High-Throughput Clonal Selection of Recombinant CHO Cells Using a Dominant Selectable and Amplifiable Metallothionein-GFP Fusion Protein

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Abstract: Transfected mammalian cells can be used for the production of fully processed recombinant proteins for medical and industrial purposes. However, the isolation of high-producing clones is traditionally time-consuming. Therefore, we developed a high-throughput screening method to reduce the time and effort required to isolate high-producing cells. This involved the construction of an expression vector containing the amplifiable gene metallothionein (MT), fused in-frame to green fluorescent protein (GFP). The fusion gene (MTGFP) confers metal resistance similar to that of the wild-type metallothionein and expression can be monitored using either flow cytometry or a fluorometer to measure green fluorescence. Expression of MTGFP acted as a dominant selectable marker allowing rapid and more efficient selection of clones at defined metal concentrations than with the antibiotic G418. Cells harboring MTGFP responded to increasing metal concentrations with a corresponding increase in fluorescence. There was also a corresponding increase in recombinant protein production, indicating that MTGFP could be used as a selectable and amplifiable gene for the coexpression of foreign genes. Using our expression vector encoding MTGFP, we demonstrate a high-throughput clonal selection protocol for the rapid isolation of high-producing clones from transfected CHO cells. We were able to isolate cell lines reaching specific productivities of >10 μg hGH/10^6 cells/day within 4 weeks of transfection. The advantage of this method is that it can be easily adapted for automated procedures using robotic handling systems.
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Keywords: Chinese hamster ovary cells; metallothionein; green fluorescent protein; clonal selection; flow cytometry

INTRODUCTION

The stable transfection of Chinese hamster ovary (CHO) cells is a commonly used system for the production of recombinant proteins. Expression of the recombinant protein requires an expression vector encoding the desired gene of interest and a dominant genetic marker, usually an antibiotic resistance gene. Transfection efficiencies range from 10–60%; however, a wide variation in recombinant gene expression exists among clones that stably incorporate the foreign DNA. This could be due to the position effect, in which different regions of the chromosome modulate the expression of the transfected gene. A search for random high producers is time-consuming and labor-intensive using conventional screening methods such as immunodetection. Many hundreds, even thousands of transfected clones are typically screened for random variation of recombinant protein production.

In addition to antibiotic selection methods for isolating high-producing clones, an amplification strategy can also be used, involving coexpression of a marker gene. Two widely used amplification systems employed with CHO cells are dihydrofolate reductase (DHFR) (Alt et al., 1978) and glutamine synthetase (GS) (Cockett et al., 1990). Selective cycling in the presence of increasing concentrations of either methotrexate (MTX), an inhibitor of DHFR function, or methionine sulfoximine (MSX), an inhibitor of GS, results in the amplification of the integrated DNA and increased expression of the desired gene product.

The CHO-DHFR expression system involves the use of a mutant CHO cell line, lacking the enzyme activity for dihydrofolate reductase (Urlaub and Chasin, 1980) and requiring growth in GHT medium (glycine, hypoxanthine, and thymidine). The GS expression system is not effective in CHO cells due to endogenous GS activity (Brown et al., 1992). In addition, there appears to be a limited amount of amplification that can be achieved due to the development of drug resistance to MTX or MSX, resulting in no further increase in recombinant protein expression (Bebbington et al., 1992; Cockett et al., 1990).

To overcome these problems associated with the development of drug resistance when generating cell lines for the

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production of recombinant proteins, we investigated the use of the amplifiable gene metallothionein (MT) (Karin and Richards, 1982). Although CHO cells do contain MT genes, they have been silenced by DNA methylation (Gounari et al., 1987; Harris, 1984; Stallings et al., 1986). Previous reports have described expression vectors containing the MT gene being used for selection and amplification of foreign genes, including expression in CHO cells (Beach and Palmiter, 1981; Kushner et al., 1990; Bailey et al., 1999).

