

# Accelerated Cell Line Development Using Two-Color Fluorescence Activated Cell Sorting to Select Highly Expressing Antibody-Producing Clones

Robert J. Sleiman,<sup>1</sup> Peter P. Gray,<sup>1</sup> Martin N. McCall,<sup>1</sup> Joe Codamo,<sup>1</sup>  
Noelle-Ann S. Sunstrom<sup>1,2</sup>

<sup>1</sup>ACYTE Biotech Pty Ltd., University of New South Wales, Sydney, NSW, Australia;  
e-mail: info@acyte.com

<sup>2</sup>NeuClone Pty Ltd., P.O. Box 6378, UNSW, Sydney, NSW 1466, Australia;  
telephone: +614-31-950-517; fax: +612-9661-3992; e-mail: n.sunstrom@neuclone.com

Received 2 January 2007; revision received 19 April 2007; accepted 23 July 2007

Published online 6 August 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21612

**ABSTRACT:** The success of engineered monoclonal antibodies as biopharmaceuticals has generated considerable interest in strategies designed to accelerate development of antibody expressing cell lines. Stable mammalian cell lines that express therapeutic antibodies at high levels typically take 6–12 months to develop. Here we describe a novel method to accelerate selection of cells expressing recombinant proteins (e.g., antibodies) using multiparameter fluorescence activated cell sorting (FACS) in association with dual intracellular autofluorescent reporter proteins. The method is co-factor-independent and does not require complex sample preparation. Chinese hamster ovary (CHO) clones expressing high levels of recombinant antibody were selected on the basis of a two-color FACS sorting strategy using heavy and light chain-specific fluorescent reporter proteins. We were able to establish within 12 weeks of transfection cell lines with greater than a 38-fold increase in antibody production when compared to the pool from which they were isolated, following a single round of FACS. The method provides a robust strategy to accelerate selection and characterization of clones and builds a foundation for a predictive model of specific productivity based upon two-color fluorescence.

Biotechnol. Bioeng. 2008;99: 578–587.

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**KEYWORDS:** CHO; fluorescence activated cell sorting; GFP; selection; recombinant antibody

past decade (Boulianne et al., 1984; Kain et al., 1995; Patterson and Lippincott-Schwartz, 2002; Reichert, 2000, 2001). Production efficiency is influenced by the choice of host cell, expression system, and method of identifying and selecting high producing cell lines. Mammalian cell lines such as Chinese hamster ovary (CHO) offer distinct advantages (reviewed in Wurm, 2004), including the ability to grow to high cell densities in serum-free media (Sinacore et al., 2000). Expression systems used in CHO include those where selection is based on expression of either dihydrofolate reductase (DHFR; Alt et al., 1978), glutamine synthetase (GS; Bebbington et al., 1992), or human metallothionein gene (hMT; Bailey et al., 1999). Moreover, the use of hMT has been extended to include a selective marker, green fluorescent protein (GFP) to facilitate both the screening of recombinant gene-positive clones and identification of high producers using flow cytometry (Bailey et al., 1999, 2002).

Since its discovery almost half a century ago (Shimomura et al., 1962), GFP, the humanized variant, eGFP (Kain et al., 1995), and other autofluorescent protein reporters have proven to be of great benefit to biological research (Chudakov et al., 2005). Recent advances include their use as photoactivatable reporters (Patterson and Lippincott-Schwartz, 2002; Verkhusha and Sorokin, 2005) and biosensors (Choe et al., 2005; Griesbeck, 2004) for in vivo fluorescent labeling in protein expression. In flow cytometry such autofluorescent proteins offer an alternate method for detecting and selecting

## Introduction

Commercial production of recombinant antibodies for therapeutic use has gained enormous momentum over the

Correspondence to: N.-A.S. Sunstrom

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cells stably expressing recombinant antibodies in a co-factor-independent fashion. A number of elegant strategies using dual-fluorescence reporter systems for high-throughput clone characterization have also been devised involving fusing genes for GFP to the *Discosoma* red fluorescent protein (DsRed) flanking foreign DNA in *E. coli* (Choe et al., 2005). Protein tagging and detection with engineered self-assembling fragments of GFP by flow cytometry has been performed in *E. coli* but not in mammalian cells (Cabantous et al., 2005a,b; Zhang et al., 2004).

The use and application of flow cytometry and in particular high-speed fluorescence activated cell sorting (FACS) in cell-based therapies has also evolved (Edwards et al., 2004; Hawley et al., 2004; Ibrahim and van den Engh, 2003; Muirhead et al., 1985; Young et al., 2005). Its utility in biotechnology to achieve high-producing clones expressing recombinant proteins for human therapeutics is also well characterized (Rieseberg et al., 2001; Sklar, 2005) traditionally relying either upon complex microdrop methodologies (Powell and Weaver, 1990; Weaver et al., 1991) or surface affinity matrix strategies to capture secreted antibody, prior to clonal isolation (Borth et al., 2000; Brezinsky et al., 2003; Carroll and Al-Rubeai, 2004; Holmes and Al-Rubeai, 1999; Manz et al., 1995). These methods, however, are labor intensive requiring multiple complex preparative steps. The use of fluorogenic biarsenical FLASH or ReASH substrates which bind to tetracysteine motifs have provided an alternate labeling strategy for improved detection of transcripts by fluorescence in mammalian cells, although it has not been successfully tested using FACS (Martin et al., 2005). The use of auto-fluorescent proteins as intracellular reporters of antibody expression in biotechnology continues to gain interest as FACS-based technologies evolve. Although simultaneous multiparametric detection of fluorescent reporters by flow has been successfully demonstrated using di- and tri-cistronic expression vectors in CHO and human embryonic 293 cells, respectively (Lucas et al., 1996; Meng et al., 2000; Zhu et al., 1999), Bailey et al. (2002) provided evidence for high-throughput clonal selection of stable cell lines using a dominant selectable and amplifiable metallothionein-GFP fusion protein (Bailey et al., 2002). The use of eGFP as an intracellular reporter of gene expression has been extended to include transient expression systems in CHO cells (Kunaparaju et al., 2005).

