pellet (2.5 ml) was resuspended in fresh medium (20 ml) and returned to the bioreactors. The nutrient medium was replaced at the same time. Cell pellets approaching 5 ml were routinely harvested from the WHEATON® CELLine™ 1000 bioreactors.

Representative harvests from a four-day culture period from the cell compartment of 4 individual bioreactors are shown below. The cultures were maintained for more than 30 days, and production levels were consistent throughout the duration.

| WHEATON® CELLine™ 1000 | Viable Cells x $10^6$/ml | Harvest Volume | Rec Protein (µg/ml) |
|---|---|---|---|
| No. 1 | 5.15 | 15 | 39.31 |
| No. 2 | 7.85 | 15 | 44.65 |
| No. 3 | 10.75 | 18 | 67.93 |
| No. 4 | 13.10 | 20 | 57.05 |

**Conclusion:** CHO cells grew and were produced when cultured as suspension cells. Although the original clone was selected from adherent monolayer forming cultures, the clone still produced at acceptable levels when cultured in suspension medium and in the absence of monolayer formation. After a brief adaption period, the clone was easily maintained in the WHEATON® CELLine™ flasks. This resulted in a much easier production method than standard monolayer propagation involving trypsinization and continued passaging on tissue culture plastic. Additionally, the production of a concentrated supernatant reduced downstream processing issues and allowed for the use of supernatant directly for specific applications.

Of the commercial medium tested, both a serum-free and a protein-free medium appeared suitable for suspension CHO cultivation. The protein-free medium was pursued due to its protein-free characteristics and slightly better production values. There is no explanation for why the other medium types tested were not as successful. This may be due to differences in adaption times, cell line-specific sensitivities, or other unknown reasons.

After six days of culture, the CHO line at confluence produced a 1.67 µg/ml concentration in a 10 ml culture volume in a standard T-25 flask. In contrast, much higher concentrations were achieved in WHEATON® CELLine™ cultures. After four days of culture, a mean of 52.23±6.42 µg/ml was obtained in 4 WHEATON® CELLine™ 1000 units. This represents a 32 fold increase in product concentration in a smaller supernatant volume. A single WHEATON® CELLine™ 1000 flask in four days produced equivalent to a six-day (544 ml) monolayer culture. This was achieved with minimal handling in a single device and significantly reduced supernatant volume for downstream processing.

Alternative medium choices for the nutrient medium may be possible with further evaluation of small molecular weight components' role in the nutrient medium. Although the addition of FBS to the RPMI-1640 nutrient medium appeared somewhat beneficial compared to the absence of any FBS supplement, the presence of ITS and Excyte VLE seemed to have a more beneficial effect in the presence of only 0.5% FBS. Further evaluation of small molecular weight components on the role of cell proliferation and survival in the WHEATON® CELLine™ is warranted and may provide significant cost savings by reducing the consumption of the more expensive complete medium. The dilution of the entire medium with a basal medium may prove to be an acceptable alternative, as it appears likely that dilution of a component across the semi-permeable membrane reduces the effectiveness of the complete commercial medium formulations when used only in the cell compartment. If a small molecular weight component that is essential to the culture's success is present in the commercial formulas. In that case, it may be present at excess concentrations and be capable of being diluted with less expensive basal medium types and used in the nutrient reservoir. Alternatively, supplementation of a basal medium with specific components may duplicate the effects achieved with the complete medium for use as a nutrient medium.


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