We also investigated the use of a selective marker, green fluorescent protein (GFP), which can be used for visually monitoring gene expression and selection of cells expressing inducible gene products (Chalfie et al., 1994; Mosser et al., 1997). We constructed an expression vector containing an in-frame fusion between the human *MT* gene and the *GFP* genes (MTGFP). The use of the fusion marker should facilitate both the screening of recombinant gene-positive clones and identification of high producers using flow cytometry. We demonstrated this by the expression of either human growth hormone (hGH) or chloramphenicol acetyl transferase (CAT), cloned into the MTGFP vector. Flow cytometry can be used to screen many millions of cells and FACS can isolate the high producers. Transfected cells responded to increasing concentrations of cadmium, leading to increased green fluorescence, which was measured by flow cytometry or with the use of a fluorometer. Analysis of clones isolated from increasing cadmium-resistant pools demonstrated that increased fluorescence correlated linearly with increased production of recombinant protein.

MATERIALS AND METHODS

Engineering the *MTGFP* Fusion Gene

The coding sequence for the *MTIIA* gene was amplified using the primers MTGFP-1 and MTGFP-2 (Table I). The reaction containing 1U Taq polymerase (Gibco Invitrogen, Carlsbad, CA), 200 μM dNTPs (Progen Industries, Darra, QLD, Aust), 1 mM $MgCl_2$, 50 pmol each of the forward and reverse primers, and 10% DMSO (v/v), with an annealing temperature of 50°C, as previously described (Dieffenbach and Dveksler, 1995). The coding sequence for the enhanced *GFP* gene was amplified from pEGFP-1 (BD Biosciences Clontech, Palo Alto, CA) using MTGFP-3 and MTGFP-4 (Table I) with a final $MgCl_2$ concentration of 2 mM. The amplified products were gel-purified and a 27-cycle primer overlap extension PCR was performed to prepare the MTGFP fusion, using the MTGFP-1 and MTGFP-4 primers, producing a final PCR product of 2,483 bp. To increase specificity, the annealing temperature was raised after three cycles from 50–55°C.

Construction of the Expression Vectors pMTGFP, pMTGFP/hGH, and pMTGFP/CAT

To create pMTGFP (Fig. 1), the PCR fusion product was initially digested with *PstI* and gel-purified. The resulting 2,467 bp fragment was then ligated into the expression vector pNKΔMT, which is a modification of the pNK vector (Bailey et al., 1999) where the *MT* gene had been removed by *PstI* digestion. To create pMTGFP/hGH, a 2,610 bp fragment containing the genomic sequence for hGH (gift from PJ Kushner, California Biotechnology, Mountain View, CA) (nt −559 to +2049 relative to the ATG start site) and flanked by *EcoRI* and *KpnI* restriction sites was ligated into pMTGFP and digested with *EcoRI* and *KpnI*. To create pMTGFP/CAT, the cDNA for CAT was isolated from pNKCAT (Bailey et al., 1999) by digestion with *HindIII* and *KpnI* and ligated into the respective sites in pMTGFP.

Cell Culture and Transfections

CHO-K1 (ATCC CCL61) were grown in a 50:50 mix of DMEM/Coons F12 (CSL, Parkville, VIC, Aust) supplemented with 10% FCS (CSL). Cells were incubated at 37°C in an atmosphere of 5% carbon dioxide and 90% humidity.

Plasmid DNA was purified using the QIAfilter Plasmid Maxi kit (Qiagen, Hilden, Germany) and a 4.4 μg / 35 mm dish was introduced into the cells using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA). Cells were put under selection pressure at 48 h in medium containing 400 μg/ml G418.