The application of chain-specific reporter pairs using two-color intracellular fluorescence by FACS in identifying high-antibody producing clones provides an attractive alternative to single-color immunocytochemical methods of detection. This is underscored by the phenomenon that sufficient intracellular concentrations of light chain are considered a critical determinant in establishing high antibody secretion rates (Bole et al., 1986; Hendershot et al., 1987; Lee et al., 1999; Strutzenberger et al., 1999).

The current work describes a one-step co-factor-independent method relying upon intracellular two-color FACS for the identification and selection of high-antibody producing CHO-cell clones. The method benefits from the

use of a proprietary pNK expression vector encoding a metal-hyperinducible promoter driving expression of the heavy or light chain of a recombinant antibody linked to an attenuated internal ribosome entry sequence (aIRES) for cap-independent translation of fluorescence reporters eYFP and eGFP.

This technique abrogates the need for complex preparative steps or labor-intensive workflows and is contrasted against the traditional 'limiting dilution' (LD) approach. The data presented here provide evidence of an efficient and robust high-throughput method for the identification and selection of high antibody-producing clones.

## Materials and Methods

### Cell Culture of CHO-K1 Cells

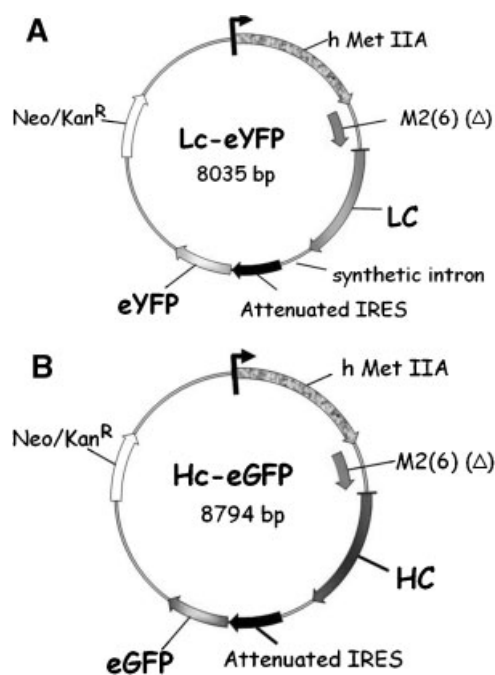
Chinese hamster ovary cell lines CHOK1 (ATCC CCL61), were grown in DMEM/F12 (JRH Biosciences Lenexa, Kansas City, KS) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). CHO-K1 pools were seeded every 72 h to achieve sub-confluent monolayer concentrations of  $5 \times 10^4$  cells per  $\text{cm}^2$  in T25 ( $25 \text{ cm}^2$ ) 'tissue culture treated' flasks (Corning, Sydney, Australia), buffered with  $\text{CO}_2$  gas and incubated at  $37^\circ\text{C}$ . Pools were maintained in continuous culture for no more than 2 months following resuscitation and prior to FACS analysis.

### Plasmids

Plasmids for transfection were constructed from a modified form of the expression vector pNK (Bailey et al., 1999) where DNA encoding a neomycin cassette and the entire human metallothionein IIA gene facilitate plasmid selection and amplification, respectively. The metal-hyperinducible promoter, M2.6 (McNeill et al., 1989), drives expression of the light heavy or light chain of a recombinant antibody. An aIRES (Jackson and Kaminski, 1995) promotes cap-independent translation of fluorescence reporters' eYFP and eGFP (Fig. 1).

### Transfection and G418-Selection

Transfections were facilitated using electroporation. The methodology included addition of DMSO to increase transfection efficiency (Melkonyan et al., 1996) and was adapted to meet the requirements of the cell line studied. Essentially, healthy mid-log CHO-K1 cells were detached using 0.2% EDTA and centrifugated prior to washing with  $1 \times$  PBS to remove residual EDTA. Cell pellets were resuspended at a concentration of  $2.3 \times 10^7$  cells/mL in fresh DMEM/F12 containing 10% FBS and DMSO (final concentration 1.25%, v/v) to achieve cell densities of approximately  $1.4 \times 10^7$  cells ( $600 \mu\text{L}$ ) per cuvette. Suspensions containing 2.5  $\mu\text{g}$  of plasmid DNA (pLc-eYFP and/or pHc-eGFP) or no DNA (mock control) were electroporated at 950  $\mu\text{F}$  and 250 V (square wave pulse),



**Figure 1.** The pLc-eYFP (A) and pHc-eGFP (B) mammalian expression vectors. Metallothionein expression, conferring resistance to Zn and Cd heavy metal ions, is under the control of the wild-type metal-responsive metallothionein IIA promoter (hMet IIA). Light (Lc) and Heavy (Hc) antibody chain expression is driven by a hyperinducible form of the hMet IIA promoter denoted M2(6) (McNeall et al., 1989). An attenuated internal ribosome entry sequence (aIRES) allows cap-independent translation of the fluorescence reporters' eYFP and eGFP (Bailey et al., 1999).

using a Gene Pulser (BioRad, München, Germany). Following electroporation, cultures were allowed to incubate at room temperature for 10-min prior to transfer into 10-mL fresh medium (also containing 1.25% DMSO). Cultures were then split into  $2 \times 5$  mL aliquots and transferred into T25 flasks and allowed to incubate at  $37^\circ\text{C}$  for 48-h prior to media replacement containing  $400 \mu\text{g/mL}$  G418 for 10 days to facilitate selection. Metal amplification of transfected pools commenced following G418-induced death of mock-transfected CHO-K1 control cultures.

