Control of Key Parameters in Developing Mammalian Production Clones

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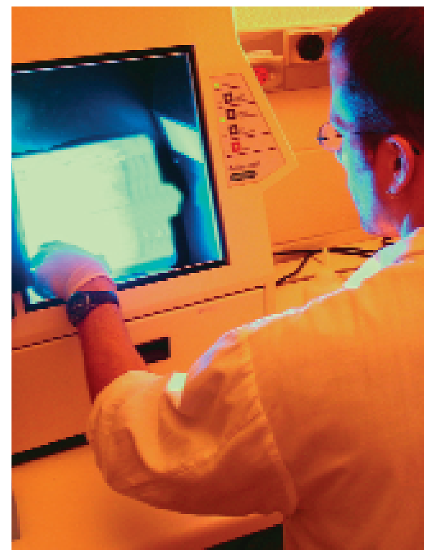
Techniques for generating expression clones to produce recombinant biopharmaceuticals have been improved considerably, especially in vector constructs, during the past 20 years (**1**, **2**). By 1995, the number of recombinant pharmaceutical proteins expressed in mammalian cells outran the number from microbial expression systems (**3**). Improving key parameters such as process control, reduction of media costs, and replacement of animal-derived components led to breakthroughs in the use of animal expression systems.

Initially, *E. coli* was used to generate human hormones and cytokines, predominantly as inclusion bodies; however, bacteria could also secrete proteins into their periplasma and culture supernatant. But refolding and proteolytic or chemical modifications are needed to reconstitute recombinant proteins for human use. The expression vectors for *E. coli*–based systems vary in the strength of their promoter genes, in the origin of replication (ori)–controlling plasmid copy numbers, and in how the gene of interest affects their translational machinery. Process control parameters govern induction of target gene transcription and cellular life span, during which translation occurs.

By contrast, mammalian cells are more complex in the 5′ and 3′ regulatory sequences of their expression vectors that regulate transcription efficacy and RNA stability. Variability in cell culture processes can significantly influence product quality and quantity. For example, nutrient starvation in complex media influences product glycosylation and may cause apoptosis, leading to the release of glycolytic and proteolytic enzymes into the culture supernatant (**4**, **5**). Other process parameters such as pH, temperature shifts, oxygen availability, and shear forces may cause cell necrosis if not carefully controlled.



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The development of a mammalian production clone is an interactive process that can result in different phenotypes that do not show up while the cells remain in a static laboratory-scale culture at low cell densities (up to 10^6 cells per mL). Clone selection occurs at a late bioreactor stage through evaluation of the stability and productivity of the cell line together with the quality of the recombinant protein. Here we report data on the development of recombinant Chinese hamster ovary (CHO) cell lines expressing a human immunoglobulin M (IgM) antibody of pentameric structure and human monoclonal antibodies (MAbs) of IgG subtype. We screened the

PRODUCT FOCUS: ANTIBODIES

PROCESS FOCUS: PRODUCTION, EARLY PROCESS DEVELOPMENT

WHO SHOULD READ: PROCESS DEVELOPERS, ANALYTICAL STAFF, QA/QC, MANUFACTURING

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LEVEL: ADVANCED

primary transfectants for secretion rates, intracellular amounts of the protein of interest (POI), and gene copy numbers (as well as the amount of specific mRNA) to get early information on the cell line. In a further experiment, we analyzed the stability of an established cell line under selection pressure and without methotrexate (MTX). The results illustrate the stability of the cell line and proper fermentation in a bioreactor.

MATERIALS AND METHODS

We used CHO cells deficient in dihydrofolate-reductase (CHO-dhfr) from the American Type Culture Collection (ATCC CRL-9096) as the host cell line. They were cultivated in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 4 mM L-glutamin, 8 mM hypoxanthine–0.8 mM thymidine (HT), and 10% fetal calf serum (FCS). For selection, the medium consisted of DMEM supplemented with 4mM L-glutamin, 0.5 mg/mL geneticin-sulfate (G418), and 10% dialyzed FCS. Gene amplification was achieved using selection medium with increasing amounts of MTX starting at 0.05 µM.

