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Optimization of cell line development in the GS-CHO expression system using a high-throughput, single cell-based clone selection system

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Therapeutic antibodies are commonly produced by high-expressing, clonal and recombinant Chinese hamster ovary (CHO) cell lines. Currently, CHO cells dominate as a commercial production host because of their ease of use, established regulatory track record, and safety profile. CHO-K1SV is a suspension, protein-free-adapted CHO-K1-derived cell line employing the glutamine synthetase (GS) gene expression system (GS-CHO expression system). The selection of high-producing mammalian cell lines is a crucial step in process development for the production of therapeutic antibodies. In general, cloning by the limiting dilution method is used to isolate high-producing monoclonal CHO cells. However, the limiting dilution method is time consuming and has a low probability of monoclonality. To minimize the duration and increase the probability of obtaining high-producing clones with high monoclonality, an automated single cell-based clone selector, the ClonePix FL system, is available. In this study, we applied the high-throughput ClonePix FL system for cell line development using CHO-K1SV cells and investigated efficient conditions for single cell-based clone selection. CHO-K1SV cell growth at the pre-picking stage was improved by optimizing the formulation of semi-solid medium. The efficiency of picking and cell growth at the post-picking stage was improved by optimization of the plating time without decreasing the diversity of clones. The conditions for selection, including the medium formulation, were the most important factors for the single cell-based clone selection system to construct a high-producing CHO cell line.

[**Key words:** Chinese hamster ovary cell; Glutamine synthetase; Cell line development; High-throughput selection; Semi-solid media]

Biopharmaceuticals (biologics) are rapidly growing products in the pharmaceutical market. In particular, monoclonal antibodies have shown remarkable sales growth and market penetration, and are currently five of the 10 top selling drugs worldwide. Mammalian cells are the common hosts for commercial production of therapeutic proteins. Among the 58 biologics approved from 2006 to 2010, 32 are produced in mammalian cells (1). Despite the availability of numerous other mammalian cell lines, such as baby hamster kidney, mouse myeloma-derived NS0, human embryonic kidney-293, and human retina-derived PerC6, nearly 70% of all recombinant therapeutic proteins are produced in Chinese hamster ovary (CHO) cells (2–4). In the top 10 selling biologics, which amassed over US $57 billion in sales during 2011, eight are produced by mammalian expression systems, of which only one, Remicade (infliximab), is not produced in engineered CHO cells (5). CHO cells currently dominate as a commercial production host because of their ease of use, strong regulatory track record, and safety profile.

CHO transfectants are selected and expanded by either dihydrofolate reductase (DHFR) in DHFR-deficient cells (6–8) or methionine sulfoximine (MSX) inhibition of the glutamine synthetase (GS) gene in CHO lines. CHO-K1SV was developed by Lonza Biologics, which is a suspension, protein-free-adapted CHO-K1 derivative employing the GS gene expression system. Many therapeutic monoclonal antibodies have been developed using the GS expression system in CHO cells (GS-CHO expression system) (9–13). CHO-K1SV expresses the GS enzyme endogenously. Thus, positive transfectants are obtained by the dual selection of MSX and glutamine-free media. In this system, transfected cells harboring one or more copies of the cloned GS gene are selected in a glutamine-free medium. There are some advantages using the GS expression system over the DHFR system. For example, the GS expression system does not require mutant host cells (9) and, compared with the DHFR system, high-producing mammalian cell lines (high producers) can be more rapidly obtained using the GS system (9). Furthermore, because the GS enzyme facilitates intracellular production of glutamine and transfectants are selected in glutamine-free medium, there is less accumulation of ammonia that is usually a toxic by-product in cell culture processes (14,15).

However, the selection of high producers is a crucial step in process development for the production of biologics. High producers are rare, and those that also satisfy product quality and other selection criteria, such as a high growth rate and expression stability, are much rarer (16). Transfection pools constructed by the random integration method contain numerous variants because of the various integration sites of the exogenous gene. This broad diversity might be caused by disrupted and dysregulated endogenous genes. Clone diversity is very important for cell line development.