### Metal Selection of Transfected Pools

G418-resistant pools were subject to  $100 \mu\text{M}$   $\text{ZnSO}_4$  and  $1 \mu\text{M}$   $\text{CdCl}_2$  heavy metal exposure. Metal-resistant pools were grown to confluence and sub-passaged three times before being subject to step-wise increases in  $\text{CdCl}_2$  of 2, 4, and  $6 \mu\text{M}$ , respectively. Once stable  $100 \mu\text{M}$   $\text{ZnSO}_4$  and  $6 \mu\text{M}$   $\text{CdCl}_2$  (6/100) metal-resistant pools were established, supernatants were collected (prior to FACS analysis) for determination of antibody titers by ELISA and expressed as volumetric (ng/mL) or specific ( $q_p$ ; pg/cell/day) antibody concentrations.

### ELISA

Antibody titers in culture supernatants were determined by ELISA using standard methodologies. Essentially, 96-well

microtitre plates (Sarstedt Australia, Ingle Farm, SA, Australia) were pre-coated with a goat anti-human IgG Fc  $\gamma$ -specific primary antibody (Jackson ImmunoResearch Labs, Inc., Baltimore, MD) and allowed to incubate at room temperature overnight. Following a series of washing steps, wells were blocked using blocking solution (milk,  $1 \times$  PBS and Tween 20) prior to washing. Bound recombinant antibodies were quantified using a TMB substrate (Sigma, St. Louis, MO) following incubation of each well with a sheep anti-kappa light chain HRP-conjugated secondary antibody (The Binding Site, Birmingham, UK) and quantified from a seven serial-dilution series standard curve of human IgG4-kappa light chain (The Binding Site). The TMB substrate reaction was terminated following a 30-min incubation at room temperature with  $2 \text{ M}$   $\text{H}_2\text{SO}_4$  prior to reading at 450 nm using a microplate reader. Data were collected and analyzed using SoftMax Pro analysis software (Molecular Devices Corporation, Sunnyvale, CA).

### FACS and Clonal Isolation

Dual fluorescing cells selected from 6/100 pools were single cell deposited using FACS into five 96-well flat bottom 'tissue culture treated' microplates (Corning) containing  $80 \mu\text{L}$  DMEM/F12 media supplemented with 10% FBS, G418 and 6/100 metal. Clones were allowed to incubate at  $37^\circ\text{C}$  and 5% carbon dioxide in a humidified incubator and supplemented with fresh media after 7 days. Surviving clones (113 in total) were transferred into 24-well plates and allowed to culture with fresh media replacement every 72 h until the monolayer occupied approximately 90–100% of the wells surface area. Light microscopy was used to corroborate for presence of cytoplasmic extensions as an indicator of viability and to estimate the extent of confluence. Confluent clones were seeded at a ratio of 1:2 (v/v) and media replaced 24-h later. Supernatants were removed following 72-h incubation for recombinant antibody quantification and clones detached for cytometric analysis.

FACS was performed using a BD FACSAria cell sorter (Becton Dickinson, San Jose, CA) equipped with an Coherent<sup>®</sup> Sapphire<sup>™</sup> solid state; 13–20 mW (488 nm) argon ion laser and an automatic cell deposition unit (ACDU) for plate sorting. The forward scatter (FSC) photodiode detector was coupled with a 488/10 bandpass and a 1.2 neutral density filter in order to establish the CHO-K1 cell line's FSC dynamic range. EGFP and eYFP emissions were separated using 502LP and 527LP dichroic mirrors (Chroma, Rockingham, VT) and bandpass-limited using HQ510/20 BP and HQ550/30 filters (Chroma), respectively. Propidium iodide (PI) staining was used to identify dead and dying cells and PI emission was collected using a 655LP mirror and a 695/40 bandpass filter. The top 1% dual fluorescing (PI negative) cells emerging from 6/100 metal-induced CHO-K1 pools were subject to low pressure FACS through a  $100 \mu\text{m}$  nozzle and single cell deposited into 96-well plates. Droplet delay values were determined using

standard operating procedures. The window extension was kept constant at 2.0 and event rates were maintained within 10% of the drop drive frequency to maintain stable droplet break-offs.

Single plasmid controls expressing single-color eYFP or eGFP reporters were used to determine the degree of fluorescence spillover. Once PMT voltages were optimized for each single-color control, the measurement was repeated using compensation values to subtract eYFP signals from the eGFP channel and vice versa. Once established, there was no a priori reason to adjust compensation (or PMT voltage) parameters as the excitation source remain unchanged. All fluorescence data were collected in log mode and analyzed using BD FACSDiva™ software (Becton Dickinson).

Frequency histogram class intervals (or bin widths) were calculated using Rice's rule,  $[2(n^{1/3})]$  where  $n$  is the number of observations and plotted accordingly (Beer and Swanepoel, 1999). Frequency histograms and locally weighted least squares (LOWESS) regression analyses were generated using GraphPad Prism (GraphPad Software, San Diego, CA).