All transfections were carried out using lipofectin reagent from Life Technologies (www.lifetech.com) in nonhomologous cotransfections of two different plasmids. With recombinant human IgM expression, one plasmid contained the IgM heavy chain gene and the dhfr gene under control of the human cytomegalovirus immediate early promoter in a bicistronic expression plasmid. The second combined the antibody's light chain and the IgM joining-chain cDNA in a similar vector. Neomycine phosphoribosyl-transferase was inserted on both plasmids.

We started the selection one day (24 hours) after transfection, and growing clones were detected two weeks later. Subsequently, the clones were treated with 0.05 µM MTX in selection medium, and upcoming clones were tested by enzyme-linked immunosorbent assay (ELISA).

ELISA, SDS-PAGE, and Western Blots: We analyzed the clones for expression of complete antibody molecules with a light- and heavy-chain ELISA, using a double-sandwich assay for quantification of the culture supernatants. IgM and IgG content were quantified in serially eightfold-diluted samples and compared with polyclonal human IgM or IgG, beginning with 200 ng/mL at the highest concentration.

In brief, Maxisorp 96-well immunosorbent plates from Nunc (www.nuncbrand.com) were coated with goat-antihuman IgM (µ-chain specific) or goat-antihuman IgG (γ-chain specific) from Sigma (www.sigmaaldrich.com) at a concentration of 2µg/mL in coating buffer (0.1 N $NaHCO_3$ at pH 9.6). After being washed three times, samples and standards were incubated for 60 min, then the plates were washed again and incubated with goat-antihuman κ-light chain conjugated with alkaline phosphatase at 1:1000 dilution (also from Sigma) for 60 min, then detected using pNPP substrate ($C_6H_4NO_6$ $PNa_2 \times 6H_2O$) in a coating buffer (pH 9.6), both from Sigma. Antibody production was calculated in µg per 10^6 cells per day.

We specified the degree of polymerization for the recombinant human monoclonal IgMs by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in Novex brand NuPAGE Tris-Acetate 7% gels from Invitrogen (www.invitrogen.com) using denaturing, nonreducing conditions. Gels were blotted to a 0.45-µM polyvinylidine fluoride (PVDF) membrane from Millipore (www.millipore.com), and polymeric IgMs were detected with alkaline phosphatase–conjugated goat-antihuman κ-chain ($f + b$) antibody from Sigma.

Flow Cytometric Analysis: To determine the intracellular content of the protein of interest, we used flow cytometry. A sample of cells (2–4 × 10^6 cells) was fixed, washed twice in Tris buffer (0.1 M Tris HCl, 2 mM $MgCl_2$, 0.1% Triton X-100 at pH 7.4) from Sigma containing 20% FCS, and then suspended in 200 µL Tris/FCS containing mouse-antihuman κ-chain antibody conjugated with Quantum Red at 1:64 dilution and goat-antihuman µ–chain antibody conjugated with FITC at 1:64 dilution or FITC-labeled goat-antihuman γ-chain at the same dilution (all from Sigma). After incubation for 60 min at 37 °C, the cells were washed, resuspended in 200 µL Tris buffer, and analyzed on a FACS Vantage flow cytometer from Becton, Dickinson and Company (www.bd.com). Cells were excited with 250-mW laser power at 488 nm, and fluorescence emission was measured with a 530/30 (Fl 1) and a 660/20 BP filter (Fl 3), respectively. We used nonproducing host cells as a negative control, and adjusted compensation of spectral overlap from Fl 1 to Fl 3 using cells stained only with the goat-antihuman γ-chain fluorescein (FITC) conjugate (**6**).

