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Scaling-Up and Production of Therapeutic Antibodies for Preclinical Studies

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Abstract

Monoclonal antibodies are increasingly used for treatment of various diseases, mostly cancer therapy and auto-immune diseases. New chimeric, humanized, or fully-human antibodies specific to novel or already validated targets are being developed in many academic labs as well as in commercial entities. A validation of antibodies in preclinical settings is an important component of the road from discovery to clinic. Preparing sufficient amount of high-quality antibodies for preclinical studies is a challenge in some cases. This article summarizes protocols used in research laboratories, and is based on our own experience in the establishment of stable clones of CHO cells to scaling-up of tissue cultures for production of antibodies in bioreactors.

Keywords

Therapeutic antibodies; CHO cells; stable clones; bioreactor; purification

1. Introduction

The two most important elements of an expression system are the vector that carries the gene of interest and the host cells. Currently, mammalian cell systems, especially CHO cells, are the major workhorse for production of recombinant antibodies, although there are reports of making Abs in other cells such as insect cells and chicken eggs (1, 2). CHO cells easily adapt to production medium, grow to high cell density, and yield stable glycosylation of antibodies (3). Mouse myeloma cells (NS0) and baby hamster kidney cells (BHK) are also widely used (*see Note 1*). The vector carrying the recombinant IgG gene determines the selection system. Glutamine synthetase (GS) and dihydrofolate reductase (DHFR) systems are the two commonly used selection systems for establishing stable clones. DHFR system allows high expression, and untransfected cells die in the selection medium with DHFR inhibitor methotrexate (MTX); however, the selection process is lengthy. In theory, the selection gene can be in different vectors or in the same vector where the recombinant Ab gene is carried. In reality, the selection gene and the gene of interest are often in the same vector. Likewise, the VH and light-chain gene may be cloned into a single binary vector or separately into two vectors (4, 5). In addition to the selection system, the ultimate expression levels of Ab in stable clones are also determined by the promoter, the site of integration (position effect) into the chromosome, the copy number (from several hundred to a few thousand copies), and type of antibodies. To ease the downstream purification, recombinant Abs are usually secreted into serum-free medium through the signal peptides.

This article will focus on production of IgG1 with pDR12 vector and GS selection system in CHO-K1 cells. pDR12 vector allows sequential cloning of VH and light-chain genes in a

single vector and it also carries GS gene (6, 7). Transcription of the heavy chain and light chain is controlled under HCMV promoter. The heavy chain and light chain are assembled into IgG before they leave endoplasmic reticulum.

The procedure described in this article represents typical processes in research labs. Industrial production facilities routinely obtain much higher final Ab yield. However, information on such procedure at commercial sites is often not reported, and is beyond the scope of this article. For a comprehensive review on the latest developments in Ab production and basis of some manipulations see the review article by Wurm (8).

2. Materials

2.1. Establishing Stable Clones in CHO Cells

1. CHO-K1 cells (ATCC, Manassas, VA).
2. *F-12 K Nutrient Mixture (Kaighn's Modification) and FBS* (Invitrogen, Carlsbad, CA): F-12 K medium is supplemented with 10% FBS and 100 U/ml penicillin/100 µg/ml streptomycin.
3. *Dialyzed FBS* (Hyclone): The FBS used for selection is depleted with glutamine.
4. *MSX (L-methionine sulfoximine)* (Sigma): Dissolved in PBS at 100 mM, then filtered and stored at -20°C.
5. 50 × GS supplement (Sigma), a mixture of amino acids (alanine, asparagine, aspartate, glutamate, proline, and serine) and nucleosides (adenosine, cytidine, guanosine, thymidine, and uridine).
6. *Selection medium*: Glasgow's Modified Eagle's Medium (GMEM) supplemented with 10% dialysed FBS, 50 × GS supplement (Sigma), and 20 µM MSX.
7. PolyFect transfection reagent (Qiagen, Valencia, CA).
8. *Cloning rings* (Corning): 6-mm or 8-mm outer diameter. Spread a layer of vacuum grease on the bottom of a glass beaker. Place cloning ring on grease and autoclave together.
9. Clean forceps with fine tips, autoclaved.
10. Trypsin/EDTA (Invitrogen, Carlsbad, CA).
11. ELISA plates (Corning Life Science, Corning, NY).

2.2. Adaptation of Stable Clones in Serum-Free Production Medium

1. *SFM4CHO* (Hyclone, Logan, UT): This medium is free of glutamine. Before use, MSX and antibiotics are added.
2. *CO₂ incubator (37°C) with shaker*: We have not found a commercially available CO₂ incubator with built-in shaker. A low heat-generating shaker is placed inside a regular CO₂ incubator. The shaker should have capacity to shake continuously for longer time. Before placing any cells on the shaker, the temperature in the incubator, especially on the platform, should be monitored.

