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# The gel microdrop secretion assay: Identification of a low productivity subpopulation arising during the production of human antibody in CHO cells

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**Abstract**

The long-term stability of high-level expression is the most important factor to consider when choosing cell lines for the expression of recombinant proteins. Declining volumetric yields in large-scale fermentation can be caused by changes affecting the cell population as a whole such as loss in viability, depletion of nutrients or accumulation of metabolites affecting cell growth. Alternatively, genetic instability may lead to the outgrowth of a less productive, metabolically favored sub-population. Currently a variety of parameters are measured to monitor the condition of cells in fermenters including glucose uptake, lactate accumulation and oxygen consumption; in addition, periodic viable cell counts allow the determination of the growth rate and viability of the population. All of these methods measure the condition of the cell population as a whole and changes must involve a significantly large proportion of the total culture in order to be detectable. Here we report on a method that allows the evaluation of the productivity of individual cells. Using the gel microdrop secretion assay, we detected the appearance of a sub-population of cells with lower productivity. Subsequent analysis of the culture confirmed the existence of lower productivity cells with a lower vector copy number. Therefore, the single cell secretion assay proved to be a rapid method to detect and isolate a low productivity variant of the producer cell line.

*Abbreviations:* DHFR, dihydrofolate reductase; EIA, enzyme immunoassay; FITC, fluorescein isothiocyanate; GMDs, gel microdrops; MTX, methotrexate; rpm, revolutions per minute

## Introduction

An important aspect of any process involved in the production of biologicals from transfected cell lines is the stability of recombinant gene expression. The ideal production process maintains a high expression level through many cell generations without the selection pressure of added antibiotics or other drugs. In practice, some candidate cell lines lose their high levels of expression even when maintained under selection pressure (Kaufman et al., 1985). During process development, careful monitoring for signs of expression instability is performed and evidence of instability may require changes in the fermentation conditions or a change to an alternate cell line. This monitoring is typically done by periodic determinations of specific productivity, for example, micrograms of product produced per million cells per day. This method, while detecting a loss of expression, is unable to distinguish between decreased expression caused by physiologically unfavorable fermenter conditions affecting the total cell population or the outgrowth of a less productive sub-population. Often additional studies are necessary to distinguish between these possibilities.

By careful monitoring of cultures over several weeks, production of a Protein C-Factor X fusion protein by a transfected CHO cell line has been correlated with oxygen consumption and lactate accumu-

lation (Sugiura and Kakuzaki, 1998). Variability in the level of production of recombinant glucoamylase was prevented by maintaining a low pH in the culture. Subsequent analysis demonstrated that the lower pH prevented the appearance of variants with a lower gene copy number and production rate (Swift et al., 1998; Withers et al., 1998).

Several groups have employed fluorescent substrates to make the task of assaying cellular productivity more rapid and less laborious (Al-Rubeai and Emery, 1993). Hybridoma cells have been stained with fluorescent probes in order to detect their antibody products on the cell surface or inside the cell (McKinney et al., 1991; Chuck and Palsson, 1992). Rao and co-workers (Albano et al., 1998) have published on the use of green fluorescent protein as a reporter of recombinant gene expression. They demonstrated that the fluorescence-based assay was as accurate as the traditional chloramphenicol acyltransferase test and easier to perform. Gandor et al. (1995) used fluorescent methotrexate together with flow cytometry to determine the level of dihydrofolate reductase in transfected CHO cells. They were able to correlate the dhfr level with the production of recombinant protein in cell lines selected for resistance to methotrexate.

In this article we report on the use of the antibody secretion assay developed by Weaver and co-workers (Powell and Weaver, 1990). The assay was used as a tool to survey CHO cells secreting recombinant human antibody in a fermenter. The study investigates the possibility that combining the ability to directly test the secretion of recombinant product by a single cell with the cell sorting capability of the flow cytometer may facilitate the analysis of cell population changes. An evaluation method that allows the researcher to monitor individual cells would enhance the process development effort by focusing the future process optimization experiments.

## Materials and methods

### *Cells and expression vectors*

The cell line described here is derived from the Chinese hamster ovary (CHO) cell line DUX B11, a mutant of CHOK1 cells deficient in dihydrofolate reductase (DHFR) (Urlaub and Chasin, 1980). 2TE4∗peaBJ, a vector which directs the expression of the heavy and light chains of a recombinant human antibody 2TE4, was used to transfect CHO cells. The vector carries the murine DHFR gene driven by the SV40 early promoter, which functions as a selection and amplification marker (Kaufman et al., 1985). G5/C and R6/N, CHO lines transfected with 2TE4 expression vector, were amplified via methotrexate (MTX) selection to increase the number of copies of the vector and therefore the level of antibody secretion. Amplification was performed by passing cultures through steadily increasing levels of MTX, starting at 20 nM and progressing to 500 nM. Cells were passed through amplification using EX-CELL PFCHO growth medium (JRH Biosciences, Lenexa, KS) supplemented with 5% dialyzed fetal bovine serum (FBS), insulin (4 mg l, Intergen Inc., Purchase, NY), and bovine transferrin (2 mg l, Intergen Inc). At each increased level of drug, cultures were allowed time to adapt and recover before the next increase in MTX concentration. The lines were subcloned by limiting dilution prior to final selection for high antibody expression as determined by ELISA and then adapted to growth in suspension conditions. (PFCHO medium without FBS). The best lines were frozen in aliquots for storage as master cell banks. A working cell bank was prepared from each master bank and all fermenter cultures were derived from these working banks.