Batch Cultivation: Specific productivity (Qp) and growth rate

Table 1: PCR primers used

Primer	Sequence
ß-actin antisense	5´-CTA GAA GCA TTT GCG GTG GAC–3´
ß-actin -150 sense	5´-CAA GAT CAT TGC TCC TCC TGA G–3´
ck antisense	5´-CTA ACA CTC TCC CCT GTT GAA G–3´
ck-150 sense	5´-CAG GAC AGC AAG GAC AGC AC–3´
cµ antisense	5´-TCA GTA GCA GGT GCC AGC TG–3´
cµ-150 sense	5´-ATG GAA CAC GGG GGA GAC C–3´
dhfr antisense	5´-TTT CTA GAT TAG TCT TTC TTC TCG TAG AC–3´
dhfr-150 sense	5´-TCA TGC AGG AAT TTG AAA GTG AC–3´

Figure 1a: Cell accumulation of three different subclones from one transfection seeded with 10^5 cells/cm^2 and cultivated for four days or seeded with 10^4 cells/cm^2 cultivated for eight days.

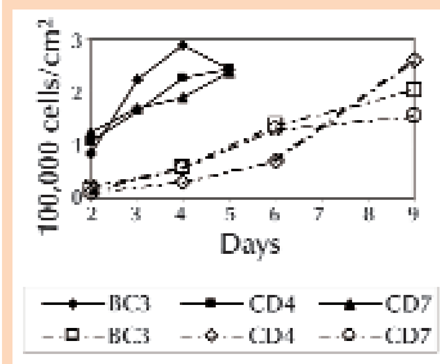
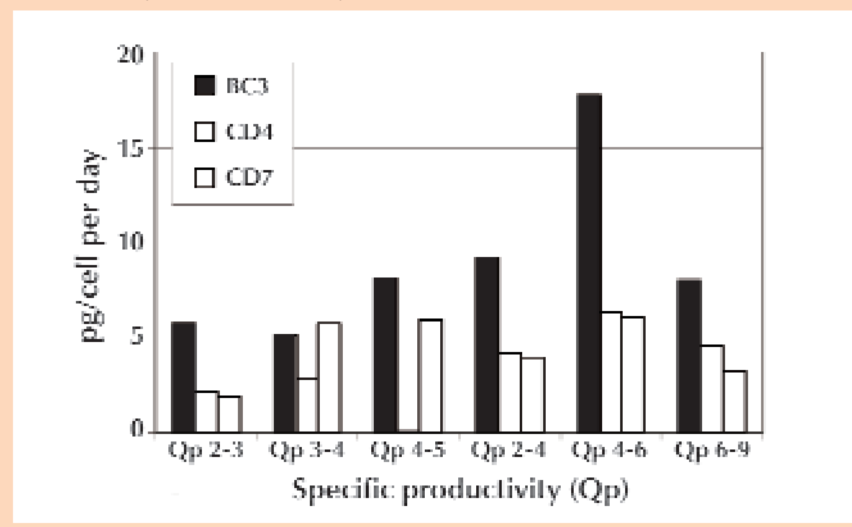


Figure 1b: Specific producivity (Qp) of three different subclones seeded in different amounts. Qp 2–3, Qp 3–4, and Qp 4–5 are values from days two to three, days three to four, and days four to five seeded with 10^5 cells/cm^2; Qp 2–4, Qp 4–6, and Qp 6–9 are values from days two to four, days four to six, and days six to nine seeded with 10^4 cells/cm^2.



of recombinant cells can be analyzed with high significance in a batch experiment using 24-well plates. We seeded the recombinant cells (clones BC3, CD4, and CD7) at an initial density of 10^4 cells/cm^2 and 10^5 cells/cm^2. Cell densities were measured by hemocytometry, and IgG titers were analyzed every second day (for those seeded at 10^4 cells/cm^2) or every day (for those seeded at 10^5 cells/cm^2). We calculated the Qp values at different times: Qp 2–3, Qp 3–4, and Qp 4–5 generated on days 3, 4, and 5 after 10^5 cells/cm^2 inoculation, each calculating the cellular increase after 24 hours; Qp 2–4, Qp 4–6, and Qp6–9 analyzed on days 4, 6, and 9 after 10^4 cells/cm^2 inoculation and calculated after 48 (Qp 2–4 and Qp 4–6) and 72 (Qp 6–9) hours.

