Journal of Bioscience and Bioengineering
VOL. 120 No. 3, 340–346, 2015

ELSEVIER

www.elsevier.com/locate/jbiosc

Efficient enrichment of high-producing recombinant Chinese hamster ovary cells for monoclonal antibody by flow cytometry

Takeshi Okumura,[1,2,*] Kenji Masuda,[1] Kazuhiko Watanabe,[1] Kenji Miyadai,[1] Koichi Nonaka,[1] Masayuki Yabuta,[1] and Takeshi Omasa[2]

R&D Division, Daiichi Sankyo Co., Ltd., Gunma 370-0503, Japan[1] *and Institute of Technology and Science, The University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan*[2]

Received 14 October 2014; accepted 7 January 2015
Available online 12 February 2015

To screen a high-producing recombinant Chinese hamster ovary (CHO) cell from transfected cells is generally laborious and time-consuming. We developed an efficient enrichment strategy for high-producing cell screening using flow cytometry (FCM). A stable pool that had possibly shown a huge variety of monoclonal antibody (mAb) expression levels was prepared by transfection of an expression vector for mAb production to a CHO cell. To enrich high-producing cells derived from a stable pool stained with a fluorescent-labeled antibody that binds to mAb presented on the cell surface, we set the cell size and intracellular density gates based on forward scatter (FSC) and side scatter (SSC), and collected the brightest 5% of fluorescein isothiocyanate (FITC)-positive cells from each group by FCM. The final product concentration in a fed-batch culture of cells sorted without FSC and SSC gates was 1.2–1.3-times higher than that of unsorted cells, whereas that of cells gated by FSC and SSC was 3.4–4.7-fold higher than unsorted cells. Surprisingly, the fraction with the highest final product concentration indicated the smallest value of FSC and SSC, and the middle value of fluorescence intensity among all fractionated cells. Our results showed that our new screening strategy by FCM based on FSC and SSC gates could achieve an efficient enrichment of high-producing cells with the smallest value of FSC and SSC.

[**Key words:** Monoclonal antibody; Chinese hamster ovary cell; Cell line development; Flow cytometry; Side scatter; Forward scatter]

The Chinese hamster ovary (CHO) cell is widely used for the production of therapeutic proteins such as monoclonal antibody (mAb) (1–6). Many expression systems in CHO cells have been developed to improve the productivity of target proteins for reducing the cost of goods. In the current available expression systems, glutamine synthetase (GS) (7), dihydrofolate reductase (DHFR) (8,9) and some antibiotic resistance genes (10,11) are used as a selective marker to isolate a transfectant which harbors an expression vector. Also, promoters such as SV40 (12,13), CMV (14,15), and elongation factor-1 (EF-1) (16,17), and functional DNA elements that enable improvement of productivity and genetic stability such as ubiquitous chromatin opening elements (UCOE) (18,19) and matrix attachment regions (MAR) (20,21) are also used to construct an expression vector. However, the expression level and genetic stability of genes introduced in the isolated transfectant are unpredictable due to a position effect by a random integration in many cases (22,23). Screening of a desired cell that is genetically stable and shows higher productivity with preferable quality is generally laborious and time-consuming. On the other hand, site-specific recombination techniques might provide a predictable expression level and genetic stability of genes of interest (GOI) in a transfectant obtained from minimum screening for the isolation of a higher producing cell (24–28). However, the cells constructed by the site-specific recombination technique have not provided satisfactory productivity because productivity from a single copy of GOI is generally lower than that from amplified multiple copies of GOI (29). So far, a random integration technique has been widely applied to isolate high-producing cells with laborious screening although it is not possible to evaluate productivity in all obtained transfectants. Therefore, high throughput screening/evaluation systems are required to isolate a desired high producing cell.

Flow cytometry (FCM) is able to perform a high throughput screening by continuous cell sorting based on intra- or extracellular fluorescein detection. In fact, some FCM-based screening strategies for the isolation of high-producing cells have been reported by some research groups (30–34). Meng et al. (30) and DeMaria et al. (31) suggested a method for selection of high-producing cell using coexpressed green fluorescent protein and cell surface protein CD20, respectively, as a reporter. It is an unfavorable method to express untargeted protein as impurity in biopharmaceutical manufacturing. Yoshikawara et al. (32) used fluorescein methotrexate, which is a chemical compound that is easy to remove and binds DHFR, as a reporter to screen high-producing cells. However, this method has efficacy only for the expression system that uses DHFR as a selective marker. Moreover, these three methods are an indirect method by using reporter. On the other hand, Brezinsky et al. (33) demonstrated that the secreted antibody as a target is transiently present on the cell surface and it can be

* Corresponding author at: R&D Division, Daiichi Sankyo Co., Ltd., Gunma, 370-0503, Japan. Tel./fax: +81 276 86 7357.
E-mail address: okumura.takeshi.m3@daiichisankyo.co.jp (T. Okumura).