### Limiting Dilution Cloning

CHO-K1 6/100 pools were single cell deposited using standard LD methods. Briefly, exponentially growing CHO-K1-cells were allowed to expand in a T25 (25 cm<sup>2</sup>) 'tissue culture treated' flask (Corning) for 48-h prior to detaching with EDTA/PBS. Cultures were centrifuged and cell pellets washed once in 1 × PBS prior to resuspension in fresh pre-warmed DMEM/F12 media (supplemented with 10% FBS, G418 and 6/100 metal) to determine cell density and viability. The cell suspension was diluted to a final cell density of  $1 \times 10^3$  cells per mL and from this, serial dilutions (1:2, v/v) were made until a final cell density of 5 cells per mL was achieved. Finally, 200 μL aliquots were dispensed using an eight-channel pipettor into five flat bottom 96-well 'tissue culture treated' microplates (Corning) and allowed to incubate at 37°C and 5% carbon dioxide in a humidified incubator and supplemented with fresh media after 7 days. Surviving clones (108 in total) were transferred into 24-well plates and allowed to culture with fresh media replacement every 72 h until the monolayer occupied approximately 90–100% of the wells surface area. Light microscopy was used to corroborate for presence of cytoplasmic extensions as an indicator of viability and to estimate the extent of confluence. Confluent clones were seeded at a ratio of 1:2 (v/v) and media replaced 24-h later. Supernatants were removed following 72-h incubation for recombinant antibody quantification and clones detached for cytometric analysis.

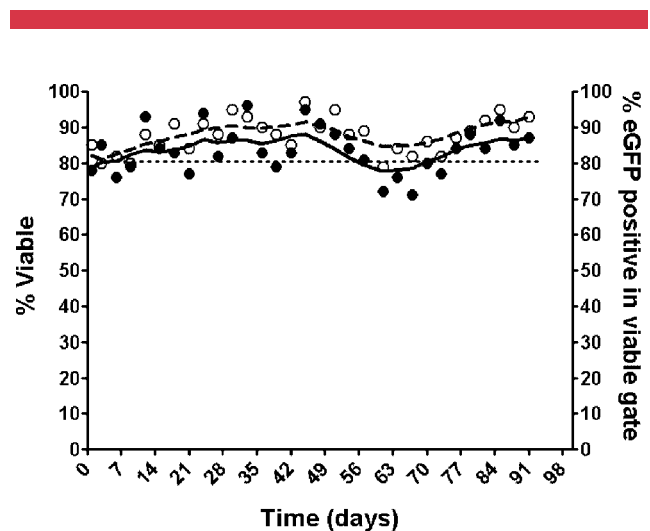
## Results and Discussion

### Continuous Fluorescent Reporter Expression Does Not Affect CHO Cell Viability

The impact of long-term intracellular fluorescent reporter expression on cell viability in an antibody-expressing CHO-

K1 clone co-transfected with pLc-eYFP and pHc-eGFP was examined. While the question of GFP-induced cytotoxicity has been examined by others in a number of cell types using retroviral vectors, these data remain equivocal, with no adverse effects reported in some cases (Hanazono et al., 1997; Kandel et al., 1997; Liu et al., 1999; Wahlfors et al., 2001). Cytotoxicity has been attributed to expression of a C-terminal peptide encoded by the multiple cloning site in pEGFP-C2 but not pEGFP-C1 vectors (Endemann et al., 2003), in 72-h transient transfections. The results presented in this study are predicated on stable eGFP expressing clones subjected to long-term culture conditions.

Plasmids for transfection were constructed from a modified form of the expression vector pNK (Bailey et al., 1999) described in 'Materials and Methods' section. Transfected CHO-K1 cells were selected with G418 over a 2-week period prior to stepwise metal amplification to 6 μM Cadmium and 100 μM Zinc. A FACS-sorted high antibody-expressing clone was maintained in culture over 3 months with supernatants and cell aliquots collected at 72-h intervals for monitoring antibody concentration, viability, and eGFP fluorescence intensity, respectively. The data presented here (Fig. 2) show neither any loss in viability nor decreased antibody production for stable CHO-K1 clones co-expressing eYFP and eGFP reporter pairs when cultured over a 3-month period. Viabilities of untransfected parental CHO-K1 cultures consistently averaged between 80% and



**Figure 2.** Cell viability for a CHO-K1 clone expressing stable eGFP over long-term culture. CHO-K1 cells were co-transfected (by electroporation) with plasmids encoding pLc-eYFP and pHc-eGFP and selected with G418 over a 2-week period prior to stepwise metal amplification. A representative stable clonal isolate (5H6) derived by FACS was maintained over a 3-month period by continuous sub-passaging with supernatant and cell aliquots removed for specific productivity and viability quantitation at 72-h intervals. Viable cell numbers were collected by PI-exclusion gating using flow cytometry to eliminate dead and dying cells. % Viable (closed circles) and % eGFP positive in viable gate (open circles) were derived from the acquisition of 10,000 cells for each time point. Locally weighted least squares (LOWESS) regression analyses for the change in viability and eGFP fluorescence over time are shown as continuous and dashed curves, respectively. Specific antibody productivities between 5 and 7 pg/cell/day were recorded for aliquots with threshold viabilities greater or equal to 80% as denoted by the dotted line.

95% throughout the study (data not shown). Specific antibody productivity as determined by ELISA was not adversely affected and typically ranged from 5 to 7 pg/cell/day over the study period for at least 70% of samples taken. The remainder where viabilities of less than 80% were observed had decreased productivities typically between 4 and 5 pg/cell/day (data not shown). In addition, a LOWESS regression analysis provided evidence for a correlate between extended eGFP expression and cell viability.