2.3. Production of Antibodies in Bioreactor

1. *Bioreactor*: Bioflo 410 (New Brunswick Scientific, NJ), with four-gas control. Similar bioreactor fitted for cell growth will also work.
2. Masterflex peristaltic pump (Cole-Parmer, Vernon Hills, IL).

3. *Tubings with luer adaptor* (Cole-Parmer, Vernon Hills, IL): packed in bag and autoclaved.
4. 5-l or 10-l Kimax glass bottle (Thomas Scientific), autoclaved.
5. *Base feed bottle with tubing and needle adapter* (New Brunswick, NJ): Packed in bag and autoclaved.
6. 8% sodium bicarbonate solution, filtered first then filled into the base feed bottle.
7. Medical-grade oxygen tank equipped with content gauge and delivery gauge.
8. *Production medium*: SF4CHO supplemented with 100–125 μ M MSX and antibiotics. SFM4CHO is formulated with anti-foaming reagent.

2.4. Purification of Antibodies from Culture Supernatant

1. Protein G resin, XK16 column, FPLC workstation (GE Healthcare, Piscataway, NJ).
2. PBS (Invitrogen, Carlsbad, CA).
3. 50 mM acetic acid, pH = 4.0. This can be replaced with 100 mM glycine, pH = 3.0.
4. *1 M Tris base pH 9.0*: Used at 1/10 of fraction volume to neutralize elutes.
5. Labscale TFF system (Millipore, Billerica, MA) with 30 kDa MWCO cassette. It can be substituted with dialysis tubing or cassette for small volumes.

3. Methods

3.1. Establishing Stable Clones in CHO Cell

1. *Transfection of CHO-K1 cells*: The day before transfection, CHO-K1 cells are set up in a six-well plate at 1 million/well in F-12 K growth medium. Cells should be at 50%–60% confluence at time of transfection. Use PolyFect to transfect cells with 2 μ g of DNA per well. The quality of DNA is critical to the efficiency of transfection. It should be free of salt and organic solvent. Follow the manufacturer's instruction for transfection. Maintain cells in 37°C incubator for 48 h (*see* Note 2).
2. To confirm transfection and expression of antibody, culture supernatant is taken 48 h post-transfection and tested on ELISA plate which is coated with the corresponding antigen. A goat anti-human Fc-HRP secondary Ab is used to detect bound antibodies.
3. Once the expression of antibody is confirmed, cells in each well are divided into 3–6 wells of six-well plate in F-12 K growth medium. The purpose of this step is to reduce cell density and avoid overcrowded foci later (*see* Note 3). After overnight incubation and the cells are attached to wells, they are rinsed with PBS three times. Each well will be replenished with 2.5 ml of the selection medium which has 25 μ M MSX.
4. The selection medium does not have glutamine, but has glutamate, which can be converted into glutamine by glutamine synthetase (GS). MSX inhibits GS. If CHO-

²We have compared transfection efficiency with intact plasmid and linearized plasmid. They do not seem to impact on transfection efficiency. Intact plasmid is soon cleaved by exonuclease or endonuclease activity in nucleus and become linearized. A complete study on topology of DNA to transfection efficiency can be found in this article (9). Some researchers believe linearized plasmid gives better efficiency (8).

³Dividing of the transfected culture before selection medium is added helps to have well-isolated foci.

K1 cell are transfected with plasmid pDR12-IgG, the GS encoded by the plasmid will overcome MSX, and cells will continue to grow, while untransfected CHO-K1 have very low chance to grow in the selection medium (see Note 5).