Real-Time Polymerase Chain Reaction (PCR): Total RNA was isolated from 5 × 10^5 cells of different cell lines using Trizol reagent from Life Technologies and further treated with Rnase-free Dnase I (from Roche) at 20–25 °C for 10 min. RNA samples were cDNA-transcribed with 200 units of Moloney murine leukemia virus (MMLV) reverse transcriptase using oligo-dT(20) primer, both from Promega (www.promega.com); MMLV buffer from Promega supplemented with 0.4-mM MgCl2, 500-µM dNTPs, and 1 IU/20 µL Rnase inhibitor from Amersham Biosciences (www. amershambiosciences.com) in a total volume of 20 µL. Reverse transcription was carried out for 60 min at 37 °C, followed by five minutes at 95 °C to terminate the reaction.

We determined the gene copy number from chromosomal DNA isolated from 5 × 10^5 cells using the Wizard SV 96 Genomic DNA Purification System from Promega. PCR primers generating a 150-bp fragment at the 3´ end of each gene of interest (and for ß-actin as an endogenous internal standard) were used for amplification. They are listed in Table 1.

Real-time PCR was carried out using a Rotor Gene 2000 thermocycler from Corbett Research (www.corbettresearch.com) and Rotorgene software version 4.2. Calibration curves were included every time, with template gene copy numbers of 10^7 to 10^2. We measured all samples in triplicate and all standards in duplicate. To prevent detection of unspecific priming, we also ran a nontemplate control (NTC) in every experiment.

Each real-time PCR reaction contained the following buffer (all ingredients from Sigma): 10 mM Tris-HCl; 3 mM magnesium chloride; 50 mM potassium chloride; 1.5% Triton X-100; 0.5 pmol sense primer; 0.5 pmol antisense primer; 200 µM each of dATP, dCTP, dGTP, and dTTP; 1 µL of 1:20,000 Sybr Green I from Roche (www.roche.com); 1 µL of Taq-polymerase; and 1 µL of cDNA — or genomic DNA — template.

We used 1 µL of undiluted or 1 µL 1:5 dilution of template in the 20-µL reaction mix for each reaction (7). A 20-µL sample of the total PCR mix was denatured at 94 °C for 90 sec and cycled 35 times at 94 °C for 10 sec, then 55 °C for five seconds, and then 72 °C for 12 sec. To visualize the melting characteristics of the products, we added a melting step: 65–99 °C increasing one degree every two seconds. The absolute number of specific genes was calculated for each sample by three measurements, resulting in a maximum standard deviation of 10%. Finally, we normalized the values, dividing them by the absolute gene copy number of ß-actin and expressing them as multiples of ß-actin.

Results and Discussion

Evaluation of Production Potential: Our goal in screening early transfectants is to select a few high-producing candidates, simplifying and minimizing further effort in the

laboratory. Figure 1 illustrates the productivity of recombinant IgG-producing CHO clones, generated in one transfection event, by showing cell accumulation (Figure 1a) and specific IgG productivity (Figure 1b) of three early transfectants (BC3, CD4, and CD7) two months after transfection. All three clones produced 2–3 µg/mL of the monoclonal antibody during normal cultivation, with two 1:3 passages weekly (data not shown), which indicated no obvious criterion to select one over the other. By contrast, the batch experiment clearly demonstrated that clone BC3 displays highest productivity independent of initial cell count and even at relatively high cell densities (Figure 1b, Qp 4–5 and Qp 6–9). Further investigations must be done to determine product quality and ensure that this clone is expressing a functional and correctly processed antibody.