Metal Amplification

G418-resistant cells were grown in stepwise increasing amounts of metal starting with an initial concentration of 2.5

Table I. Primers used to prepare the MTGFP fusion gene

Primer	Sequence	Position/gene	Modifications
MTGFP-1	TACTCTTCCTCCCTGCAGTCTCTA	−758[1]/MT[2]	Contains PstI restriction site
MTGFP-2	CACCATGGGCCCGGCGCAGCAGCTGCA	+630/MT	Removes TGA stop codon
MTGFP-3	GCCGGGCCCATGGTGAGCAAGGGCGAG	−9/GFP[3]	
MTGFP-4	ATTTACGCCTGCAGATACAT	+954/GFP	Contains PstI restriction site

[1]The number of nucleotides from the ATG start site.
[2]MT, metallothionein.
[3]GFP, enhanced green fluorescent protein.

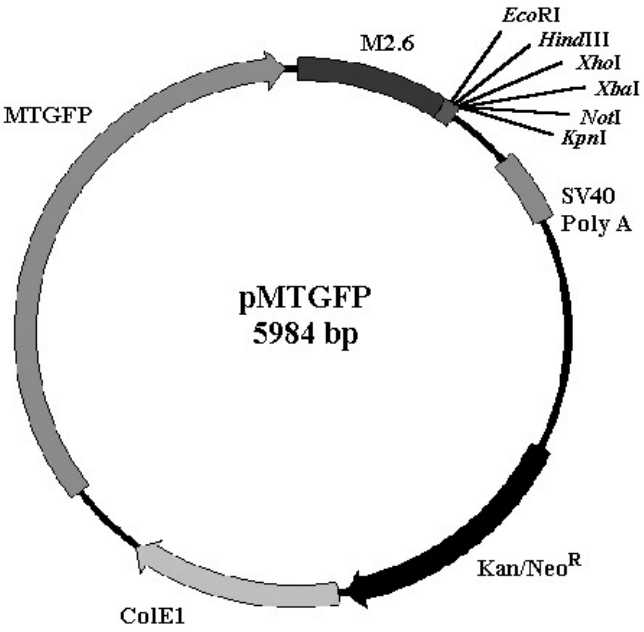

Figure 1. The pMTGFP mammalian expression vector. The fusion gene *MTGFP* is under the control of the wild-type metallothionein IIA promoter. A target gene of interest can be cloned into the multiple cloning site downstream of the M2.6 promoter and is followed by a sequence coding the SV40 polyadenylation site for downstream processing of mRNA. The gene encoding neomycin and kanamycin (Neo/Kan) confers resistance to G418 and kanamycin in mammalian and bacterial cells, respectively, under the control of their respective promoters (Bailey et al., 1999).

μM $CdCl_2$ and 50 μM $ZnSO_4$ (Bailey et al., 1999). The cells were passaged to 90% confluency four times before the $CdCl_2$ concentration was doubled. The $ZnSO_4$ was maintained at 50 μM. At each level of cadmium resistance the fluorescence was monitored using flow cytometry and specific productivity of the recombinant protein was determined by ELISA.

Metal Selection

G418-resistant cells were seeded into 100 mm dishes in 7 ml media and were allowed to attach for 6 h. Metal (1–10 μM $CdCl_2$ and 100 μM $ZnSO_4$) was added to the medium in the presence or absence of 400 μg/ml G418 and the cells were monitored daily for emergent colonies of metal-resistant cells. Approximately 6 days after metal was added cells were refed with medium containing 2 μM $CdCl_2$ and 100 μM $ZnSO_4$. Cells were then grown to confluency and analysis of recombinant protein production was performed by flow cytometry and ELISA.

Fluorescence Measurements

Flow cytometry, fluorescent-activated cell sorting (FACS), and single-cell deposition were performed on a MoFlo Cytometer (Cytomation, Fort Collins, CO) equipped with a multiline 200 mW argon ion laser-emitting light at 488 nm. Cell emission spectra was detected on FL-1. Analysis was performed on viable cells, gated by forward and side scatter, and verified by propidium iodide staining; 10,000 events were acquired. Acquisition and data analysis was performed using the CyCLOPS Summit operating system (Cytomation). Cell sorting also required the programs Sortmaster (Cytomation) and CyCLONE (Cytomation). Flow calibration and optical alignment was performed with the aid of Flow-Check Fluorospheres (Beckman Coulter, Fullerton, CA) before each analysis.