5. Following addition of the selection medium, cells will still grow for 1–2 days and most cells start to die after 5–6 days. There will be a lot of debris in the culture at this point. Small foci will appear soon after. Two weeks after addition of the selection medium, foci are clearly visible to eyes. The position of foci can be marked on the bottom of the plate to facilitate foci picking later.
6. *Preliminary cloning:* Cloning ring is used to physically separate different clones. Mark well-isolated foci on the bottom of the plate. Remove the culture medium and rinse cells gently with PBS once. With clean forceps, place a cloning ring on each focus and push the ring down so the vacuum grease seals well. Add 100 μ l trypsin/EDTA solution to the inside of each ring. It is a good idea to have all reagents and plasticwares ready by the side, so cells will not dry out while reagents are searched. Trypsinization of cells can be monitored under microscope. Once cells are detached from plate, 100 μ l of selection medium is added to each ring, and cells are collected by pipette tips. Cells from each ring are cultured in separate wells of 24-well plate in selection medium.
7. *Further cloning:* Limited dilution is used for this step. When cells grow to confluence, the culture supernatant is taken for ELISA test to confirm the presence of IgG expression. It is possible to have some negative clones at this stage even though cells are in the selection medium. Cells from wells of 24-well plate are harvested and counted; calculate and seed a single cell in each well of 96-well plates in the selection medium. The rest of cells can be frozen and stored, in case there is need to repeat this step. On the 96-well plate, the cell number each well receives varies. The wells with a single cell are marked. It takes 1–2 weeks for cells to grow to enough density. At this point the plates should be carefully inspected under microscope and the wells with a single focus of cells are marked and matched with the earlier marking. The supernatant from these marked wells is taken for ELISA test. The wells with expression of IgG and with a single focus are selected as starting cells for monoclonal antibody production (see Note 4).

3.2. Adaptation of Stable Clones in Serum-Free Production Medium

CHO-K1 stable clones selected after limited dilution are propagated in selection medium. When sufficient cell numbers are reached, some cells are frozen and stored away as backup. The rest of cells are to be adapted in serum-free medium. Cells can be adapted to grow in serum-free medium in two methods described below.

Method 1, Direct adaptation—When cells growing in the selection medium are nearly confluent, change medium into SFM4CHO medium supplemented with 20 μ M MSX. Cells will keep dividing and reach confluence. After confluence is reached, cells will grow in suspension. At this point, cells are maintained in flask till sufficient number of cells in suspension is accumulated. The floating cells are collected and viability is monitored. These cells are now maintained in shaker flask at a concentration $>2.0 \times 10^5$ cells/ml and let grow

⁵MSX is required in all selection and production media. We have found that 25 μ M MSX is a good starting concentration for obtaining reasonable number of clones. Higher concentrations of MSX make it hard to get clones initially. Once positive clones are identified, MSX concentration may be ramped gradually to achieve higher recombinant antibody expressions.

⁴With many positive clones, selection of certain clones for further adaptation becomes an art. Initial high expressers are not necessarily the best choice because these cells often grow slowly and may not adapt well in SFM4CHO. We have found that the clones with morphology similar to that of native CHO-K1 cells often adapt well and in the end express recombinant IgGs well. It is the combination of high expression and fast growth that makes the suitable clone for production.

in CO₂ incubator with shaker. Cells will grow slowly while they adapt to SFM4CHO. Depending on the IgG they carry, it could take 1–2 weeks for cells to go into active growth phase. When cells grow rapidly in SFM4CHO medium, the MSX concentration can be increased at 10 μ M increment to boost expression of IgG. Usually 100–125 μ M can be reached without affecting the growth of cells and high levels of IgG expression is reached in these cells.

Method 2, Step adaptation—When sufficient cells are accumulated in the selection medium, they are resuspended in 2.5% dialyzed FBS/SFM4CHO in shaker flasks. When cells grow regularly and are able to reach the density of 5×10^5 cells/ml without clumping or generating debris, they can be resuspended in SFM4CHO. Some clones may benefit from further adaptation in 1.25% FBS/SFM4CHO. Allow three passages before reduction of serum concentration. Again, the time taken to adapt cells into SFM4CHO varies depending on different IgGs and clones.

For most stable clones, step adaptation works well, even though it is lengthy.