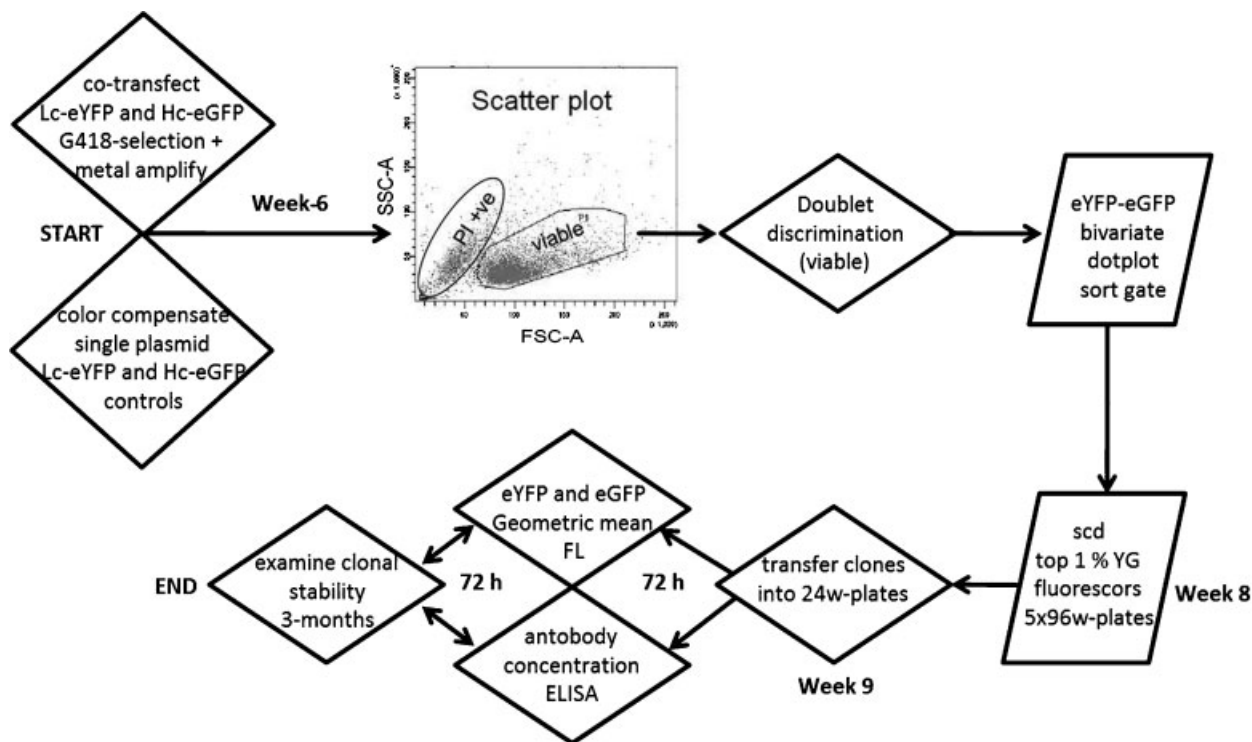
### Use of Stable Intracellular Fluorescence and FACS Reduces Clonal Isolation Workflow

The rapid method for selecting high-producing clones using two-color intracellular fluorescence and FACS is outlined in Figure 3. It is distinguished from other FACS-based methods by abrogating the requirement for complex microdrop or surface affinity matrix preparations to capture secreted antibody, prior to clonal isolation (Borth et al., 2000; Brezinsky et al., 2003; Carroll and Al-Rubeai, 2004; Holmes and Al-Rubeai, 1999; Manz et al., 1995). The workflow

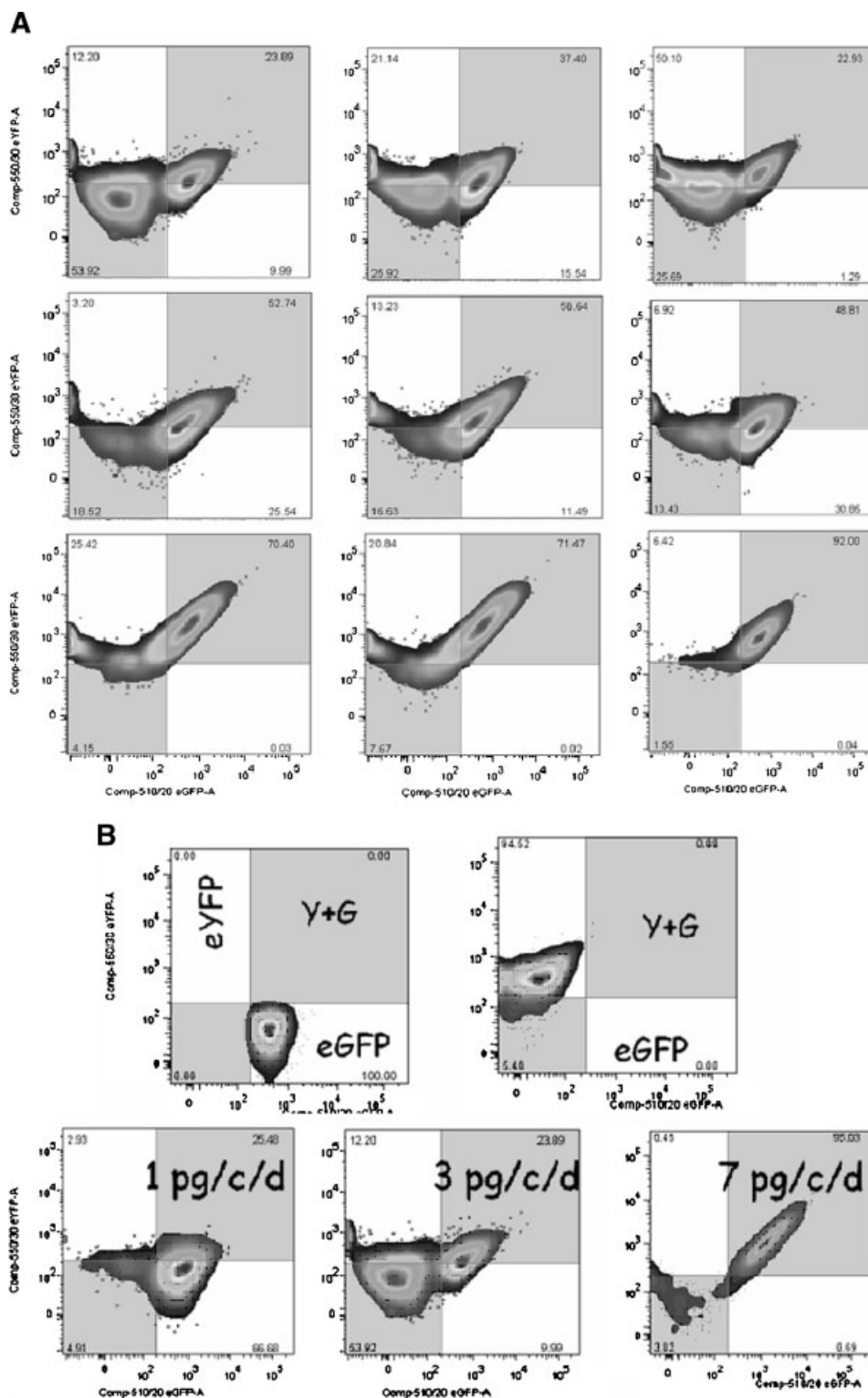
presented here is both rapid and simple, requiring only a single FACS-sort step to derive clonal cell lines and an ELISA step to measure rates of antibody production. The workflow was designed to limit the number of cell-based manipulations required to derive clonal lines from stable transfected pools.