Cells were harvested for FACS by trypsinizing and then filtered through a 70-μm nylon mesh nozzle. The collection rate was 1,000 cells/sec and single cells were dropped into wells of 96-well plates containing 100 μl medium. The medium also contained 200 μg/ml G418 and 100 μM $ZnSO_4$.

Fluorescence intensity of GFP in the clonal populations was measured on an *fmax* fluorometer (Molecular Devices, Sunnyvale, CA), equipped with a quartz halogen lamp with filters for 485 and 538 nm, sufficient to excite and detect GFP. Data analysis was performed using SOFTmaxPRO (Molecular Devices).

ELISA

Quantification of hGH was measured from conditioned media collected from cells grown in 96-well plates for 10 days using an hGH ELISA kit as described by the manufacturer (Roche Applied Science, Mannheim, Germany). CAT protein levels were determined using a CAT ELISA kit (Roche Applied Science) as previously described (Bailey et al., 1999).

RESULTS AND DISCUSSION

Expression of MTGFP

The expression of the fusion protein MTGFP was examined in CHO cells transfected with the plasmid pMTGFP (Fig. 1). pMTGFP was constructed from a modified form of the expression vector pNK (Bailey et al., 1999), where the DNA encoding methallothionein IIA was replaced with the fusion gene but retaining the entire promoter region for the methallothionein IIA gene. The metal-hyperinducible promoter, M2.6 (McNeall et al., 1989), drives expression of a target gene cloned into the multiple cloning region.

Initially, the level of fluorescence was examined by flow cytometry in cells exposed for 8 days to increasing concentrations of cadmium (1–10 μM) in the absence or presence of G418. The data presented in Figure 2 shows the mean relative fluorescence of cells as a function of increasing metal concentration. At low (100 μM Zn^{2+} alone) or no metal selection there exists a twofold difference in mean fluorescence, depending on the absence or presence of G418. It appears that the addition of G418 alone is more effective at enriching a selected population than just using low concentrations of metal (1–2 μM Cd^{2+} + 100 μM

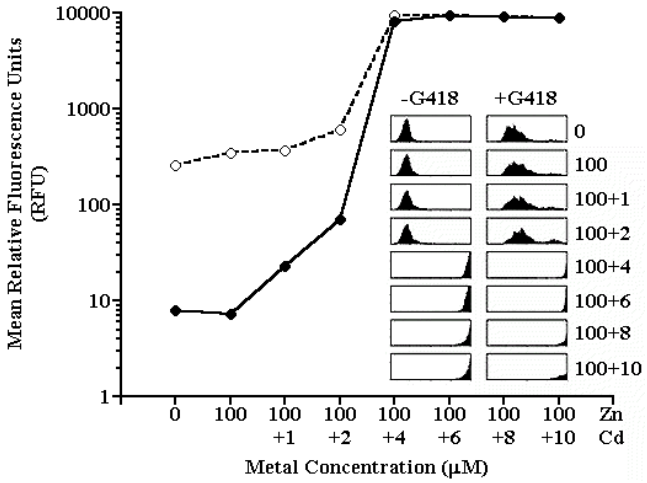

Figure 2. Expression of MTGFP: CHO-K1 cells were transfected with pMTGFP and after 48 h were exposed to 100 μM $ZnSO_4$ and increasing concentrations of $CdCl_2$ ± G418 for 8 days. Flow cytometric analysis was then performed to determine the level of GFP expression. The mean relative fluorescence units (RFU) of MTGFP were plotted against the metal concentrations used to select the cells. Background fluorescence equivalent to that of nontransfected cells has been subtracted. Open symbols (○) represent in the presence of G418 and closed symbols (●) represent in the absence of G418. The inset shows the flow cytometry profiles of cells that have survived metal ± G418 selection.