1389-1723/$ – see front matter © 2015, The Society for Biotechnology, Japan. All rights reserved.
http://dx.doi.org/10.1016/j.jbiosc.2015.01.007

stained with a fluorescence anti-IgG antibody. It is also useful for screening high-producing cells, however, cell size is not regarded in this method. Shi et al. (34) focused on cell size and intracellular density, and set a gate based on forward scatter (FSC) versus side scatter (SSC) for screening, however, this was only for removing dead cells. We predicted that fluorescence intensity might have depended on the cell size because larger cells could present a significant amount of expressing mAb on a cell surface and we could improve the screening strategy to enrich high-producing cells by regarding cell size and intracellular density.

In this study, we report a new effective screening strategy for the isolation of higher mAb producing cells using FCM by gating based on FSC and SSC.

MATERIALS AND METHODS

Host cell line CHO−O1, which is derived from CHO−K1 ATCC CCL-61 (American Type Culture Collection, Manassas, VA, USA) and has been adapted to serum-free and suspension culture conditions, was used as a host cell. Cells used for transfection were maintained in CD-CHO (Life Technologies Corporation, Grand Island, NY, USA) with 4 mM alanyl-glutamine (Sigma, St. Louis, MO, USA), 0.2% anti-clumping agent (Life Technologies Corporation) at 37 °C in a 5% CO_2 atmosphere.

Expression vector The double gene vector carrying light chain and heavy chain genes of an mAb, pDSLH4.1, was designed and constructed as an expression vector for a model mAb and used for transfection. An ampicillin resistance marker was used for plasmid propagation in *Escherichia coli*. A neomycin resistance gene was used for transfectant selection in CHO cells. Vector configuration is provided in supportive data (Fig. S1).

Transfection and stable pool preparation The expression vector pDSLH4.1 was transfected to CHO−O1 using Neon Transfection System (Invitrogen, Carlsbad, CA, USA). The transfected cells were cultured in C/E medium, composed of CD-CHO (Life Technologies Corporation) with 40% (v/v) EX-CELL 325 PF (SAFC, St. Louis, MO, USA), 2% (v/v) CHO Feed Bioreactor Supplement (SAFC), 4 mM alanyl-glutamine (Sigma), 10 mg/L HT supplement (Life Technologies Corporation), and 0.2% anti-clumping agent (Life Technologies Corporation) in a T-25 tissue culture flask at 37 °C in a 5% CO_2 atmosphere. Twenty-four hours after transfection, geneticin (Life Technologies Corporation) was added to the culture for transfectant selection. Six to eight days after transfection, the cells were transferred to a 125-mL Erlenmeyer flask and cultured at 37 °C in a 5% CO_2 atmosphere. Fourteen days after transfection, a stable pool which showed a resistance to Geneticin was obtained.

Flow cytometry Cell sorting was performed using BD FACSAria Fusion sorter (Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA). 2×10^8 cells were pelleted by centrifugation for 3 min at 200 ×*g* 4 °C and suspended in cold wash buffer, phosphate-buffered saline (PBS) w/o calcium and magnesium (Life Technologies Corporation) with 2% (w/v) of bovine serum albumin (BSA) (Bovogen Biologicals, Essendon, Victoria, Australia). After recentrifugation, the resulting cell pellet was resuspended in cold wash buffer. The recovered cells were stained with 1328 μg fluorescein isothiocyanate (FITC)-conjugated Goat $F(ab')_2$ Fragment Anti-Human IgG (H + L) (Beckman Coulter Company, Marseille, France) or phycoerythrin (PE)-conjugated Goat $F(ab')_2$ Fragment Anti-Human IgG (H + L) (Beckman Coulter Company) for 30 min at 4 °C. The stained cells were washed and resuspended with cold wash buffer at a density of 5×10^6 cells/mL. The resulting cells were sorted on BD FACSAria Fusion sorter using a laser diode emitting at 488 nm and detecting FITC emission with a 530/30 bandpass filter.