### *Preparation of gel microdrops and antibody secretion assay*

The cells' level of antibody secretion was determined using the hybridoma secretion assay protocol supplied by One Cell Systems Inc. and modified as follows. CHO cells were incorporated into gel microdrops (GMDs) using a CellSys 101® microdrop maker (One Cell Systems, Inc., Cambridge, MA, USA). The instrument consists of a set of rotating blades that form an emulsion from the oil and aquaeous solution of buffer and melted agarose added to the glass container. As the emulsion was cooled in an ice water bath, the agarose solidified to form microdrops approximately seventy microns in diameter. Two million harvested cells were encapsulated by resuspending in 100 $\mu$l warm PBS, 100 $\mu$l pluronic acid solution (Gibco/Life Technologies, Gaithersburg, MD), and 350 $\mu$l of molten CelBioGel, biotin conjugated agarose (One Cell Systems), warmed to 37 °C. The resulting GMDs were isolated from the emulsion solution by centrifugation for 10 min at 2200 rpm, washed twice with cold PBS, and incubated with streptavidin (Gibco) at 90 $\mu$g/ml. GMDs were then washed again, and incubated with biotin-conjugated goat anti-human IgG

(Organon Technika, Durham, NC) at 35 $\mu$g/ml, 4 °C for 15 min. After washing, the GMD-encapsulated cells were returned to growth medium in a 37 °C incubator with a 5% $CO_2$ atmosphere for a period of time previously determined to be sufficient to allow an accumulation of secreted antibody within the drop. For this study, a 30 min incubation was performed. At the end of the incubation, GMDs were incubated with FITC-conjugated goat IgG fraction to human IgG FC (Organon Technika) at 3.75 $\mu$g/ml, 4 °C for 15 min. After washing, the GMDs were analyzed using a FACScan flow cytometer (Becton-Dickenson, San Jose, CA) equipped with a 15 milliwatt 488 nM argon ion laser and a 180 micron nozzle. Forward scatter and side scatter gates were chosen such that only GMDs containing cells were analyzed.

*Fermentation conditions and sampling strategies*

2TE4 was produced via a harvest/rebatch strategy. A seed train was initiated from a vial of the working cell bank. The culture was grown in PFCHO medium (JRH Biosciences) supplemented with insulin (4 mg/l, Intergen Inc., Purchase, NY), bovine transferrin (2 mg/l, Intergen Inc.), and Primatone RL (1.5 g/l, Quest International, The Netherlands). The seed train was used to inoculate a 75l bioreactor (MBR Bioreactor AG, Switzerland). The 75l culture was used as the inoculum for a 1000l fermenter with an 850l working volume. The culture was maintained at a temperature of 37 °C, agitation of 55 rpm, 40% dissolved oxygen and was sparged at 8 l $min^{-1}$. Under these conditions the number of CHO cells doubles every twenty hours. Antibody production used a repeated batch and refeed process. Approximately 85% of the fermentation volume was harvested once every three days. The fermenter was replenished with fresh medium and incubated another three days before another harvest was made. This harvest/rebatch process was repeated until eight harvests, designated A through H were recovered. The production process was run under two different protocols. For the AB102 fermentation, the G5/C cells were cultured without MTX from the cell bank vial through culture in the 1000l fermenter. For the AB205 run, MTX at a final concentration of 500 nM was included in the cell culture medium until the inoculation of the 75l fermenter; however no MTX was added to the cell culture medium used in 75l fermenter or in the 1000l fermenter used for production of 2TE4. Cells harvested from the fermenter were washed twice with phosphate buffered saline and used for DNA extractions or secretion assays as described above. R6/N cells were cultured in fermenters using the latter protocol that includes MTX.

*Analysis of antibody production by cells recovered from the encapsulation assay*

Sub-populations of cells displaying either high or low fluorescence in the antibody secretion assay were separated using a FACStar PLUS cell sorter (Becton Dickinson, San Jose, CA, USA) equipped with a 100 micron nozzle and a Coherent 90–5 200 milliwatt Argon laser tuned to 488 nM. Cell lines established via the outgrowth of cells from individual GMDs were cultured in spinner flasks using the same medium used in the fermenters. Samples were taken daily and assayed for the number of viable cells, and for the concentration of 2TE4 antibody using an EIA specific for human IgG.