Zn^{2+}). However, when cells were selected in media containing 4 μM Cd^{2+} + 100 μM Zn^{2+}, mean fluorescence increased three orders of magnitude above background levels. The background fluorescence of the untransfected CHO cells had a relative fluorescent unit (RFU) of 7 (inset, Fig. 2).

Following the 8 days of metal selection, the level of fluorescence for all cells was above 10^4 RFU both in the absence or presence of G418. In fact, there was no peak corresponding to background fluorescence. Results were similar for those cells selected at higher concentrations of metal. However, there was considerable cell death with 8–10 μM Cd^{2+}, necessitating recovery of surviving cells in medium containing 2 μM Cd^{2+} and 100 μM Zn^{2+} in order to obtain enough viable cells to analyze.

Due to the high fluorescence (>10,000 RFU) observed with the GFP transfectants, it was sometimes necessary to attenuate light without discriminating wavelengths (data not shown). This can be achieved using a neutral density filter ND 1.3, which absorbs light over the entire visual spectrum. The filter limits the light reaching the photo multiplier tube and reduces the signal considerably.

The results examining MTGFP expression demonstrated that it could be used as an effective dominant selectable marker. Cells transfected with the expression vector encoding the MTGFP fusion protein can be efficiently and rapidly selected in medium containing 4 μM Cd^{2+} and 100 μM Zn^{2+}. Selection in 4 μM Cd^{2+} resulted in a pool of cells with a significantly increased level of fluorescence than cells selected in G418 alone.

The heavy metal ions, cadmium and zinc, were used in this system to induce the expression of our target gene. They are also used to apply selection/amplification pressure on cells to isolate high producers. Cadmium, however, is a toxic heavy metal, which can have detrimental effects on cell growth and, from a regulatory viewpoint, would be good to avoid addition of during the production phase. Studies performed in our laboratory have shown that expression of hGH using the pNK expression vector can be maintained in large-scale culture without the addition of cadmium (Huang et al., 2002). The production of hGH was preserved with the addition of zinc (150 μM), with no detrimental effect on cell growth or productivity.

MTGFP can be used as a dominant and visual selectable marker for coexpressed genes. To evaluate the use of MTGFP as a selectable marker for enriching high-producing clones, CAT was coexpressed with MTGFP under the control of the metal-inducible M2.6 promoter (pMTGFP/CAT). After transfection into CHO cells, clones were selected in medium containing various concentrations of zinc and cadmium with or without G418, as described in the previous section.

The flow cytometry profiles of surviving transfected cells following the 8-day selection were similar to those presented in Figure 2. There was also a dramatic enrichment of fluorescent cells with concentrations of 4 μM Cd^{2+} / 100 μM Zn^{2+} or higher. However, at the higher concentrations of cadmium (>4 μM) cytotoxicity was also too great, as previously observed. Therefore, subsequent selections were carried out using only 4 μM Cd^{2+} / 100 μM Zn^{2+}.

CAT expression also increased at these concentrations of metal (Fig. 3), reflecting the fluorescent measurements. However, in the presence of G418 CAT expression was significantly higher. These results indicated that cells transfected with the gene encoding the MTGFP fusion protein could be efficiently selected in metal, giving enrichment of cells with high fluorescence and high productivity. This

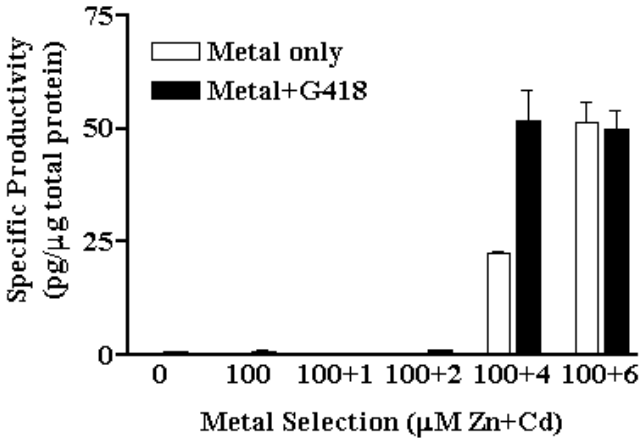

Figure 3. Expression of MTGFP/CAT: Cells were transfected with pMTGFP/CAT and the level of CAT expression was determined by ELISA, from cells selected in metal − G418 (white bars) or metal + G418 (solid bars). The metal concentrations include 100 μM $ZnSO_4$ and 0, 1, 2, 4, and 6 μM $CdCl_2$.