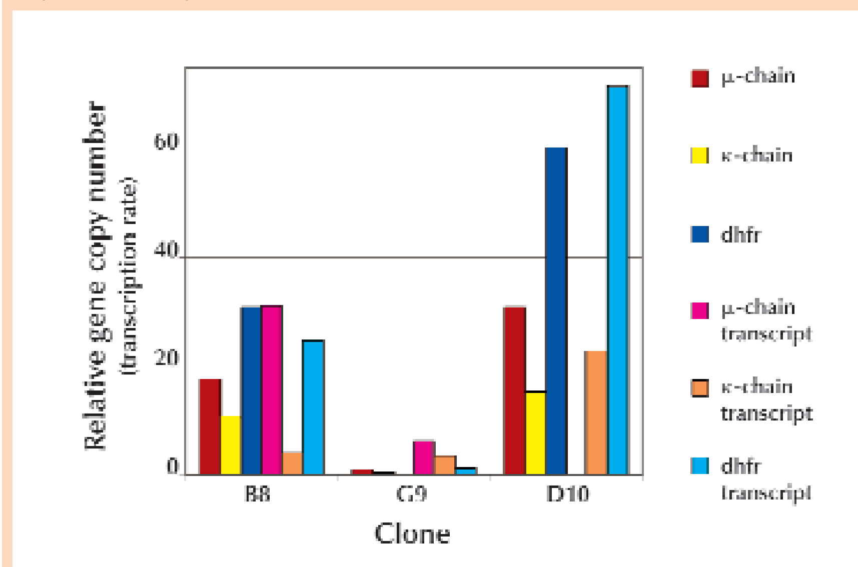
In a different transfection experiment, early transfectants expressing the human IgM antibody HB617 were analyzed for IgM secretion and intracellular IgM content. The clones B8, G9, and D10 were simultaneously controlled for expression titer and intracellular content of µ- and κ-chains detected by immunofluorescence staining. B8 specifically secretes into the culture supernatant 23.8 pg IgM/cell per day, D10 15.9 pg IgM/cell per day, and G9 0.6 pg IgM/cell per day. ELISA results and flow cytometric data are summarized in Table 2. About three-fourths (72%) of the B8 cell population stained positive in immunofluorescence — compared with only a third (34%) in clone D10 and just 5% positive cells in clone G9, which exhibited the lowest productivity. This indicates the high production potential in a distinct subpopulation of clone D10, which was responsible for IgM found in the supernatant.

Additionally, we tested the genomic and transcriptional status of the three clones: B8, G9 and D10 (Figure 2). The two parameters, specific DNA and RNA content of the target genes, were higher in clone D10 than in B8 and G9. ELISA and flow cytometric data were supported by the minor amounts of exogenous DNA and RNA of clone G9. However, the dhfr gene seems to be already amplified to a higher degree in clone D10. That could lead to a diminished antibody gene amplification during increments of MTX. Both clones B8 and D10 must be analyzed again after gene amplification and selection of a homogenous population to choose between them.

Table 2: Control of early transfectants for expression of recombinant IgM into cell culture medium and intracellular accumulation. Specific productivity was measured by ELISA; percentage of cells contributing to the expression titer are shown in the second column; the geo mean represents the average µ-chain (Fl 1) and κ-chain (Fl 3) content in each population.

Clone	Specific Titer (pg/cell per day)	Percentage of cells >blank	Geo Mean Fl 1/Fl 3
B8	23.8	72	7.3/12.2
G9	0.6	5	3.9/2.6
D10	15.9	34	5.8/7.2

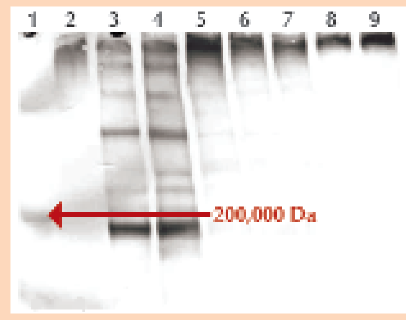
Figure 2: Real-time PCR analysis of early clones B8, G9, and D10 recognizing gene copy number and content of transcribed RNA. All results relate to a standard ß-actine nucleic acid of the individual clone. For Qp and further information, see Table 2. D10 was not analyzed for µ-chain transcript.