The EIA for human IgG was performed as follows. Ninety-six well flat bottom ELISA plates (Corning Inc., Corning, NY, USA) were coated for 1 hr at 25 °C with a solution consisting of five micrograms of goat antibody raised against human IgG (Southern Biotechnology Associates, Birmingham, AL, USA) per milliliter of PBS. Coated plates were blocked for 30 min at 25 °C with a solution of 2% skim milk powder in PBS. Samples for assay were added to the wells and incubated at room temperature for one hour. After incubation the plates were washed with PBS containing 0.05% Tween 20. Goat antibody conjugated with horseradish peroxidase (Southern Biotechnology Associates), diluted to 0.25 $\mu$g/ml PBS, was added to each well and incubated at room temperature for one hour. After washing the plates, signal was developed using K-Blue Substrate (ELISA Technologies, Lexington, KY) and read at 650 nm on a microplate reader. Antibody concentration was determined from a standard curve using known concentrations of purified 2TE4 antibody.

Productivity, expressed as picograms of human antibody per cell per day, was determined by linear regression of the mass of antibody accumulating in culture medium with the integral of viable cell density over the same period of time. The integral of viable cell density was determined by integrating the area under the viable cell density curve over a certain time period.

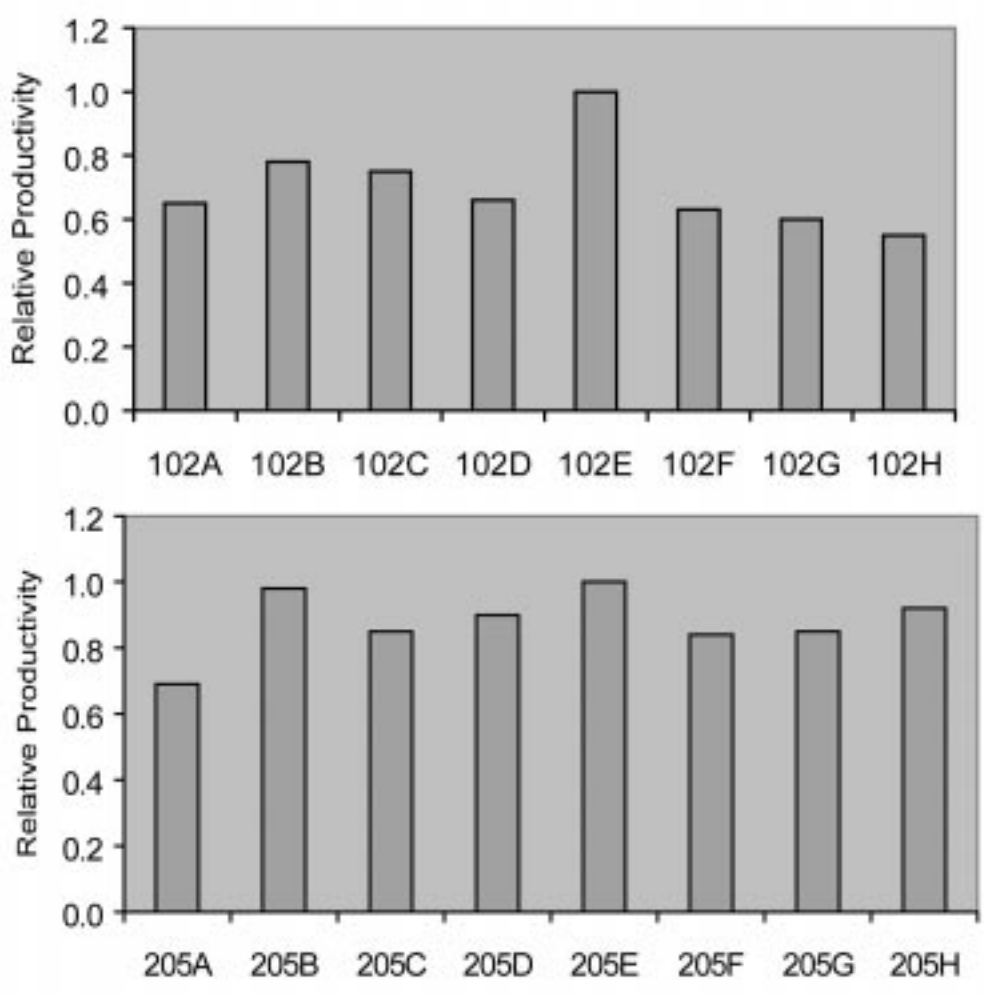


*Figure 1.* Productivity of CHO cells expressing human antibody during fermentations AB102 and AB205. Secreted antibody was harvested from 1000L fermenters using a harvest/rebatch method. After three days of incubation, 85% of the culture is removed for harvest and replaced with fresh growth medium. Productivity, expressed as picograms of antibody produced per cell per day, was calculated as described in the Section Materials and Methods. Relative productivity is expressed as a fraction of the maximum observed value, which is set equal to 1.