dramatic increase in expression is presumably due to the positive selective pressure provided by the metal rather than by the induction of M2.6, since cadmium concentrations of less than 2 μM are sufficient to induce this promoter (Bailey et al., 1999). It appears that a subpopulation of cells may have been selected at 4 μM cadmium, demonstrating that the rapid selection of a small but relevant subpopulation can be achieved using 4 μM Cd^{2+} / 100 μM Zn^{2+}.

By further increasing the exposure of transfectants to higher concentrations of cadmium, productivity of the recombinant protein can be increased. Previously, we have expressed CAT in the pNK vector (Bailey et al., 1999) and pools of cells resistant to 120 μM cadmium showed a 500-fold increase in *CAT* gene expression over the initial levels. However, stepwise selection to such high levels of cadmium can give rise to resistant populations, with loss of productivity (Kushner et al., 1990). A similar pattern is also seen with the DHFR system, where increases in MTX concentration often resulted in loss of specific productivity (Wurm, 1990; Kaufman et al., 1985). The advantage, however, with the MTGFP fusion system is that amplification with higher concentrations of cadmium can still be performed and the GFP component allows identification of the high producers and isolation through flow sorting.

Expression of hGH Correlates With GFP Fluorescence

CHO cells were transfected with pMTGFP/hGH and subjected to selection in either G418 (400 μg/ml) or metal (4 μM Cd^{2+} / 100 μM Zn^{2+}). After 5 days selection, cells were analyzed by flow cytometry and gated according to their relative fluorescent intensities of 10^1, 10^2, 10^3, and 10^4. The data presented in Figure 4A shows that metal selection resulted in an average relative fluorescence of 4500, whereas G418 selection resulted in only an average relative fluorescence of 345. This again indicates that metal selection is faster and more efficient at isolating higher fluorescent cells than G418 alone.

MTGFP/hGH transfected cells were also sterile sorted into 96-well plates, at one cell per well, within the gated regions shown in Figure 4A. Fifty-two clones with varying fluorescence intensities were collected and the cells grown to confluence. The level of hGH was then measured from the conditioned media by ELISA and plotted against the respective GFP fluorescence as measured using the fluorometer. Figure 4B shows hGH productivity for each clone as a function of GFP fluorescence. The data clearly identified an association between hGH-specific productivity and GFP fluorescence (correlation coefficient, $R^2 = 0.6137$), where cells sorted on the basis of high GFP fluorescence showed high specific productivity of hGH and cells sorted on lower fluorescence produced lower levels of hGH.

The data in Figure 4B also show that the individual clones originally sorted within the four gated regions seemed to maintain their division of fluorescence. Subsequent rounds of flow cytometry confirmed that each clone