Expression of Pentameric High–Molecular-Weight MAbs: We analyzed the quality of recombinant protein produced in different IgM-producing CHO cell lines. Two recombinant MAbs of IgM subclass (with different specificities) were expressed in CHO dhfr cells. MAb 4E10 IgM is a class-switched isotype of an anti-HIV-1 antibody (8), whereas MAb HB617 IgM was originally expressed by an EBV-transformed cell line and is directed against a glycosphingolipid. Both displayed the same constant regions in their genotypes. The expression vector sequences were verified by DNA sequencing (data not shown).

We identified the presence of complete polymeric antibodies in culture supernatants using denaturing, nonreducing SDS-PAGE and Western blots. The immunoblots revealed a mainly pentameric and monomeric form for the IgM isotype at an estimated ratio of at least 1:2 for 4E10 IgM (Figure 3, lanes 3 and 4) in contrast with the almost exclusively pentameric form for HB617 IgM (Figure 3, lanes 5–9). Our data correspond with results published

Figure 3: Recombinant IgM expression in CHO cells. SDS-PAGE–Western blot immunostaining was detected with antihuman κ-chain antibody. 4E10 IgM formed mainly monomeric molecules, whereas HB617 IgM expressed a homogenous protein migrating at the same rate as the polyclonal human IgM standard. Lane 1 is the molecular weight standard, lane 2 the human polyclonal IgM standard, lanes 3 and 4 different subclones of recombinant 4E10IgM, lanes 5–7 different subclones of HB617 IgM, and lanes 8 and 9 different subclones of a serum-free transfection of HB617IgM.



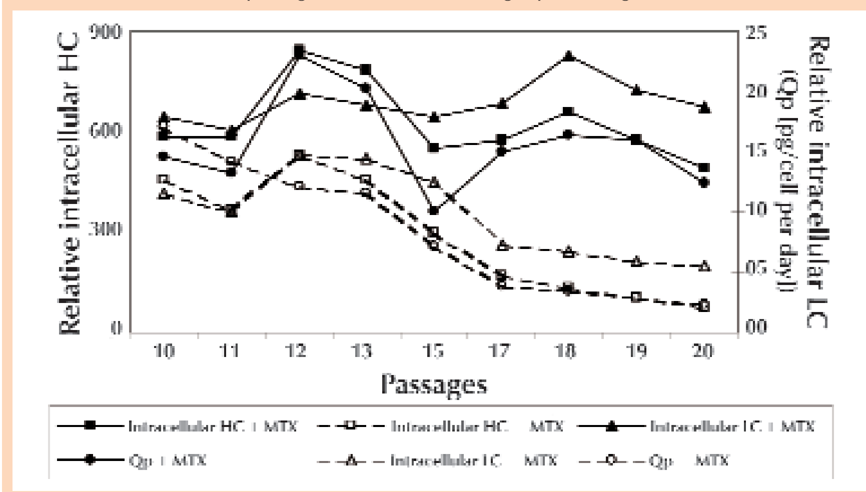
by others on the expression of polymeric IgM in lymphoid (9) and nonlymphoid cell-lines (10, 11). The IgM joining chain (J-chain) has been termed crucial for pentamer formation of IgM (12) and was coexpressed in both IgMs. Because a suitable antibody for detection of incorporated J-chain was not available, we confirmed it at the mRNA level (data not shown).

We have performed different transfection experiments with the HB617 IgM plasmids with (Figure 3, lanes 5–7) and without serum (Figure 3, lanes 8 and 9) in the cultivation medium and found the pentameric structure in all supernatants. From those findings, together with the results for other recombinant human IgMs, we assume that the potential to generate pentamers is determined by the variable region of the antibody. In other experiments (13), we expressed IgM and IgA class-switched antibodies of original IgG isotype and also found different distributions of oligomeric structures, specific for each antibody. Therefore, it seems plausible that antibodies of native IgM isotype (including human polyclonal IgM, Figure 3, lane 2) exhibit predominantly pentameres expressed even in CHO cells, in contrast with the class-switched immunoglobulins.