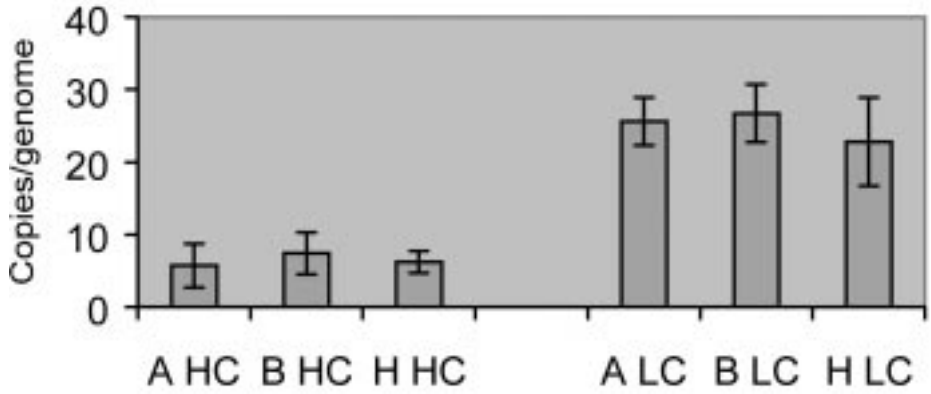


*Figure 2.* Heavy chain (HC) and light chain (LC) copy numbers for fermentation AB102. Gene copy numbers are for the cells from the fermenter harvests A, B, and H. Values are the average of three determinations. Error bars represent ± one standard deviation.

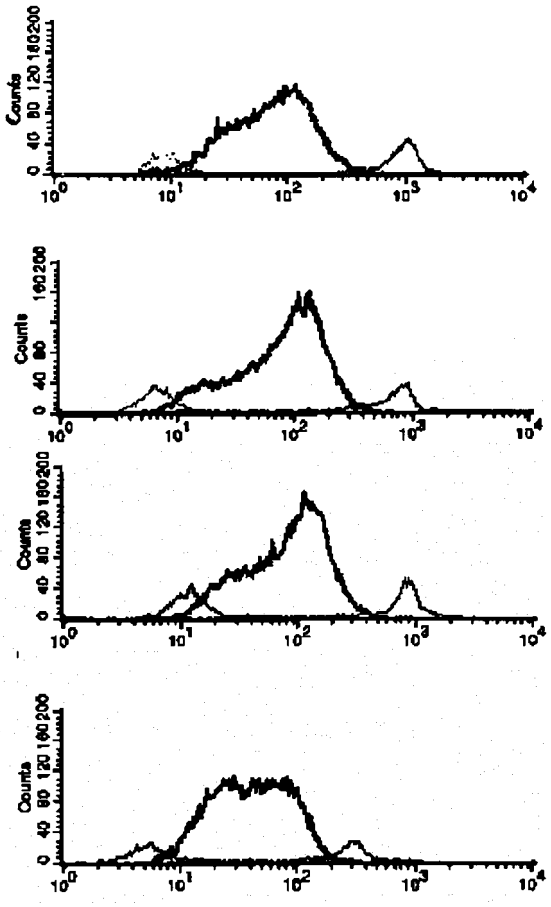


*Figure 3.* FACS histograms of microdrop secretion assays. Cells were taken from (top to bottom panels) the fermenter at the time of inoculation, at the first harvest; at the fourth harvest, and at the eighth harvest. Smaller flanking peaks represent the positive and negative control samples that were prepared in parallel with the secretion assay. These samples show the background fluorescence and the maximal signal attainable from the microdrops. At the time of the eighth harvest the cell line had been in culture a total of 6 weeks.

*Copy number determination*

Genomic DNA was extracted from the recovered cells (Ausubel et al., 1990). Ten micrograms of DNA isolated from each culture were digested with restriction endonucleases. Digested DNAs were subjected to agarose gel electrophoresis. Each gel was loaded with a series of standards containing known amounts of 2TE4 heavy chain and light chain DNA. The standards were prepared as follows. The 2TE4 expression vector was digested with *Eco*RI and *Not*I to release fragments carrying the heavy chain or light chain coding regions along with their upstream adenovirus promoters. The digested plasmid was diluted to concentrations that allowed the loading of 2TE4 DNA at levels equivalent to standard copy numbers. These standards were DNAs in amounts equivalent to 100, 20, 8, 4, or 2 copies of these genes per genome.

For each sample five micrograms of genomic DNA were digested with EcoRI. Duplicate 0.8% agarose/TAE gels were run for each set of samples. Half of each sample was run per gel. The resulting gels were blotted onto nylon membranes (HyBond N+, Amersham, Arlington Heights, IL, USA) and hybridized to probes radio-labeled with $^{32}$P as described (Ausubel et al., 1990). Duplicate blots were hybridized with probes that consisted of either the 2TE4 heavy or light chain DNA encoding the full-length coding regions. These DNA fragments were isolated from 2TE4 expression vectors by restriction endonuclease digestion with *Srf*I and *Not*I. DNA fragments were isolated and purified by gel electrophoresis and subsequent extraction. Isolated DNA fragments were radioactively labeled with $^{32}$P using the Rediprime labeling system (Amersham Pharmacia Biotech. Inc. Piscataway, NJ, USA). This system employs a random primer technology and nick translation reaction to produce probes with high specific activity. Blots were exposed to radioactive probes in hybridization solution (Ausubel et al., 1990) at 65 °C. Blots were washed under conditions of high stringency (0.1X SSC, 0.1% SDS, 65 °C) and exposed to Kodak X-OMAT photographic film (Eastman Kodak Co. Rochester, NY, USA) or to BioRad Molecular Imager screens (Bio-Rad, Hercules, CA, USA). In order to correct for variations in the loading of sample wells, blots were stripped of residual probe and probed with human S14 gene probes radiolabeled with $^{32}$P (Rhoads et al., 1986). The S14 sequence has a high degree of homology with its homologue in other mammalian species. Each sample's signal with the S14 probe was used to normalize the signal seen with the 2TE4 probes. Standard curves and copy number values were assigned using the Molecular Imager's Multi Analyst software.