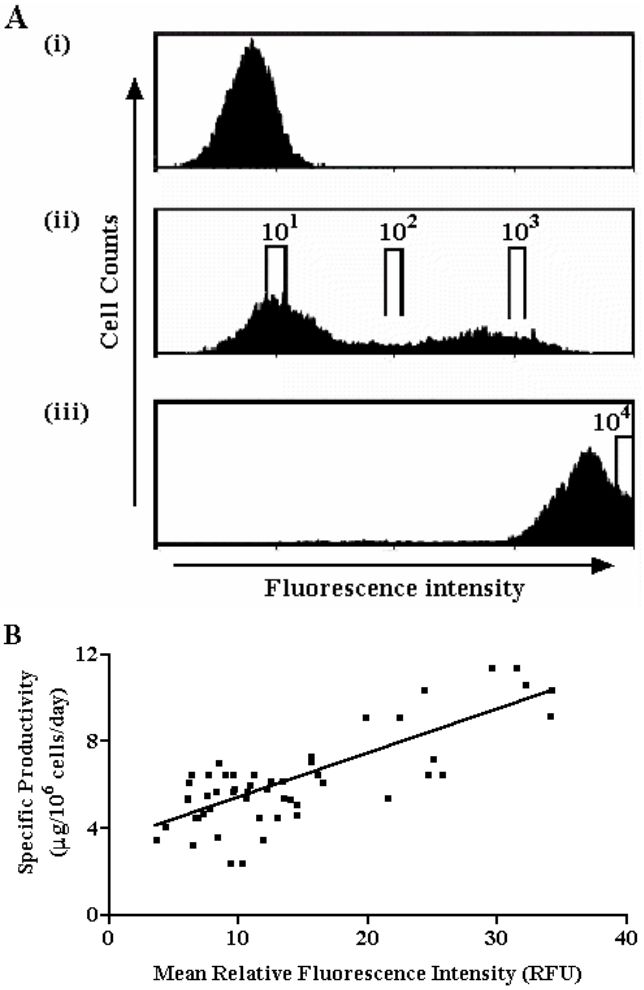

Figure 4. Expression of MTGFP/hGH: Cells transfected with pMTGFP/hGH were selected in media containing metal ± G418. **A:** Flow cytometric profiles for GFP expression were obtained from (i) untransfected cells, (ii) cells selected in media + G418, or (iii) cells selected only in metal (4 μM $CdCl_2$ + 100 μM $ZnSO_4$). Gates were set at 10^1, 10^2, 10^3, and 10^4 and cells were sorted within each gate into 96-well microtiter plates. **B:** Conditioned media was collected from the sorted clones and the level of hGH was determined by ELISA. The specific productivity of 52 sorted clones was plotted against the RFU for each clone, which was determined using the fluorometer (correlation coefficient, $R^2 = 6.6137$).

displayed a well-defined fluorescence peak at the intensity that it was originally sorted (data not shown). The profiles remained the same after a further 2 weeks in culture and did not change when frozen and recultured.

Relative Measure of Fluorescence – Flow Cytometer vs. Fluorometer

Another observation made with the MTGFP/hGH clones was that the mean fluorescence as measured by flow cytometry seemed to correspond well with the fluorescence as measured by the fluorometer. A further experiment was then performed to demonstrate the potential for using the fluorometer to measure differences in fluorescence of the clonal isolates. If the fluorescence determined by the flow cytom-

eter could be reproduced in the fluorometer, this would simplify and speed up the screening process.

Figure 5 shows the average fluorescence values obtained from clones after flow cytometric analysis compared to the data from the fluorometer, following selection in the different media. Although the units of fluorescence are different, the data shows that the average fluorescence correlates very well between the flow cytometer and fluorometer. This demonstrates that the fluorometer is an extremely useful tool that complements the screening and sorting capabilities of flow cytometry. Cells can be analyzed in vitro, allowing measurements of fluorescence intensities to be determined noninvasively, and since we have shown a correlation between fluorescence intensity and recombinant protein expression levels, the fluorometer would speed up the screening of high-producing clones.

This study has also established that the expression of GFP is nontoxic to CHO cells. In fact, viable cells were successfully sorted based on GFP expression and maintained in culture. Although the presence of GFP may cause concern, it is not secreted in our system and any release of GFP from dead cells could be successfully removed by conventional purification procedures.

High-Throughput Selection Protocol for High-Producing Clones

In this investigation, we have described a quick method for selecting high-producing clones. A flow diagram depicting this process is presented in Figure 6. This method presumes correlation between GFP fluorescence and specific recombinant protein productivity, which must be tested for every gene subcloned into pMTGFP. In summary, CHO cells transfected with a vector pMTGFP also containing a desired gene are selected in metal in the presence or absence of G418. The recommended concentration of metal would be 4 μM Cd^{2+} and 100 μM Zn^{2+}. Highly fluorescent cells are then identified and sorted by FACS into microtiter plates and grown to confluence. An alternative protocol avoiding the requirement of FACS would involve limiting dilution into the microtiter plates. Further amplification of gene expression using metal could be performed at this stage to boost specific productivity, as described in Bailey et al. (1999).