The identification of a viable population prior to sorting using scatter parameters, FSC and SSC, was crucial for improving plating efficiencies and minimizing the incidence of false positive clones due to autofluorescence emerging from dead and dying cells (data not shown). Once decided, all gates remained fixed in position to facilitate easier identification of variances between FACS-derived clones. Plating efficiencies averaged 60% with approximately 8% of clones emerging as single-color fluorescours. (The timeline from initial transfection to isolation of high antibody-producing stable cell lines was 10 weeks, with a further 2 weeks required for expansion from 24-well to 6-well plates of representative clones for validation of clonal stability.)

The method described in Figure 3 relies upon a testable hypothesis which assumes a correlation between reporter protein fluorescent intensity and the specific recombinant



**Figure 3.** FACS workflow. 1. Parental CHO-K1 cells were either co-transfected with pLc-eYFP and pHc-eGFP encoding plasmids for single cell deposition (scd) by FACS or with single plasmids as fluorescence compensation controls. Metal amplification following G418 selection continued in a step-wise fashion until the emergence of a dual (YG) fluorescing population was observed in co-transfected parental pools, using flow cytometry. 2. Viable populations were identified using back-color-gating of PI positive cells into the FSC-SSC scatter plot. 3. Singlet populations were identified using pulse-width/height doublet discrimination and eYFP-eGFP sort gates projected, accordingly. 4. Single cell depositions by FACS of the top 1% YG fluorescours were prepared into 96-well plates and emerging clones allowed to subculture in the presence of metal for 7-day prior to transfer into 24-well plates (5). 6. Supernatants were removed 72-h later to evaluate antibody concentrations using ELISA. Cell suspensions from sorted clones were prepared to evaluate changes in geometric mean fluorescence intensities of each reporter and these data were correlated against antibody productivities, accordingly. 7. Highest producing clones (5H6 and C68) were subject to reiterative subculture for antibody production and reporter fluorescence analysis over a 3-month period to ascertain clonal stability.

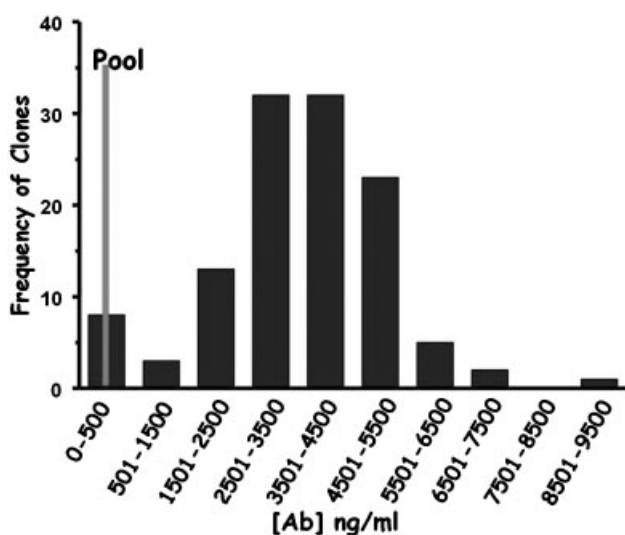


**Figure 4.** Contour density plots of eYFP (ordinate) and eGFP (abscissa) fluorescence from FAC-sorted antibody expressing clones. **Panel A:** Three representative clones from each of low (top row), median (middle row), and high (bottom row) dual fluorescence intensities are shown for comparison. **Panel B:** Specific productivity data (expressed as pg/cell/day and derived by ELISA) from single plasmid pools (top row) and co-transfected clones. All fluorescence data are shown compensated against single-color controls.

protein production. Indeed, the requirement for co-expression of autofluorescent reporters is a necessary and sufficient prerequisite for identifying antibody-producing clones by FACS.

