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Methotrexate gene amplification for development of erythropoietin with 2 additional N-link producing cell lines

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Abstract. As a workhorse for manufacturing recombinant proteins, Chinese Hamster Ovary (CHO) cells are becoming an extremely valuable cell for producing recombinant proteins having glycosylation patterns like in human system. Development of cell line, basically, focused on achieving the maximum amount of active proteins by screening the cell line to select higher producing clone. Currently, one of mammalian cell line development technologies used by most biopharmaceutical companies is based on gene amplification technology with methotrexate (MTX) to increase protein expression. With that in mind, the aim of this study is part of an effort to develop cell line capable of producing erythropoietin with 2 additional N-links. Gene amplification was performed on CHO-DG44 cell pools expressing EPO through addition of different concentrations of MTX to medium. The cells were cultured using orbital shaker at the speed of 130 rpm at 37°C and 5% CO₂ condition. Stepwise increasing MTX concentrations from 200 and 300 nM to 4000 nM were carried out. To measure the EPO produced from the culture, ELISA analysis was performed. The whole process of this amplification took approximately 60 days. From this study it was found that the use of 4000 nM of MTX gave the highest titers of EPO which was approximately 170 mg/liter. This data shows that stepwise increasing MTX concentration is very powerful step for gene amplification and subsequently for cell line development as a whole.

Keywords: Methotrexate, MTX, gene amplification, erythropoietin, EPO.

1. Introduction

To have a high producing stable cell line is extremely crucial for production of recombinant proteins in large scale setting. With this, there is a need for a robust and efficient protein expression system when developing these cell lines. Following the first approval of Chinese hamster ovary (CHO)-derived tissue plasminogen activator (tPA, Activase) in 1986, the application of mammalian cell culture for the manufacturing of protein therapeutic product has been revolutionized [1]. For more than 25 years, productivities for recombinant cell lines have increased about 20-fold as the result of improvements in media and bioprocess design. However, the progress that has been achieved in mammalian cell is still much lower than what can be achieved using microbial host systems [2]. The effort to further develop the yield involves gene delivery, genetic selection and high-throughput cultivation systems to simplify medium design and bioprocess development [3]. In addition, selection of stable cell clones with high productivity and large scale manufacturing development via culture medium and process optimization are extremely important [1]. Thus, overall, development and optimization methods which include media, feed, clone selection and processes are very important variables in the production of protein therapeutic products.

Nowadays, technology of cell line development that are used by most biopharmaceutical companies are based on two systems that make use of specific drug to inhibit a selectable enzyme



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marker essential for cellular metabolism: 1) MTX inhibits dihydrofolate reductase (DHFR) in the MTX amplification system, and 2) methionine sulfoximine (MSX) inhibits glutamine synthetase (GS) in the GS system [4] [5] [6] [7] [12]. Following transfection with expression vectors that contains the expression cassettes for the recombinant protein and selection marker genes, the transformed cells are selected and gene-amplified [11] with the selection drug, for example MTX or MSX. The presence of these drugs in the medium, then, will increase recombinant gene copy number in the cell [8]. MTX or MSX concentration can also be increased in a step-wise application to further increase cell protein productivity by further gene amplification. Following gene amplification, single cell cloning is performed to ascertain that the selected cell for recombinant protein production is obtained. One of the major problems with cell line generation and selection is that the recovery for cell lines that has high specific productivity is low. This necessitates the screening of hundreds if not thousands of individual cell lines to obtain a sufficient number that have the desired phenotype with regard to protein productivity and cell growth rate. Analysis of protein titers is subsequently used to choose the clones for progressive expansions. Finally, selected clones are evaluated in controlled bioreactors and banked for future use [9] [10].

In this study, we attempted to develop a cell line capable producing EPO with two additional N-links. Following transfection, gene amplification technology by using MTX technology was applied to the transfected cells. In order to have strong impact on MTX gene amplification, several stepwise increasing MTX concentrations from 200 and 300 nM to 4000 nM were carried out.

2. Material and Methods

2.1 Cell Culture and Reagents

The CHO DG44 cells were obtained from Life Technology (USA). L-glutamine, Pluronic F68, Freestyle Max, CD DG44 medium and CD OptiCHO medium were all obtained from (Life Technology). MTX was purchased from Sigma (USA). Erlenmeyer 125-ml polycarbonate disposable sterile flasks were ordered from Corning (USA).

Initially, before transfection the cell was grown in CD DG44 medium supplemented with hypoxanthine and thymidine (Life Technology, USA). Transfection was performed using Freestyle Max reagent as described by manufacturer (Life Technology, USA). Following transfection, for MTX screening work, the cells were grown in CD OptiCHO in the presence of MTX. The cell was cultured in an incubator in orbital shaker at 130 rpm with the condition of 5% CO₂ and 37°C temperature. The following in Figure 1 is the flowchart of major steps of gene amplification using MTX.

2.2 Transfeksi

Transfection was carried out in 125 ml of flask containing 30 ml of medium CD DG44 medium with a total of 1.5×10^7 viable cells. The number of plasmids used for the transfection process is 18 µg. As much as 600 µl of OptiPRO SFM were mixed with 18 µg plasmid DNA (solution 1). At the same time 600 µl of OptiPRO SFM was mixed with 15 µl of FreeStyle MAX (solution 2). Solutions 1 and 2 were then mixed and incubated for 10 minutes at room temperature. The mixture of the two solutions is then dripped into CHO DG44 cells for transfection reaction to occur. Cells were then cultured in the orbital shaker (130 rpm) at 37°C with 5% CO₂ concentration.

3. Result and Discussion

Process development, using CHO cell lines, focuses on achieving the maximum amount of active product. By combining the gene of interest with a selectable gene, increased production levels can be accomplished. One of the most frequently used gene amplification systems used in CHO cells are the dihydrofolate reductase (DHFR) system using methotrexate (MTX) resistance. The DHFR enzyme catalyzes the conversion of folate to tetrahydrofolate [7]. The flowchart below depicts the major steps in gene amplification using MTX (Figure 1).

Inside the cell, MTX is converted to a high molecular weight polyglutamate metabolite by folypolyglutamate synthase, which binds to DHFR and inhibits its activity. With this inhibition activity, to make sure that the cells under study can grow as expected, increased MTX concentration was carried out after cell viability reaches at a minimum of 90%. Media replacement is done twice a week. If cell growth was seen to be very slow, the media replacement can be carried out earlier.

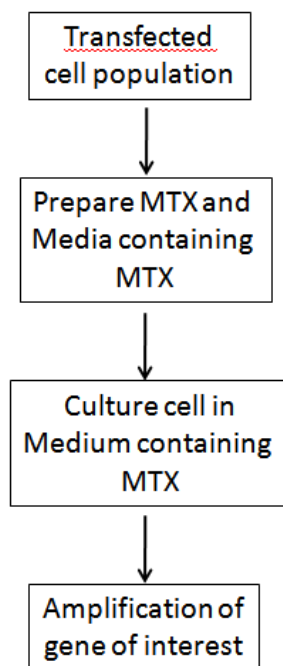


Figure 1. The flowchart of major steps of gene amplification using MTX.

Gene amplification was carried out on EPO expressing CHO-DG44 cell pools through two stepwise increasing MTX concentrations starting from 200 and 300 nM to 4000 nM were carried out (Figure 2). In this process transformant cells were cultured in a 30 ml CD OptiCHO medium containing 4 mM L-glutamine in 125 ml of erlenmeyer flask. In the presence of several MTX concentrations, transformant cells were cultured in orbital shaker and the samples were taken for analysis at day 8 of incubation.

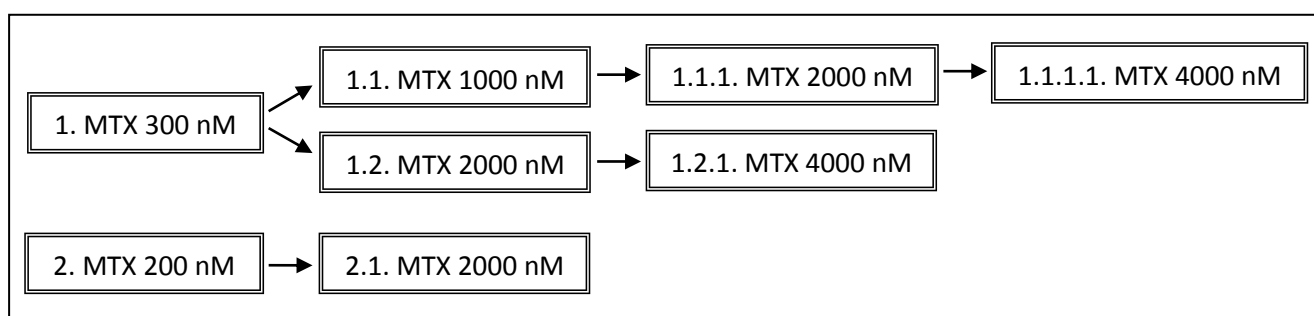


Figure 2. The different strategies of stepwise increasing levels of MTX concentrations to achieve gene amplification.

After MTX gene amplification, the protein of interest produced in supernatant were then determined. The yield obtained from the gene amplification process were then analyzed by using ELISA and the data is presented in Table 1. A clear pattern in protein expression levels was identified. Generally, the

higher the MTX concentration administered the higher the EPO protein expressed. In this study we found variations of titer in each concentration of the same MTX concentration. However, it shows clearly that the highest titer was obtained at the highest MTX concentration. The lowest (15.0 mg/liter media) and the highest (170.0 mg/liter media) titers were obtained at 200 and 4000 nM of MTX concentration, consecutively.

By setting up 8 different MTX amplification strategies, a suggestion of an amplification method can be generated. The results from this study shows that protein titer increased with the increase of MTX concentrations.

Table 1. Titers of EPO protein after being grown in the presence of several MTX concentrations in medium.

No.	Code of sample	MTX Concentrations (nM)	Titers of EPO Protein (mg/L)
1	1.	300	24.9
2	1.1.	1000	95.2
3	1.2.	2000	64.8
4	1.1.1.	2000	98.2
5	1.2.1.	4000	117.8
6	1.1.1.1.	4000	170.0
7	2.	200	15.0
8	2.1.	2000	22.8

Methotrexate (MTX) is widely used as a folic acid antagonist that is transported into cells by the folate transporter. Inside the cell, MTX is converted to polyglutamate metabolite by folylpolyglutamate synthase, which finally attach to DHFR and blocks its activity. The presence of MTX in the medium will stimulate the cell to increase the DHFR copy number in order to overcome inhibition by MTX. Since the gene of interest is integrated into the same genetic locus as DHFR, the gene of interest will get amplified as well and, subsequently, leading to increased production of the protein of interest [13] [8]. Thus, ultimately this selection strategy is expected to increase the number of high producing cell lines.

In this study it is clear that MTX gene amplification increased protein expression. However, variations between the same MTX concentrations were clearly observed. Due to this variations, we suggest to perform the MTX gene amplification in more than one transfected and selected cell pool. One of the problems with this method of cell line development is that the specific productivity of many of the recovered cell lines is rather low. This may be due to the productivity of each clone depends upon the integration locus of the plasmid(s), the response to amplification using MTX, and the nature of the protein. With regard to this, screening of thousands of clonally-derived individual cell lines that have the desired phenotype with regard to protein productivity and cell growth rate may be necessary [3].

4. Conclusion

This research shows that MTX is a very powerful agent for the use of gene amplification process so that it is extremely valuable in cell line development technology. This research shows that stepwise increasing MTX concentrations from 200 and 300 nM to 4000 nM was successful in amplifying the

gene of interest where the use of 4000 nM of MTX gave the highest titers of EPO which was approximately 170 mg / liter.

5. Acknowledgement

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