## Results

Figure 1 shows the productivity of the cell line during each of the eight harvests. Data are presented for two different production runs. AB102 was run without any MTX addition to the medium at any time. AB205 was a production run performed under identical conditions except that the seed train growth medium contained

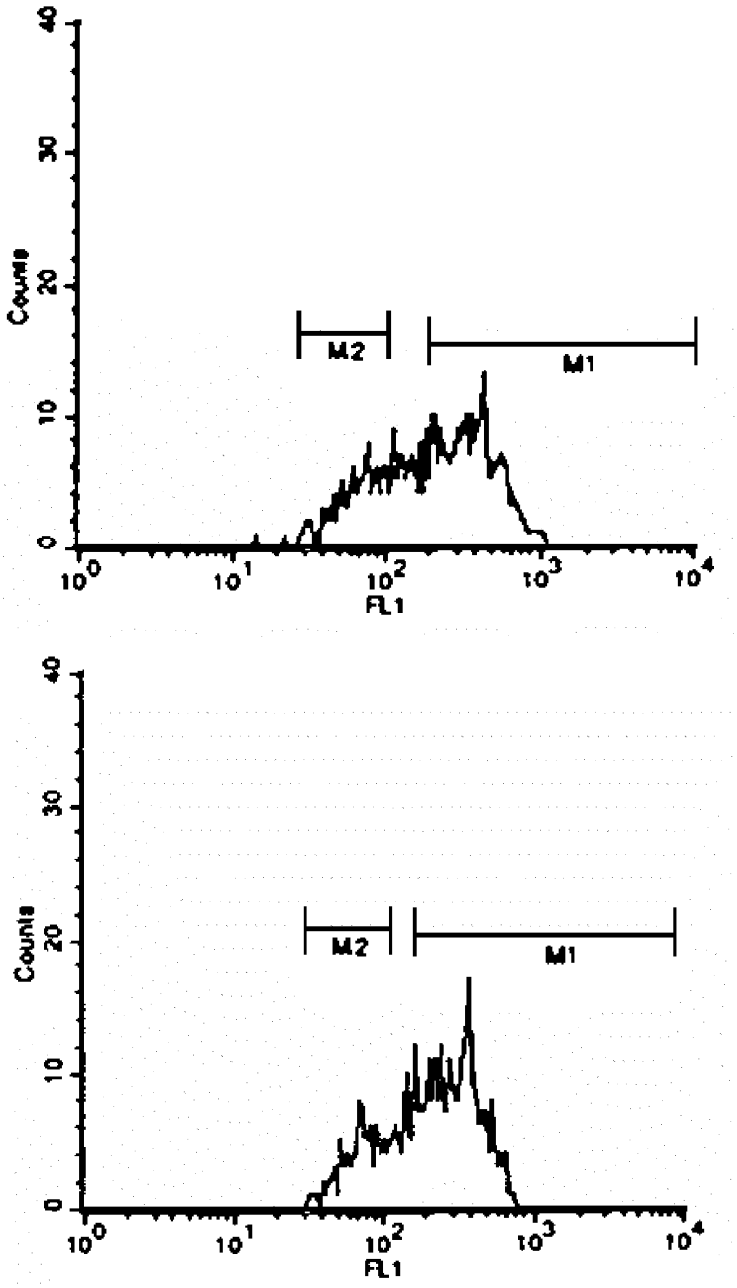


*Figure 4.* Antibody secretion profiles of cells from the end of fermenter runs AB102 and AB205. Cells appearing in regions M2 and M1 were sorted into separate populations using the FACStar cell sorter, then cultured to determine each population's productivity.

MTX. Values are expressed as milligrams of antibody produced per million cells during each 72 h incubation. Upon calculating the productivity of the cells harvested from the fermenter, it appeared that the cells' ability to produce 2TE4 antibody decreased during the later harvests. The overall yield of product increased when MTX was added to the seed train medium. However, the level of production continued to vary.

Cells recovered from the first, second, and eighth harvests were tested to determine their 2TE4 heavy chain and light chain copy number. Three determinations of copy number were performed for each sample. Southern blots probed for the recombinant human an-