The plates are then monitored using a fluorometer and since the fluorescence resulting from the expression of GFP seems to be a reliable indicator of productivity, the highest fluorescent clones can be picked for expansion and further analysis. At this stage, an ELISA could be used to determine the expression levels of recombinant protein from the conditioned media. Using the protocol depicted in Figure 6, we isolated clones with a specific productivity of >10 μg hGH/10^6 cells/day within 4 weeks of transfection.

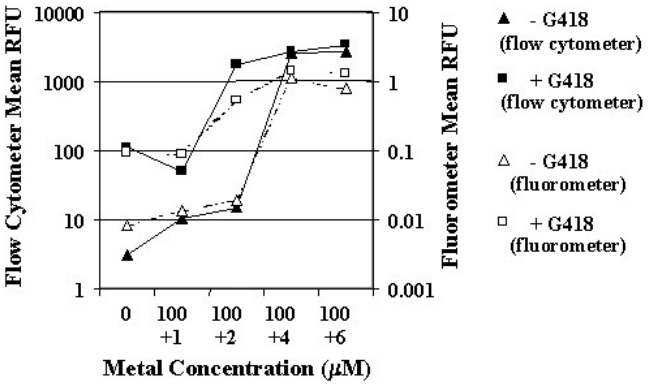

Figure 5. Comparison of fluorescence: MTGFP transfected cells were selected in media containing 100 μM $ZnSO_4$ and increasing concentrations of $CdCl_2$ (1–6 μM) ± G418. Following selection, cells were harvested and fluorescence was measured using either flow cytometry or cells were seeded into microtiter plates and analyzed in the fluorometer.

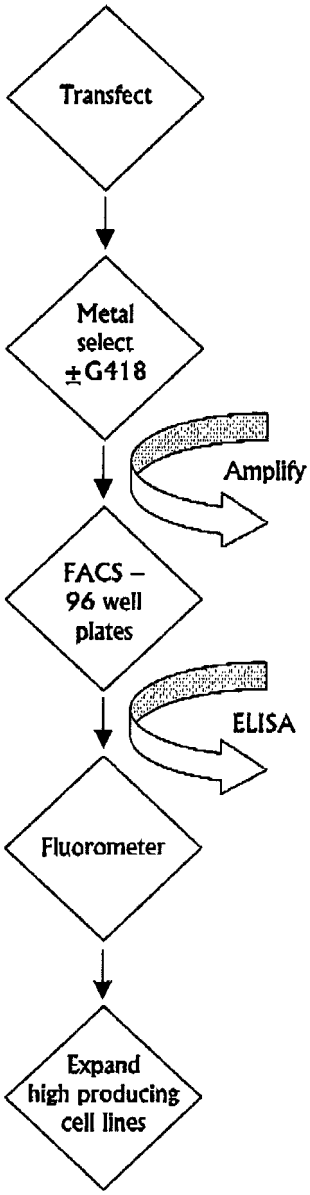

Figure 6. Figure 6. Schematic of the selection protocol. This is a representation of the high-throughput screening protocol developed in this investigation to rapidly isolate high-producing cell lines.

CONCLUSION

We have described a high-throughput screening method for identification and isolation of high-producing clones using the MTGFP marker and flow cytometry. The use of this fusion marker combines selection and amplification properties, allowing efficient visual monitoring of foreign gene-positive clones with high expression levels. The advantage of this method is that it can be easily adapted for automation using robotic systems capable of selecting the highest producing clones among tens of thousands of transfected cells. We have also demonstrated that fluorescence correlates with productivity, where high-producing cells can be identified according to their fluorescent levels.

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