### Chain-Specific Fluorescence: A Quantitative Determinant of Antibody Production

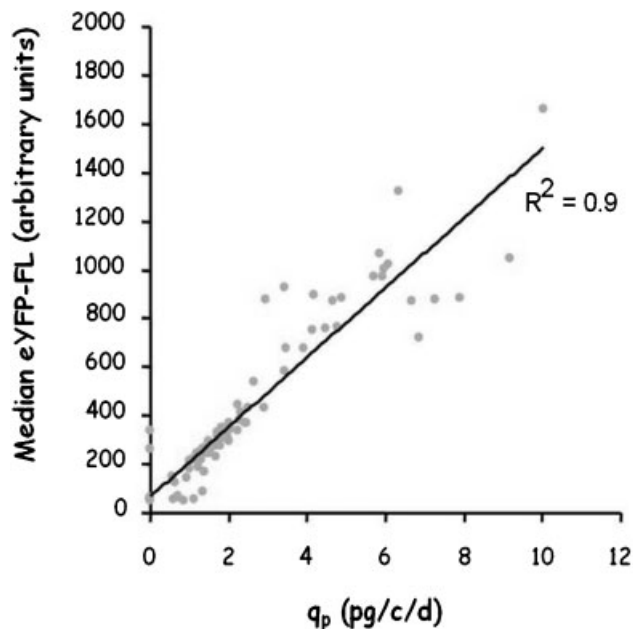
Several clones (113) were selected for further classification from a total of 280 clones isolated following single cell deposition by FACS. A representative proportion of these are displayed as contour density plots showing low, median, and high dual fluorescence intensities in Figure 4A. These data correlate with the 25th, 50th, and 75th percentile intervals for antibody production represented in the frequency histogram in Figure 5. Metal amplified pools encoding a single antibody chain act as single-color controls and do not express antibody, while clones displaying an emerging dual fluorescence profile, show a commensurate increase in antibody production (Fig. 4B). Accordingly, these data (particularly the 1 pg/cell/day plot) provide evidence for asymmetric distributions of fluorescence amongst some clones. This phenomenon may be a function of variables such as gene copy number, mRNA stability, posttranslational modification, kappa-chain concentration, and epigenetic factors such as oxidative stress. Taken together, these data provide evidence for intracellular reporter fluorescence occurring in tandem, although not necessarily in equimolar ratio, as quantitative determinants of antibody production.



**Figure 5.** Frequency distribution of antibody concentrations from 113 FACS-sorted clones. Clones from 24-well plate cultures were seeded at approximately  $3 \times 10^5$  cells per mL and supernatants removed 72-h later to establish antibody concentrations (ng/mL) by ELISA. Parental pool productivities are denoted by a vertical line.

### Clonal Isolation by FACS: Dual Intracellular Fluorescence Intensity as a Predictor of High-Antibody Producing Clones

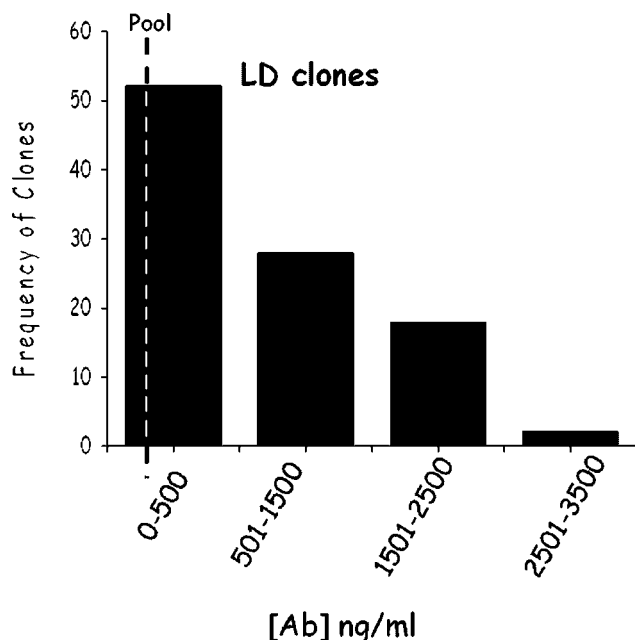
FACS-sorted clones were expanded into 24-well plates and supernatants removed 72-h later to establish antibody concentration. Accordingly, these data were ranked and plotted in a frequency histogram format to assist in characterizing the antibody productivity among clones (Fig. 5). The data describe a normal or 'Gaussian' distribution with clones within the 25th, 50th, and 75th percentile intervals yielding antibody concentrations at 2,500, 4,000, and 9,500 ng/mL, respectively. Antibody concentrations comparing the parental pool with the 75th percentile interval were increased 30-fold. The relationship between the median fluorescence intensity for each intracellular fluorescent reporter and specific productivity was examined for each FACS-derived clone (Fig. 6). As the median fluorescence intensity increased, specific productivity also increased. Pearson correlation coefficients provide evidence that the relationship is correlated. Interestingly, a large proportion of clones also had slightly lower median fluorescence intensities for eYFP compared with eGFP. Clones intersecting the  $y$ -intercept represent single-fluorescing candidates and not surprisingly, their  $q_p$  were zero indicating that simultaneous eYFP and eGFP intracellular fluorescence is a necessary determinant of antibody production.