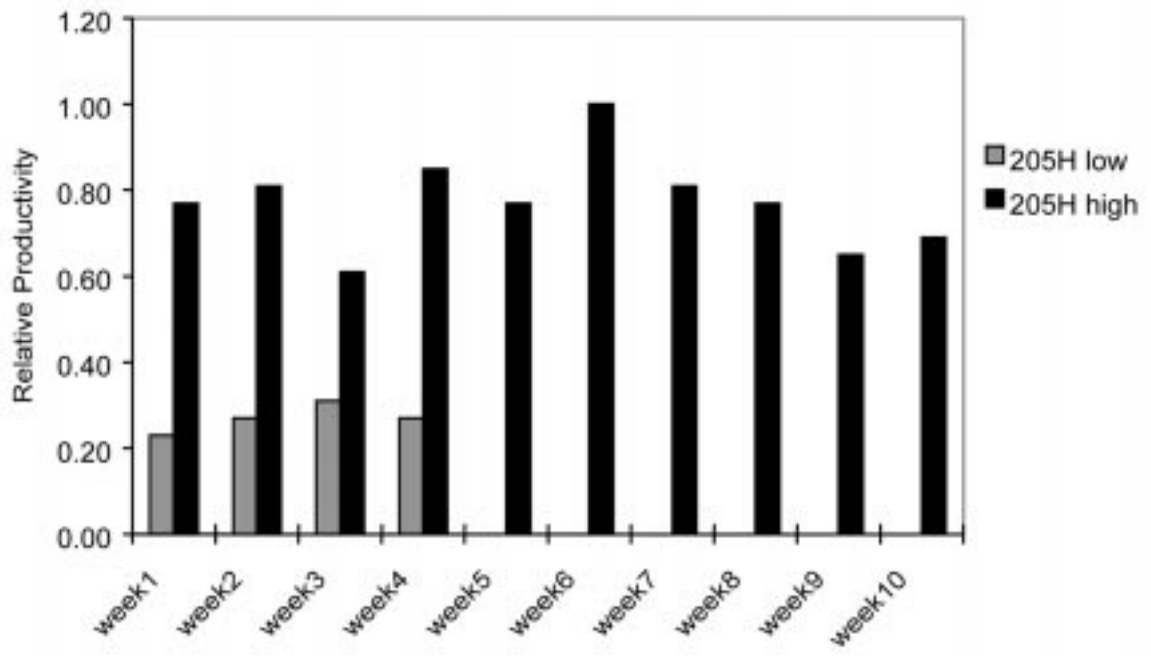


*Figure 5.* Long term productivity of AB205H high and low expression subpopulations. Cell lines were cultured in 50 ml volumes in spinner flasks and sampled daily to determine specific productivity. All cultures were transferred to fresh medium twice a week. The high expression line was maintained in culture for ten weeks. Productivity is expressed as a fraction of the maximum observed value, which was set equal to 1. Each productivity value is the average of the daily values over a 4 day period of growth.

tibody heavy and light chains detected no significant difference in vector copy number among the different harvest populations (Figure 2). Because the copy number experiments were not able to identify a loss of vector copies, cells recovered from a subsequent production run were tested using the antibody secretion assay. The encapsulation method produces single CHO cells imbedded in an agarose matrix to which is bound a capture complex specific for human IgG. This assay system allows the testing of large numbers of individual cells for their capacity to secrete antibody. Thus the histograms produced by this assay display the number of cells in the sample population that secrete antibody at any one of a range of different levels. Results of the monitoring of successive harvests are shown in Figure 3. All of the samples tested showed a major peak of high fluorescence, indicative of a high level of secreted antibody, and a secondary peak of lower fluorescence. This secondary peak represented a larger proportion of the total population in the final harvest sample. Therefore, the GMD secretion assay suggested the appearance of a less productive population of G5/C cells. To confirm this hypothesis, we set out to isolate the subpopulations that produce different levels of fluorescence in the assay. These subpopulations were tested for their rate of antibody production and for the number of vector copies in the genome.

### *Analysis of subpopulations identified via secretion assay*

Cells recovered from the last harvest of fermentation runs AB102 and AB205 were cultured in spinner flasks. For each culture the FACS histogram showed two sub-populations: one which appeared to be composed of poorly expressing cells, and one made up of cells with robust expression (Figure 4). The FACStar Plus was used to isolate representatives of each population. The AB205H sort recovered $1.3 \times 10^5$ cells and $1.5 \times 10^5$ cells for the low and high fluorescence cell lines respectively. For the AB102H culture, $0.5 \times 10^5$ and $1.5 \times 10^5$ cells were collected by sorting for the low and high level fluorescence cell lines respectively. Each pool of sorted cells was incubated in tissue culture plates containing fermenter culture medium supplemented with penicillin-streptomycin and gentamycin. Upon growth to confluence, cells were transferred into spinner flask suspension culture. After adaptation to growth in suspension, the cultures were tested for their level of productivity. The low and high