3.3. Production of Antibodies in Bioreactor

1. When cells grow in SFM4CHO without forming any clumps and are able to reach at least 2×10^6 cells/ml in shaker flasks, and the MSX concentration has been increased to 100–125 μ M, cells are ready for production of Ab in bioreactor. The viability of seed cells is critical to success of the production of IgG in bioreactor (see Note 6).
2. *Bioflo 410 operation*: Between 5–12 l of culture can be maintained in Bioflo 410. The initial seeding density should be $\sim 1 \times 10^5$ cells/ml. Fill the vessel with 5 l of PBS. Check the inoculation septum and calibrate the pH meter and dissolved oxygen (DO) probe of the bioreactor. Assemble them accordingly and make sure they are air-tight when Bioflow410 is sterilized (121°C $\times$ 45 min); there should be no escaping of air or buffer.
3. The sparge line of the bioreactor is at the “up” position for sterilization. After the sterilization cycle is completed, lower the sparge to the “down” position. Drain the PBS from bottom harvest port. Close the port tightly once PBS has been drained.
4. Prepare production medium in a biosafety cabinet and pool all medium into a clean Kimax bottle. Put rubber stopper with tubing back to the Kimax bottle. On the end of tubing adaptor, connect a 18-gauge needle. At this point keep the sheath of the needle on. Transport the bottle carefully to near the bioreactor.
5. Sterilize the inoculation septum with 70% ethanol. Insert the needle through one of the inoculation port. Use Masterflex pump to pump culture medium to the vessel of Bioflo410. Allow the temperature and other parameters of medium stabilize to set point: 37°C, 50% DO, and pH 7.2. Ideally, Bio-flo410 should be in a clean room, but it is often difficult for a research lab to have access to such a room. It is acceptable to install Bioflo410 in a regular lab space. If all the procedures are performed with sterile techniques, it is feasible to have clean culture in the end.
6. Collect seed cells from shaker flask culture. Cells are concentrated in SFM4CHO and filled in a 60-ml syringe with 18-gauge needle. Cells are inoculated into the bioreactor through the inoculation port on the septum. Avoid using the same port that has been used for transferring of culture medium.

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⁶Low viability of cells in bioreactor increases the possibility of DNA or other minor contaminants in the final product.

7. During the growth period, DO will be maintained at ~50% by automatic adjustment of the four-gas control with nitrogen, compressed air, and oxygen. The delivery pressure for all four gases is set at 20 psi. Other than the proportion of the four gases, the total gas flow rate can also be changed within a range, so long as the flow does not cause foaming of the medium. The pH of the culture will be maintained by CO₂ intake and base feeding through the pH control loop. The base feed bottle is filled with 8% sodium bicarbonate and connected to the vessel through an 18-gauge needle. The tubing of the feed bottle is in the base feed pump which is controlled by the pH loop. The agitation speed is to be maintained at 100 rpm to avoid shearing and foaming. The DO value tends to fluctuate on the first day of culture, and will become stable later.
8. Cell samples can be taken from the bottom harvest port. Viability and density of culture should be monitored throughout the period. Depending on IgG and clones, the culture density can reach $2-3 \times 10^6$ cells/ml with batch procedure. For some clones, the density may not increase after 5–7 days of culture. As long as the viability is >50% and not decreasing, IgG is continuously being enriched in the medium and culture is worth maintaining (see Note 7).
9. The bioreactor is to be stopped when the total viability is below 50% and decreases steadily, or desired IgG concentration has been reached in medium. The culture is harvested from the bottom port through clean tubing to centrifugation bottles or continuous centrifuge. Supernatant is recovered and filtered through 0.45- μ m filter. If supernatant is not immediately used for purification, it is stored at –80°C.

3.4. Purification of Antibodies from Culture Supernatant

This part of the procedure is done at 4°C.

1. XK16 column is packed with protein G resin at the flow rate 5 ml/min. The height of the packed column determines the capacity of the column. Each ml of packed protein G resin binds up to ~20 mg human IgG.
2. The column is first equilibrated with PBS, and then the culture supernatant is loaded to the column at flow rate of 2–4 ml/min through FPLC workstation. The flow-through is collected in a clean vessel.
3. After loading of the supernatant is complete, the column is washed extensively with PBS till the absorbance OD280 returns to the base line, and the IgG is eluted with eight-column volume of 50 mM acetic acid. Prepare to neutralize the eluate with 1 M Tris base, pH 9.0. Precipitation of IgG occurs in some cases due to extremely high concentrations of IgG in the peak. This can be minimized by dividing the supernatant into several runs of column.
4. The recovered IgG is buffer-exchanged to a buffer that is compatible with downstream use, such as PBS. This can be achieved by dialysis (if the total volume is manageable) or diafiltrated on LabScale TFF system if sample volume is >50 ml. When TFF system is used, the sample can also be concentrated to desired IgG concentration so that injection volume is reduced at animal studies. The final IgG sample is recovered, filtered through 0.2- μ m filter, and aliquoted to desired volume and vials.

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⁷Bioflo410 does not support spinner filter; therefore, the final culture density is limited by nutrients in the batch medium. If cells are adapted in the production medium, Ab yield at 0.25 μ l g/L, is achievable. If a bioreactor is not available, shaker flask with 25% occupancy of the volume should work fine. It is possible to have culture at 2 million cells/ml and Ab at 200 mg/l without spending excessive amount of time in adapting cells.

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Abbreviations

Ab	antibody
IgG	immunoglobulin
Fab	antigen-binding fragment
CDR	complementarity-determining regions
CHO	Chinese hamster ovary
HRP	horseradish peroxidase

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