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During the early phase of cell line development, it is difficult to evaluate the various characteristics in cell culture, such as monoclonal antibody quality, genetic stability (17), cell growth capability under stirred culture conditions, and the properties of metabolite profiles. Moreover, post-translational modifications differ among biopharmaceutical products (18). These properties are supposed to be evaluated at the late phase of cell line development, but they are mostly determined at the initial transfection (19).

Historically, cloning by the limiting dilution method has been used to isolate monoclonal CHO cells. However, limiting dilution is a laborious and time-consuming process that has a low probability of monoclonality. The limiting dilution method cannot precisely separate high- and non-producers, because the productivity of limiting dilution has to be evaluated after long-term cell culture from a single cell to an appropriate number of expanded cells. The efficiency of this method is significantly dependent on the number of clones that can be feasibly screened (16,20,21). To minimize the screening duration and increase the probability of obtaining high-producing clones with high monoclonality, the most common high-throughput method is fluorescence-activated cell sorting (FACS) (22–24). Although FACS is technically difficult and expensive equipment is necessary, it is a more suitable method for selection of high- and non-producers than limiting dilution. This is because it is a single cell-based clone selection method and directly evaluates cell productivity based on the surface protein expression. However, FACS is not an optimal method for cell clone selection, because FACS measures productivity based on cell surface expression. Cell-surface-based productivity was not exactly the same as the productivity of secreted proteins. For example, through kinetic analyses of hybridoma clones, it has been reported that there is no correlation between the amount of cell surface antibody and the level of antibody in cell culture supernatants (25). Some other improvements for FACS-based cloning were reported. The green fluorescent protein (GFP) or CD20 which are co-expressed with gene of interest are used as reporter marker. This method shows a good correlation between objective protein and reporter protein (26). However, cells need to express marker proteins which forced additional burden to cells. Other improvements are gel microdrop technology and biotin-avidin affinity matrix-based secretion assays for FACS. The gel microdrop technology requires the use of machines to create the microdrop and the method requires many optimizations for each step to ensure single cell occupancy in the microdrops. The biotin-avidin affinity technology required several optimizations for biotinylation because of the different content of amine on the cell surface. Therefore, it is desirable to develop a novel single cell-based clone selection method.

Recently, semi-solid media have been employed to retain secreted proteins in the vicinity of clones (27–29). Semi-solid media have high viscosity and minimize diffusion of secreted proteins and migration of cells. Single cells are immobilized in agarose or methylcellulose-based semi-solid medium and form colonies. Secreted proteins are retained in the vicinity of the associated colony because of the medium viscosity. The advantage of using semi-solid medium is that the secreted protein can be visualized by addition of a fluorescently labeled capture antibody or measuring the immune precipitation by the interaction between the recombinant protein and capture antibody.

The selection of high producers is a crucial step in process development for the production of therapeutic antibodies. It is expected that the combination of a single cell-based clone selection system and semi-solid medium will be a very effective method for cell line selection (29–32). However, the detailed procedure of such a combination with serum-free adapted CHO cells and/or the efficient conditions of clone selection has not been established yet.

In this study, we used the automated colony picker, ClonePix FL as a single cell-based clone selection system. This system is able to obtain normal and fluorescent images of semi-solid media. On the basis of obtained images, the single cell-based clone is able to be picked up. Obtained image parameters are colony size, roundness and proximity to neighbors from normal images and relative protein secretion from fluorescent images. We integrated a high-throughput, single cell-based clone selection system and semi-solid medium for cell line development using serum-free-adapted CHO cells and investigated efficient conditions for the selection system. The serum-free-adapted CHO cell growth at the pre-picking stage was improved by optimizing the formulation of the semi-solid medium using spent medium. The efficiency of picking and cell growth at the post-picking stage was improved by optimization of the timing of plating without decreasing the diversity of clones. The selection conditions, including the medium formulation, were the most important factors for single cell-based clone selection to construct a high-producing CHO cell line.