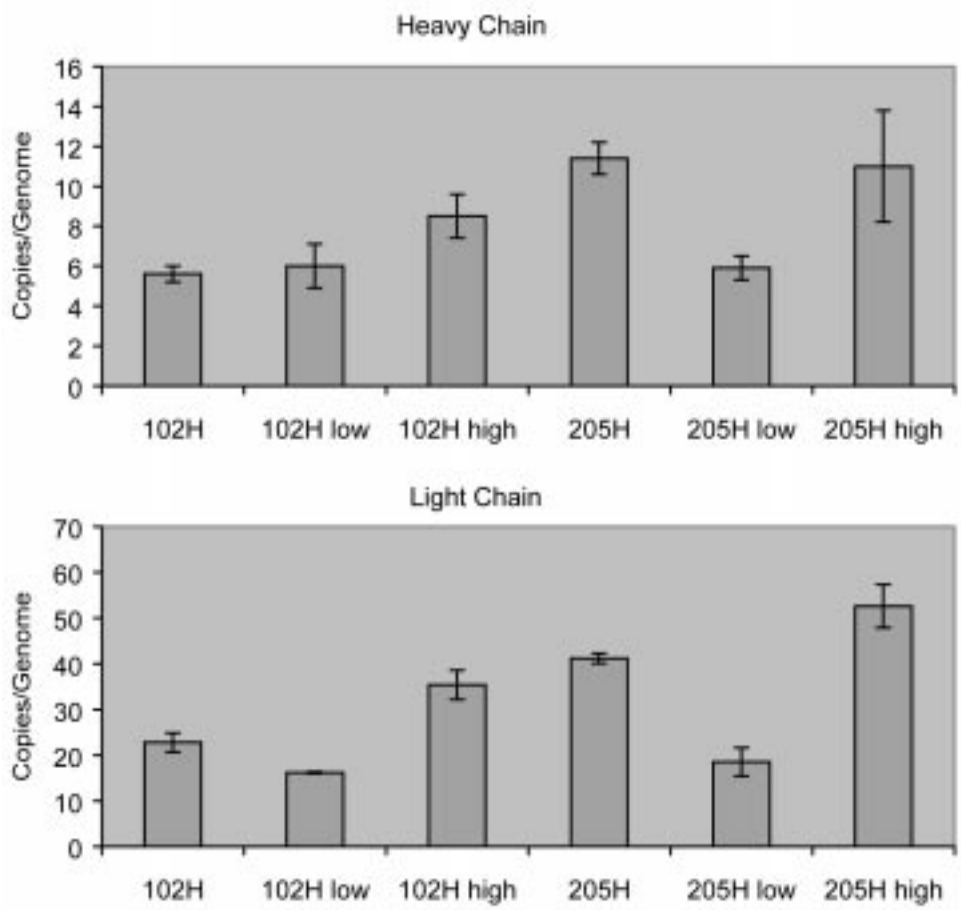


*Figure 6.* Antibody heavy chain (HC) and light chain (LC) copy numbers. Copy numbers were determined by Southern blots on cells recovered from the final fermenter harvests (AB102H and AB205H), and from the sorted cells selected for low fluorescence (102H low and 205H low) or high fluorescence (102H high and 205H high).

producer lines were cultured in spinner flasks for 4 weeks with daily cell counts and samples taken for productivity determinations (Figure 5). AB205H High was grown in spinner culture for an additional 6 weeks to test for expression stability. During this extended period of culture, the high level of antibody production was maintained. Productivity of the high expression population was greater than three-fold higher than that of the low expression cells. Copy number assessments of each population show that the high and low cultures have gene copy numbers that correspond to their level of 2TE4 production (Figure 6). The heavy and light chain copy numbers of the AB102H populations differ by 24 and 58%, respectively. Likewise the AB205H populations differ by 35 and 70%, respectively. The copy number determination assay has a coefficient of variation of 30%. Therefore three out of four of the determinations represent real differences in copy number. Based on these data it appears that the GMD secretion assay method detects the appearance of a low producer subpopulation derived from the original producer line but which has lost copies of the expression vector from its genome.

This population variance was not seen in the other amplified CHO line examined. Cell line R6/N was cultured under conditions similar to those used for fermentation AB205. The antibody secretion profile showed a uniform level of antibody secretion from cells taken at the second, fourth and eighth harvests (Figure 7).

## Discussion

The harvest/rebatch method of recombinant protein production allows the accumulation of significant