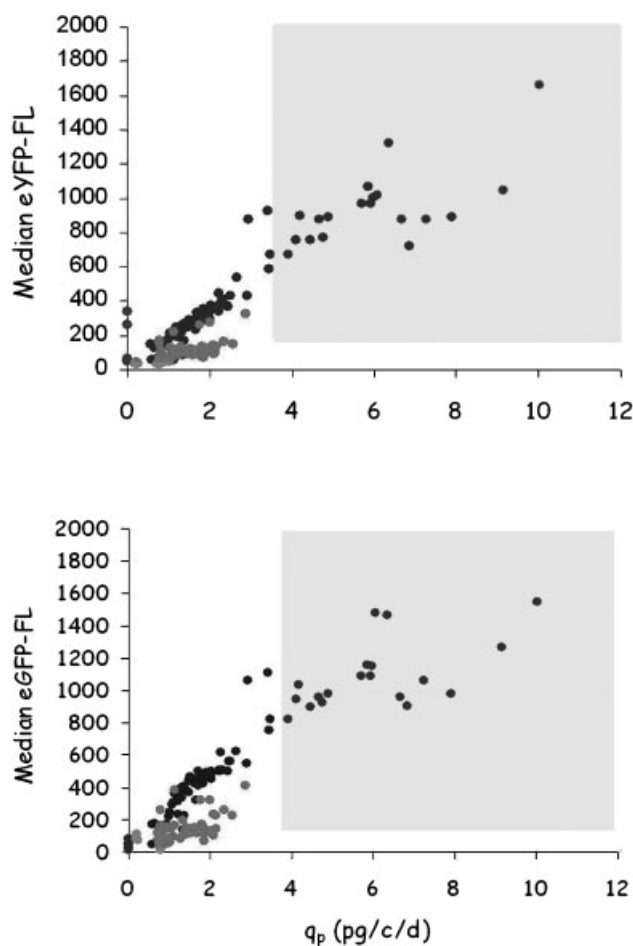
**Figure 6.** Relationship between specific antibody productivity and median intracellular reporter fluorescence in FACS-sorted clones. Supernatants from 24-well plate clones were removed 72 h after seeding and subject to ELISA to determine specific productivity ( $q_p$ ). Median intracellular reporter fluorescence data were derived by flow cytometry and  $x, y$  ( $Q_p, FL$ ) pairs for each clone were plotted to establish correlation coefficients.

## Limiting Dilution Is Less Efficient Than FACS for the Isolation of High Antibody-Producing Clones

For comparison, an analysis of antibody concentrations from clones derived by LD was made (Fig. 7). Accordingly, 108 clonal isolates were seeded at approximately  $3 \times 10^5$  cells per well into 24-well plates and supernatants removed 72-h later to determine clonally-derived volumetric antibody concentrations (ng/mL) compared to the unsorted parental pool. Productivity data were then paired with the dual color fluorescence data for each clone. The LD data were not normally distributed, as expected, failing the Kolmogorov–Smirnov Test for normality (KS Distance 0.243, data not shown). Approximately half of all clones examined had productivities between 0 and 500 ng/mL with the remainder expressing between the 501 and 2,500 ng/mL. Only 1.8% of clones fell within the 2,501–3,500 ng/mL antibody concentration interval compared to 33% in the same interval for FACS-sorted clones (Fig. 5). Of particular interest, no LD clones produced greater than 3,500 ng/mL compared to 58% of FACS-derived clones producing antibody concentrations between 3,501 and 9,500 ng/mL. Indeed, the distinction is most apparent when an overlay of the mean fluorescence and specific productivity data are made (Fig. 8). LD derived clones are distinguished by the absence of antibody producing clones greater than 2.4 pg/cell/day. However, FACS-derived clones' using two-color intracellular fluorescence as a determinant of antibody production extended well beyond the LD-threshold and in one case reached 10 pg/cell/day.



**Figure 7.** Frequency distribution for antibody concentrations from clones derived using limiting dilution (LD) method. All clones were seeded at approximately  $3 \times 10^5$  cells per mL into 24-well plates and supernatants removed 72-h later to determine volumetric antibody concentrations (ng/mL) by ELISA. Parental pool productivities are denoted by a dashed vertical line.



**Figure 8.** Comparative analysis of mean intracellular eYFP and eGFP reporter fluorescence and specific antibody productivity between LD and FACS-derived clones. LD and FACS-derived clones are depicted by light and dark circles, respectively. Shaded squares demarcate high antibody-producing clones.

Of interest, is the variability in mean fluorescence intensity (and therefore heterologous antibody expression) between clones, irrespective of the clonal isolation method. While the diversity is less marked in LD-derived clones, the phenomenon nevertheless may be attributed to a number of factors. A physiological determinant of heterologous protein expression is considered by some to be a result of differential growth rates between cells with different expression levels, or the slow adaptation of high producers when cultured at low density (Brezinsky et al., 2003). Another possibility considers that heavy chain overproduction, if not secreted, associates with BiP a HSP70 molecular chaperone causing their retention within the ER, possibly leading to increased toxicity (Hendershot et al., 1987). In another embodiment, light chain synthesis is thought to play a critical role in removing BiP from the first constant domains of heavy chains, thereby allowing correct folding and antibody assembly to proceed (Bole et al., 1986; Lee et al., 1999).

Another determinant of intracellular antibody expression is cell cycle status. While cell-cycle specific rates of

immunoglobulin protein accumulation in exponentially growing murine hybridoma cells have been demonstrated (Kromenaker and Srienc, 1994), the observation in other mammalian cells is less clear (al-Rubeai et al., 1992; Lloyd et al., 1999). Although correlations between proportion of S phase cells and intracellular antibody accumulation and specific productivity in CHO cultures have been reported, in batch cultures these are only achieved at late stages of exponential phase with the batch phase/medium being the major determinant of maximum specific productivity and intracellular protein concentration (Lloyd et al., 1999). Indeed, the FACS-based strategy described here is predicated on the identification and selection of clonal candidates emerging from asynchronously dividing pools. Asynchronously dividing clones, however, provide a closer estimation of the association between maximum specific productivity (assuming steady-state secretion kinetics) and antibody chain-specific reporter fluorescence.

## Conclusion

We have described a robust high-throughput screening method for the identification and isolation of high antibody-producing clones using two-color intracellular fluorescence reporters and FACS. The method is rapid and co-factor-independent relying only upon standard selection/amplification strategies and the availability of a single 488-nm wavelength excitation source prevalent in all modern-day cytometers. Moreover, the association of one fluorescent reporter for each antibody chain provides a single-step approach for the identification and isolation of high-producing clones without the need for complex antibody-antigen-specific manipulations. Finally, the method provides preliminary evidence for establishing a predictive model of antibody expression by FACS.

The authors would like to thank Mr. Frederick Luk for his assistance in the preparation and maintenance of limiting dilution clones.

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