

Establishment of Stable Transfectant of CHO Lec Cells

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Purpose and Backgrounds

CHO lec 3.2.8.1 cells

CHO Lec 3.2.8.1 cells have four independent mutations in the N- and O- glycosylation pathways (Stanley, 1989). N-linked carbohydrates produced by CHO Lec 3.2.8.1 cells are all of the high mannose type, but differ in the number of mannoses, ranging from Man₉ to Man₅. O-glycosylation is homogenous, with only a single GalNAc residue attached per site. When cultured in the presence of the alpha-glucosidase I inhibitor N-butyl-deoxynojirimycin (NB-DNJ), glycoproteins produced in CHO Lec 3.2.8.1 cells are almost completely susceptible to Endo H digestion (Davis, 1995; Ikemizu, 1999). Endo H cleaves chitobiose, leaving a single N-linked N-acetylglucosamine per site, which is ideal for maintenance of protein solubility and special carbohydrate-protein interactions, such as between the first N-acetyl glucosamine residue and tryptophan. (On intact proteins, Endo H cleaves (Man)₅₋₉ chains but (Man)₃₋₄ chains are resistant.)

Vectors

Use expression vectors with strong promoter such as immediate early CMV (pcDNA3.1 etc.) or human EF-1a (pEF1/V5-His etc.). pBJ-5/GS vector also gives you high expression but requires additional cloning strategy.

Materials

Medium

Sigma provides special serum-free medium for CHO cells (C-1707). Add glutamine, nonessential amino acids, penicillin/streptomycin, and 10% FCS. FCS can be omitted but switching must be done over a couple of medium changes (i.e., change to 5% FCS once and to 2% twice followed by completely switching to serum-free medium etc.). In the case of Sigma's media being discontinued (which happened before), you can prepare basic medium (J/J) by yourself but this cannot be used as serum-free medium.

Recipe for 10 lt of J/J medium:

To 9 lt of milli-Q water, add

1. MEM w/o Gln (GIBCO, #11700-077). Pwdr for 10 lt. NaHCO₃, 27.75g; glucose: 30 g; nucleosides: adenosine (Sigma A-4036), 70mg; guanosine (Sigma G-6264), 70mg; cytidine (Sigma C-4654), 70mg; uridine (Sigma U-3003), 70mg; thymidine (Sigma T-1895), 24mg
glutamic acid (G-1626) and asparagine (A-0884); 600 mg each; sodium pyruvate solution (100X, 100mM, GIBCO #11360-021), 100ml; nonessential amino acid solution (100X, 10mM, GIBCO #11140-019), 200ml
2. Penicillin/Streptomycin (100x), 100ml

Adjust pH to 7.15 and filter sterilize.

Selection marker

CHO Lec 3.2.8.1 cells are relatively resistant to neomycin (GENETICIN G418; GIBCO #11811-031). It is better to draw kill curve for each batch of cells (and G418) every time you do transfection, but generally you can start with 1 mg/ml. Even with this high concentration, it will take more than a week to kill all of

the non-transfected cells. Puromycin (Sigma, P-7255) kills CHO Lec cells within a couple of days at a range of 4-8 $\mu\text{g/ml}$, usually use 10 $\mu\text{g/ml}$. Always include negative control transfection (electroporation without DNA) to know whether antibiotics works OK.

Others

- Electroporation PBS (**EPBS**):
NaH₂PO₄·H₂O 96.6 mg;
Na₂HPO₄·7H₂O 482.4 mg;
NaCl 2.197 g in 250ml water, sterile filtered
Electroporation cuvette (4mm gap, BTX #01-000196-01)
- 96-well tissue culture plate (low evaporation)

Procedure

Day -1

Split confluent CHO Lec cells into 75 cm² flasks (1/3 dilution).

Day 0

Preparation of DNA

1. take 10 μg DNA/construct add 1/10 vol 3M NaOAc (pH5.2), mix add 2X vol 100% EtOH, mix place on dry ice, 5-10min Spin 10min at max speed Remove supernatant
2. Wash pellet with 70% EtOH

Electroporation

1. Cells should be 50~70% confluent. Detach cells with trypsin/EDTA, wash once w/ complete medium, and count cell number. Suspend cells in EPBS at $\sim 1 \times 10^7/\text{ml}$ (Typical yield is $\sim 2 \times 10^7$ cells/flask) Add 800 μl of cells to cuvette plus DNA (dissolved in 10 μl of sterile water)
TIPS: You can't use DNA dissolved in TE because Tris will kill cells after electroporation. On ice, 15min Electroporation using Gene Pulser (Set voltage: 370V, Capacitance: 960 μF)
 - i. wipe off solution outside the cuvette place into the holder
 - ii. press two red buttons simultaneously until you hear beep**TIPS:** Resulting time constant should be around 15 msec. If electroporation was successful, you will see fine air bubbles over the solution
On ice 15min Using tiny pipet included in the cuvette pack, transfer cells into 9ml complete medium in 15 ml tube. Briefly suspend cells and plate into 10cm dish
2. Culture 24h

Day 1 (Most of cells will not be attached)

1. Transfer medium (together with floating cells) into centrifuge tube and immediately add fresh medium to the dish
TIPS: If you let the dish dry up, cell will die. Spin down and resuspend cells in fresh medium, and put back to the original dish.
2. Culture 24 h.

Day 2 (Still you will see most of the cells floating)

1. Collect medium (and cells), detach any adherent cells from dish with trypsin/EDTA, combine them, and spin down.
2. Resuspend cells in selection medium containing appropriate antibiotics. Dispense into 96-well plate at 200 $\mu\text{l}/\text{well}$. Culture at 37°C. You don't have to change or add medium until you harvest the supernatant for screening.

You should vary numbers of cells that you plate into each well. In some cases, the transfection efficiency is so high and you get multiple colonies in each wells necessitating you to re-clone the cells from the positive well. Try 3×10^4 cells/well (equivalent to 3 plates (300wells)/transfection) as a highest density and go down to 1×10^3 /well. If you do double transfection using two different vectors (as in the case of two integrin subunits), the number of colonies will be low and you might need more cells in each wells.

~Day 5

Cells from negative control transfection will start to die.

Day 7-10

Almost all of control cells will be dead by this period. You will be able to see tiny colonies in the transfected wells.

Day 13-16

Medium of the wells containing colonies turn to yellow. For secreted proteins, take those culture sup that had changed their color and screen them for the expression level (by ELISA etc.). For membrane proteins, detach cells by adding $50 \mu\text{l}$ trypsin/EDTA, transfer to 48- or 24-wells, and check expression by FACS after growing the cells.

In some cases, you may need additional re-cloning by limiting dilution.

References

1. Stanley, Mol. Cell. Biol. 9, 377 (1989) Davis, JBC, 270, 369 (1995) Ikemizu, PNAS, 96, 4289 (1999)
2. Casanovas and Springer, JBC, 270, 13216 (1995)