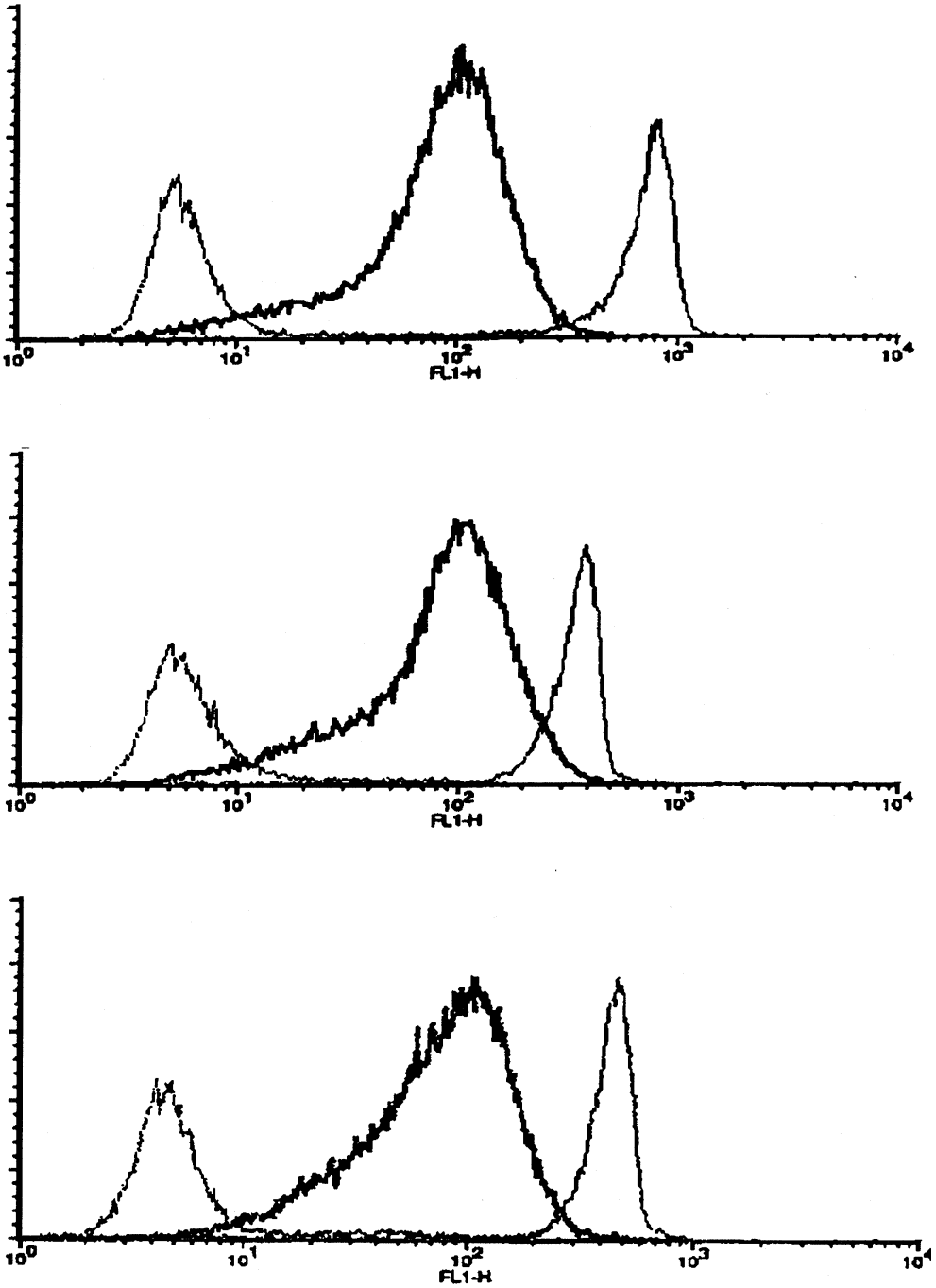


*Figure 7.* FACS histograms of microdrop secretion assays of cells taken from a 1000L fermention of cell line R6/N. The top, middle and bottom panels represent histograms of the second, fourth, and eighth harvests, respectively. Flanking peaks were generated from the positive and negative control samples as described for Figure 3.

quantities of product while maintaining the producer cell line in a condition of rapid growth. A consequence of this strategy is that the CHO cell line is in culture for a period of several weeks. Under these conditions, a variant low productivity cell can have an advantage over the majority of cells, translating into a higher rate

of growth. The lower levels of productivity seen in the later fermenter harvests suggest that such a variant population may have arisen. This study confirms that the gel microdrop secretion assay can be used to monitor the stability of recombinant antibody secretion from the transfected CHO cells in large-scale fermentation. The method allows us to choose between production cell lines based on their expression stability. For example, the R6/N cell line displayed a uniform level of antibody secretion over the course of the fermentation. The assay documents the superiority of the R6/N line over G5/C under harvest/rebatch conditions. The method could be applied to cells producing other secreted proteins by using a different set of antibodies in the microdrop capture complex. The method had been used by other groups to sort hybridomas based on antibody expression levels in tissue culture (Powell and Weaver, 1990; Weaver et al., 1997).

Furthermore, the gel microdrop (GMD) technology facilitated the recovery of subsets of cells with different rates of antibody secretion. The GMD method enabled us to detect the nature of this instability in 6 hr, rather than relying on the Southern blot approach that was less sensitive and required several days to perform. The GMD method showed that a subset of the cells had lost their ability to produce antibody at high levels while others express 2TE4 at the original level. Subsequent genetic analysis confirmed that this loss of antibody expression coincided with a reduction in expression vector copy number that did not effect the expression of product from the remaining copies. Other groups have reported instability in the expression of recombinant proteins from clonal amplified cell lines (Kaufman et al., 1985; Lubiniecki et al., 1992); and at least one model of drug resistance gene amplification also accounts for the subsequent loss of amplified DNA by the same mechanism (Toledo et al., 1992).

The data presented here suggest that in isolating the high production cell population, the method may recover especially stable cell clones from the end of production. Additional experiments are necessary to determine if these clones are inherently more stable in the next round of fermentation.

## Conclusions

The gel microdrop secretion assay allows us to distinguish between factors which inhibit the metabolism of all cells in the culture, such as depletion of a critical nutrient or accumulation of waste products, from factors that may affect only a subset of cells, such as genetic instability. Furthermore, the method can be used to recover a population with stable expression of the protein of interest. Additional tests must be conducted in order to determine if such populations are more stable in fermentations than their parent line.

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