Fed batch culture Sorted cells from a stable pool were evaluated by a fed-batch culture in a 125 mL Erlenmeyer flask. Cell count and viability analyses were conducted using Guava PCA (EMD Millipore Corporation, Hayward, CA, USA). Initial cell density was adjusted at 3×10^5 viable cells/mL in production culture of which the medium was custom-made basal media DA1 (Life Technologies Corporation) with 20 mM HEPES (Life Technologies Corporation) and 4 mM L-glutamine (Life Technologies Corporation). A production culture was performed at 37 °C and 120 rpm in a 5% CO_2 atmosphere for 14 days. Custom-made feed media DAFM3 (Life Technologies Corporation) was added to the production culture at 10% of the working volume on days 4, 6, 8, and 10. Cell density analyzed by Guava easyCyte HT (EMD Millipore Corporation), viability, and antibody concentrations analyzed by ProteinA-HPLC were monitored during cultivation.

Cell cycle analysis Cell cycle analysis was performed by a flow cytometer with Guava Cell Cycle Reagent (EMD Millipore Corporation) according to the manufacturer's instructions. Tested cells were pelleted by centrifugation and suspended in PBS. The recovered cell pellet was resuspended in residual PBS and fixed in ice-cold 70% ethanol for an hour at 4 °C. The fixed cells were washed by PBS and stained with Guava Cell Cycle Reagent. The cell cycle in the stained cells was analyzed by Guava easyCyte HT (EMD Millipore Corporation).

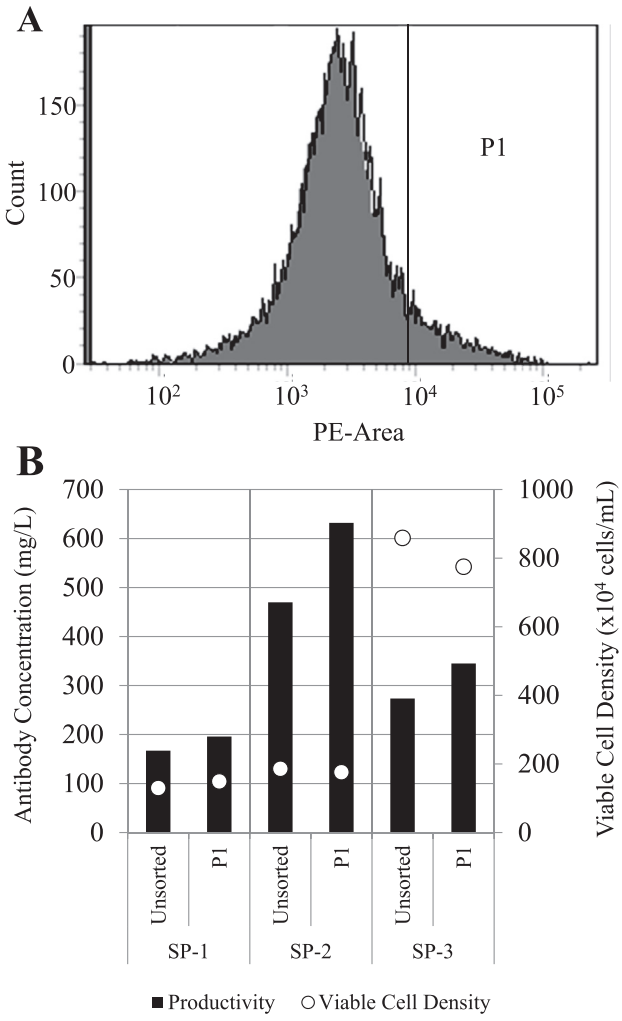

FIG. 1. Simple gate sorting of a stable pool by FCM. (A) A histogram of fluorescence intensity obtained from cells stained with a PE-conjugated anti-IgG antibody in the stable pool (SP-1). P1 means a fraction showing the top 10% fluorescence intensity. (B) Evaluation of final product concentration (closed bars) and viable cell density (open circles) in a fed-batch culture of unsorted (stable pool) and sorted cells (P1 gate fraction) in three stable pools (SP-1, -2, -3).

RESULTS

Cell sorting by simple gating After pDSLH4.1 was transfected to CHO−O1, a stable pool that has a huge variety of GOI expression levels was obtained under selection pressure by Geneticin and sorted by FCM. Transfection was conducted three times, and three stable pools named SP-1, -2, -3 were obtained and sorted. The fraction (P1 gate) which showed the top 10% fluorescence intensity in the cells stained with PE-conjugated anti-IgG antibody was collected and independently expanded (Fig. 1A). The mAb final product concentration of the expanded P1 gate fraction was compared to that of the unsorted cells in a fed-batch culture. The final product concentration of the sorted cells (P1) was 1.2−1.3-times higher than that of unsorted cells (Fig. 1B).

Cell sorting by gating based on FSC and SSC As shown in Fig. 1, the P1 gating fraction, showing the top 10% fluorescence intensity, gave us the possibility to isolate the higher mAb producing cells. However, fluorescence intensity might have