MATERIALS AND METHODS

Cell line and culture medium CHO-K1SV cells (Lonza Biologics, Slough, UK), which are serum-free-adapted CHO-K1 cells, were used as host cells for cell line construction. The CHO-K1SV cells were cultured in serum-free CD-CHO medium (Invitrogen, Carlsbad, CA, USA) supplemented with 6 mM L-glutamine (Invitrogen). The cell concentration was measured by the trypan blue exclusion method using a ViCell-XR cell counting instrument (Beckman Coulter, Brea, CA, USA) or estimated by microscopic images of the cultures using Clone Select Imager (Molecular Devices, Sunnyvale, CA, USA). The final selection medium was CD-CHO supplemented with 25 μM MSX and without L-glutamine. To prepare glutamine-free spent medium, host cells cultured in CD-CHO medium with glutamine were transferred to CD-CHO medium without glutamine. The transferred cells were maintained for two passages, and then the culture supernatant was collected by centrifugation and filtration. The supernatant was aliquoted and stored at −40°C. Spent media, albumin (InVitria, Fort Collins, CO, USA and EMD Millipore, Billerica, MA, USA), and glutamine synthetase expression medium (GSEM) supplement (Sigma–Aldrich) were used for semi-solid medium supplementation. To optimize the formulation, we used a combination of antibody-producing cell lines that were established by the limiting dilution method.

Plasmid construction and transfection Antibody expression vectors were constructed by cloning fully humanized heavy and light chain cDNAs into GS expression vectors (Lonza Biologics) (9). Subsequently, the two vectors were combined to create a double gene expression vector (33). The constructed plasmid was purified and linearized by restricted enzyme digestion for transfection into CHO-K1SV cells according to the manufacturer's protocol. Transfections were performed under the optimized electroporation conditions determined by Lonza Biologics with a Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA, USA). At 24 h post-transfection, an appropriate concentration of MSX (typically 25 μM) was added to the culture medium for the first step of selection.

Clonal isolation using a single cell-based clone selection system A ClonePix FL (Molecular Devices) was employed as a single cell-based clone selection system. Methylcellulose-based semi-solid media, Clone Media CHO-G or Clone Matrix (Molecular Devices), were used for clonal isolation. Stable cell pools at 3–14 days after transfection were plated at an appropriate cell seeding concentration (100–10,000 cells/mL) in 6-well glass bottom tissue culture plates (W1150, Molecular Devices) for fluorescence-based detection. The semi-solid media for plating were Clone Media CHO-G (K8730, Molecular Devices) or Clone Matrix (K8510, Molecular Devices), based on the original formulation. The semi-solid medium was supplemented with Clone Detect (fluorescein isothiocyanate-conjugated sheep anti-human secondary antibody, K8200, Molecular Devices) to detect the accumulated fluorescent signal by the ClonePix FL. The semi-solid medium and CHO cells were agitated and vortexed well to generate a uniform solution (32). After plating, we confirmed single cell separation under a microscope. The plates were incubated at 36.5°C with 5% CO_2 for 10–14 days.

Individual 6-well plates were then analyzed using ClonePix FL analysis software. Briefly, each plate was divided into 6 × 6 sections. Each section was imaged under white light, followed by a fluorescence measurement with the light emitting diode. Picked cell colonies were selected based on the picking parameters including colony size, colony compactness, distance between neighboring colonies, and exterior mean fluorescence intensity. Colonies possessing the desired picking parameters were deposited into single wells containing 200 μL of rescue medium (CD-CHO medium) in a 96-well plate. Cell growth was analyzed using the Clone Select Imager. Confluent cell growth in each well was observed at 6–17 days, and clones showing high antibody production were subsequently expanded in 24-well plates to obtain parental cell lines. Established parental cell lines were applied to further analyses. The colony formation ratio (%) was evaluated by the following equation: