

# SEQUENTIAL ADAPTATION IN MAMMALIAN CHO-K1 CELLS PRODUCING HUMAN ERYTHROPOIETIN

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## Abstract

The production of recombinant proteins for clinical applications using mammalian cell technology has become a prevalent system because of its capacity in assembling functional proteins. One of the main problems with CHO-K1 cells is that this cell has to grow in the presence of serum. However, the presence of serum will complicate the downstream step for protein production. Thus, protein produced in media without serum, theoretically, would be easier to purify. Technically, this type of cell can be produced by growing the CHO-K1 cells in serum-free media by using adaptation method in suspension condition. This research showed that through sequential adaptation using conditioned media, the CHO-K1 cell line that produces the human erythropoietin gene (hEPO) was able to grow in suspension culture using serum-free media. Based on Western blot analysis, it showed that the protein (hEPO) was able to be expressed in suspension culture with molecular mass of about 47 kDa.

**Keywords :** *Adaptation, CHO-K1, hEPO, Serum-free media*

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## Introduction

Animal cell culture media are usually made up of mixed, defined chemicals such as carbohydrates, amino acids, vitamins, minerals, lipids, buffers and proteins such as growth factors. They often contain, in addition, some undefined or semi-defined components known as hydrolysates. The complexity of such composition provides many opportunities to optimize individual components or entire classes of ingredients. The emergence and growth of the biotechnology industry fuelled efforts to improve cell culture media for maximizing product yields and lowering cost of goods. Typically now, media for the biotechnology industry are serum-free and have much higher nutrient concentrations than classical media had because of the need to sustain high cell densities and increase cellular productivity (Jerums and Yang, 2005). Media optimization requires consideration of the product, cell line, and manufacturing process involved. Defined serum-free, as well as proteinfree

formulations, are available matching the needs of anchorage-dependent or suspension cell lines. The optimal culture conditions result in high cell densities and maximum protein yields.

Chinese hamster ovary (CHO) is the mammalian cell that commonly used as a host cell for the production in human therapeutic. Currently, around 70% the biotechnology-based drug products from mammalian cells and largely produced using CHO cells (Matasci *et al.*, 2008). Besides CHO cells are relatively easy to use in research technologies protein expression and have a clear track record, *glycosylation machinery* in CHO cells also resemble the pattern of glycosylation in humans (Bragonzi *et al.*, 2000, Ribela *et al.* 2003; Baldi *et al.* 2007). In addition to the pattern of glycosylation, the advantage of using CHO cells is because most of the pathogenic human virus can not replicate in these cells (Jayapal *et al.* 2007). By adapting cell lines to serum-free suspension conditions processing and scale-up can be greatly

simplified. The objective of this study was to adapt a serum-dependent adherent CHO cell line to serum-free suspension culture conditions.

## Material and Methods

### Cell culture and reagents

The CHO-K1 (NAIST) cells were grown in F-12 Ham media contained Nutrient Mixture (Sigma N6658) with supplementation with 10% of fetal bovine serum (FBS, Sigma/Gibco) 100U of penicillin and 100µg/µL of streptomycin (Invitrogen) with 0,5 % anti-clumping agent was obtained from Canada (Gibco01-0057AE) and 1.000ng of G418 antibiotic. The cell culture was grown in a CO<sub>2</sub> incubator with the condition of 5% CO<sub>2</sub> and 37°C temperature and passaged cell every 3-4 days.

### Transfection

The recombinant plasmid (pcDNA3) containing human *epogene* with five N-linked carbohydrate chains (Septisetyani *et al*, 2012) was transfected into the CHO-K1 cells using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. The pooled stable transformants were screened in the presence of 1,000 ng of G418 antibiotic. Limiting dilution cloning method was used for single cell cloning. Initially, the cell was cultured in 96-well plate with the density of approximately 1 cell per well and incubated for 10 days. To ascertain that only a single cell was presence in each well, on day 2 or 3, the wells were observed. After 10 days, the cell colonies that grown from a single cell were transferred to 24-well plates. Following confluency, the cells were then transferred to a bigger plate (6-well plates) and finally to 10 cm dish.

### Adaptation of cell cultures to a serum-free media

Adaptation process was performed using adaptation with sequential method from Invitrogen's preferred method. In this adaptation process, the passages were divided into four stages (Table 1).

### SDS-PAGE and Western blotting

The purity of the protein was analysed by SDS/PAGE as previously described in a 12%

separating gel with a 5% stacking gel using the Mini-PROTEAN-3 apparatus (BioRad, Hercules, CA, USA). Proteins were transferred to Amersham Hybond ECL (GE Healthcare) by electroblotting. Western blots were performed using polyclonal anti human EPO antibody (Sigma, St. Louis, MO, USA) as the primary antibody and anti-rabbit IgG alkaline phosphatase linked whole antibody (Promega, Madison, WI, USA) as the secondary antibody. The bands were detected by BCIP/NBT color development substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) (Promega, Madison WI, USA).

### Evaluation of Productivity (Assessing Productivity)

Protein expression evaluation was performed by culturing the transformant CHO K1 cells with seed cells 3 x 10<sup>5</sup> viable cells per mL in 30 mL SFM complete (antibiotic G418 and anti clumping agents) in suspension culture. To evaluate the expressed protein, the medium was taken at interval of days 0, 2, 4, 6, 8, 10, 12, and 14.

## Results

In this research, the pcDNA3 recombinant plasmid containing codon optimized human *epogene* with five N-linked oligosaccharide chains was transfected into CHO-K1. The singlesclones were isolated and screened for the expression of hEPO protein. Adaptation the adherent monolayer cells (CHO-K1) to grow in SFM with sequential treatment adaptation and serum-supplement media was performed. To do this, four passages were performed in which the culture of each passage used 75% media from the previous passage (Table 1). To have a good perform of cells culture, before adaptation period, the cell were grown until attaining of 100% confluency and at least we have three times sub-culture of cells. Once the condition was attaining 100% confluency, a seed 3 x 10<sup>5</sup> cells/ml were cultured in 10 ml F12 media culture (10% FBS, 1% P/S, 1% Antibiotic)

and grown in an incubator with 5% CO<sub>2</sub> and temperature 37°C. The media used from the previous passage acted as serum-supplement media which was essential in this treatment.

Table 1. The sequential adaptation steps with serum-supplemented media and SFM

PASSAGE	MEDIA COMBINATION
1	75% serum-supplemented media : 25% SFM
2	50% serum-supplement media: 50% SFM
3	25% serum-supplement media: 75% SFM
4	100% SFM

Observation on the progress of the adaptation from adherent monolayer to suspension culture showed that, as seen in Figure 1, initially, the CHO-K1 cells grown as adherent culture and with the advance of adaptation process the cells became so clumpy and less attached to the base of the flask. As soon as the cell was less attached to the base, the culture was transferred to a non-treated T25 flask. At the end of adaptation period, the cell culture was transferred to 30 ml medium in 125 mL Erlenmeyer flask and grown in a shaker at 135 rpm the condition of 5% CO<sub>2</sub> and 37°C temperature. To avoid cell clumping, anti-clumping agent was applied. As seen in Figure 1B, before the addition of clumping agent the cell culture looked very clumpy and after the addition of clumping agent (Figure 1C) the cell clumping disappeared.

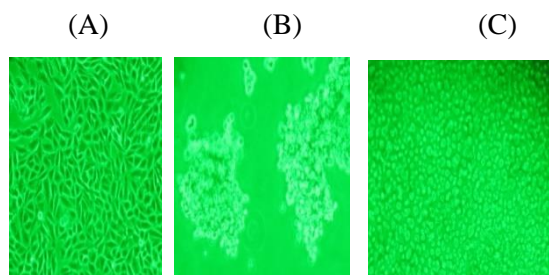


Figure 1. Microscopic evaluation of cells during adaptation period. (A) Cells in adherent culture, (B) Cells during adaptation process, and (C) Cells in suspension culture.

To follow and ascertain the adaptation process, the analysis using Western blot with polyclonal anti-human EPO antibody as the primary antibody and anti-rabbit IgG alkaline phosphatase-linked whole antibody as a secondary antibody (Sigma, St. Louis, MO, USA). As seen in that figure, the expression of the protein of interest (rhEPO) with molecular weight about 47 kDa was clearly shown. Overall, the protein expression increased with the peak expression at passage number three. During this adaptation process, an expression level of rhEPO in each passage was analyzed in 5 ml and T-25 flasks media with a seed of cells 3 x 10<sup>5</sup> cells/ml.

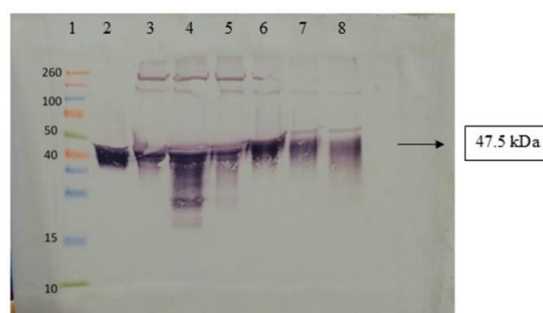


Figure 2. Western blot analysis of cell cultures. L1: protein ladder, L2: positive control, L3: adherent culture (10 cm dish with 10 ml media), L4: 1<sup>st</sup> passage, L5: 2<sup>nd</sup> passage, L6: 3<sup>rd</sup> passage, L7: 4<sup>th</sup> passage, L8: 5<sup>th</sup> passage (30ml in 125 erlen), For each passage suspension culture, time for culture is 3 days and 5ml in T25 flasks.

Assessing productivity can be analyzed by the conditions specified by the cell growth rate and viability. To improve the condition and viability of cells, in this research the addition of glucose to the suspension media needs to be done on day 4 and 7. The purpose is to determine the extent of glucose can affect the growth rate and viability cells, and then cells were grown in 125 ml plastic flask with a volume of media 30 ml with a seed 3 x 10<sup>5</sup> cells/mL. Cells were grown for 14 days and every two days the samples were taken for analyzed protein expression productivity (Figure 3).

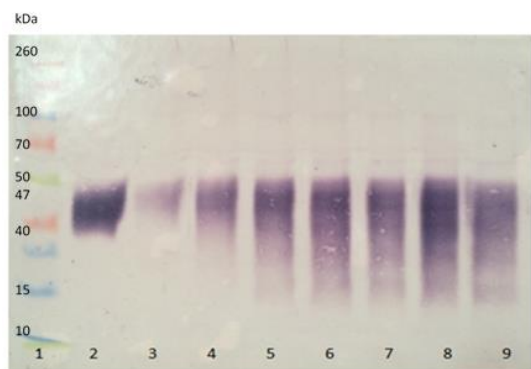


Figure 3. Western Blot Analysis for Assessing Productivity (AP) of CHO-K1 Cells in Suspension Culture. L1: Marker, L2: Positive Control, L3: AP in day 2, L4: AP in day 4, L5: AP in day 6, L6: AP in day 8, L7: AP in day 10, L8: AP in day 12, L9: AP in day 14.

## Discussion

Adaptation adherent monolayer cells of CHO K1 producing human erythropoietin to become serum-free media suspension system was performed successfully. The suspension CHO cells produced comparable recombinant protein to the complete media containing media. Daily use of CHO-K1 cells as production machine requires a lengthy time for adaptation process from the native type adherent clone to clones capable of proliferation in a suspension environment depleted of exogenous growth factors and cell-matrix contacts. In this experiment, we report an alternative way to growing the cell previously grown in adherent monolayer to grow in suspension cells culture system prior to protein production. In this study, adaptation method was performed by using “serum-supplement media”. In first passage, we were able to adapt cell that was previously grown as an adherent monolayer to finally grow as suspension cells culture. It is potential that conditioned media in which the previous cell has grown has released or secreted proteins, cytokines, and any other chemicals (Hannoun *et al.*, 2010) that subsequently enhance the adaptation process to occur successfully. However, in this suspension culture, a combination of new media (75%) was also important, since the presence of FBS is still essential, especially in early time of adaptation period.

Chinese Hamster Ovary (CHO) cells are widely used in large-scale culture. These cells have conventionally been grown as attached

cultures, but they will also grow in suspension, a property which has allowed for relatively simple scale up in stirred tank and airlift bioreactors. Unfortunately, some CHO-derived lines which have been adapted to suspension growth can form large, tightly bound aggregates in culture. In this study we have the problems from media that often faced in adaptation to serum free media is clumping. Clumping will make cell counting becomes very difficult if not impossible. Approaches to solve the problem of cell aggregation by using anti-clumping agent may be necessary (Reinhart *et al.*, 2013). Our preliminary adaptation works without using anti clumping agent showed that clumping inhibited adaptation. Based on this, in order to increase cell density, viability and protein expression, anti-clumping agent was added. We notice that the addition of clumping agent significantly reduced cell clumping.

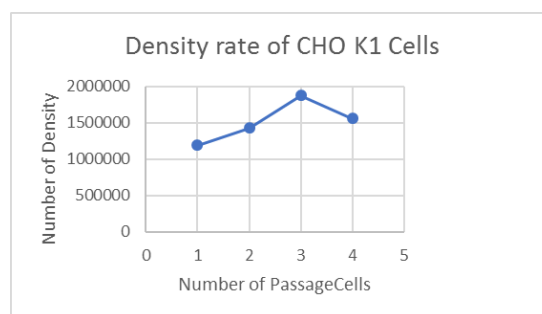


Figure 4. Cells Density of CHO K1 cells from passage 1 until the final step of sequential adaptation (passage 4).

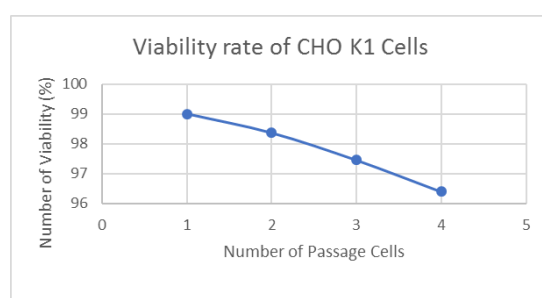


Figure 5. Percentage of Cell Viability CHO K1 cells from passage 1 until the final step of sequential adaptation passage 4.

Figure 4, cell density of CHO K1 cells, the result showed cell growth increased from the beginning until the end step of sequential adaptation. Although in late passage cells had decreased slightly, this condition is normal, during the cell of density are  $1 \times 10^6$  cell/mL as well as cell viability in 90% of life (Figure 5).

Western blot analysis was performed to evaluate how the adaptation process and passage number affect the expression level of rhEPO protein. The data showed that protein expression peaked at passage number four and six, after that decreased. As seen in Figure 2, it is interesting to note that the protein expression level, in a given culturing time (in this case 3 days), was higher in smaller volume and flask (5 ml in T25 flask) than in higher one (30 ml media in 125 ml flask). In our previous study (data not were shown), using suspension cell line CHO-DG44 in 30 ml media, the protein expression was performed successfully. Thus, the obtained adapted cell line can be utilized for large scale protein production.

## Conclusion

The sequential adaptation in mammalian CHO-K1 cells producing human Erythropoietin in serum-free media suspension can be achieved successfully without significant loss in protein production.

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