Correlation of Intracellular with Expressed Protein: We used our recombinant CHO cell line expressing MAb 4E10IgG (8) to monitor intracellular accumulation of antibody chains and product released to the culture supernatant. Continued passages over 21 splits represent a stability over 50 generations after thawing the master cell bank (MCB). Figure 4 correlates secreted (Qp) and intracellular protein of interest (intracellular HC and LC). During the period under consideration with selection pressure, intracellular content of heavy- and light-chain molecules analyzed by flow cytometry remained constant. ELISA quantification featured Qp values in the range of 11–23 pg/cell per day with no indication for a decrease. By contrast, cells cultivated without MTX continuously lost Qp (from 15 to 3 pg/cell per day), which was reflected in a decrease of intracellular heavy- and light-chain antibody.

As published previously (14), the long-term cultivation study of 2F5IgG recombinant CHO cells (15) indicated a significant reduction of intracellular light chain while heavy chain was accumulated in the cell. Because those data were generated over a period of 150 passages, they cannot be directly compared with our 21 passages. However, with incorrect folding or mismatch formation of heavy and light chain, the CHO 4E10 IgG cell lines might also accumulate heavy chain in the cytoplasm over long-term cultivation (more than 15 months). In a different report, the production rate of recombinant clones was compared with the gene copy number, and the results demonstrated different behavior of the individual clones, suggesting that stability must be carefully analyzed for each production clone (16).

Figure 4: ELISA quantification and intracellular content of μ- and κ-chain molecules over the course of 21 consecutive passages of a recombinant IgG-producing CHO cell line MCB.



The Case for Multidimensional Analysis

The development of production cell lines must be approached from several angles. Screening for only one parameter (such as high production titer) risks isolating clones that have already reached their maximum level of performance (physiological and genotypic potential), whereas others might be more suitable for selecting subpopulations, gene amplification, and cultivation in high–cell-density systems.

Data showing intracellular content of the HB617 IgM–producing transfectants B8, G9, and D10 showed that the highest expression rates detected in the culture supernatant do not

necessarily reflect the potential of the clone because the cell population at such an early stage of development is often incongruous. Clone B8, secreting the highest antibody titers into the culture supernatant, displayed a fraction of 72% producers in fluorescence cytometry. By comparison, clone D10 (with a slightly reduced productivity) showed less than half of the cellular fraction staining positive in immunofluorescence that B8 showed. Thus, fluorescence cytometric sorting might select a more potent clone from the primary transfectant D10 than from B8 (17).

Another aspect worth considering is the genomic status of the transfectants during selection and amplification. In our example, all three HB617 IgM clones (B8, G9, and D10) were amplified with 5×10^{-8} M MTX, but significant differences in gene copy number and the amount of transcript were detected. The weak producer G9 was found to have only minor amounts of specific nucleic acids. The best producer (B8) was not amplified in its dhfr gene copy number compared with clone D10, indicating that the potential to increase the copy number for the gene of interest by MTX amplification is higher in B8. However, neither real-time PCR nor Southern blot hybridization nor in situ hybridization specifically detect the integration site of the exogenous DNA. If the microenvironment of the integration site is so important for transcription efficacy, then genome-walking sequencing must be the method of choice for further investigation of recombinant production clones.

Our results demonstrate that growth rates and specific product accumulation for early transfectants is accurately tested in miniature batch experiments using 24-well plates. Parallel intracellular staining of cells provides evidence about the homogenicity of the cell populations. Additionally, determining the gene copy number and transcription rates will significantly contribute to the selection of recombinant cell lines with technological relevance. Furthermore, product quality must be carefully controlled from the earliest possible stage to ensure that essential posttranslational modifications are performed.

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