Video Article

A Method For Production of Recombinant mCD1d Protein in Insect Cells.

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URL: <http://www.jove.com/index/Details.stp?ID=556>

DOI: 10.3791/556

Citation: Khurana A., Kronenberg M. (2007). A Method For Production of Recombinant mCD1d Protein in Insect Cells.. JoVE. 10. <http://www.jove.com/index/Details.stp?ID=556>, doi: 10.3791/556

Abstract

CD1 proteins constitute a third class of antigen-presenting molecules. They are cell surface glycoproteins, expressed as approximately 50-kDa glycosylated heavy chains that are noncovalently associated with beta2-microglobulin. They bind lipids rather than peptides. Although their structure confirms the similarity of CD1 proteins to MHC class I and class II antigen presenting molecules, the mCD1d groove is relatively narrow, deep, and highly hydrophobic and it has two binding pockets instead of the several shallow pockets described for the classical MHC-encoded antigen-presenting molecules. Based upon their amino acid sequences, such a hydrophobic groove provides an ideal environment for the binding of lipid antigens.

The Natural Killer T (NKT) cells use their TCR to recognize glycolipids bound to or presented by CD1d. T cells reactive to lipids presented by CD1 have been involved in the protection against autoimmune and infectious diseases and in tumor rejection. Thus, the ability to identify, purify, and track the response of CD1-reactive NKT cell is of great importance. The generation of tetramers of alpha Galactosyl ceramide (alpha-Galcer) with CD1d has significant insight into the biology of NKT cells. Tetramers constructed from other CD1 molecules have also been generated and these new reagents have greatly expanded the knowledge of the functions of lipid-reactive T cells, with potential use in monitoring the response to lipid-based vaccines and in the diagnosis of autoimmune diseases and other treatments.

Protocol

I. Thaw the Cells

Take the frozen vial of TN5 cells, and thaw it in a 37°C water bath. Add 5ml of insect Express medium (Invitrogen, catalog number-10486) in 25cm² T.C flask. Note that Express five comes without Glutamine; need to add 10ml of 100X glutamine pen strep or Glutamine alone to 1 lt media. Add TN5 cells into it and incubate for 30 minutes in a 27°C incubator. Then, aspirate the medium with DMSO gently without disturbing the cells attached to the bottom of flask. Add 5ml of Fresh medium.

II. Growing and Expanding the Cells

Monitor the cells every day. When flask is nearly 70% confluent, bring the cells in suspension by banging the flask on both sides and transfer the cells to 175cm² flask by adding 21 ml of Insect express medium and 5ml of cell culture. Each T175 flask should have around 25-30ml medium as a final volume. Split confluent flask (approx 1 million/ml conc.) 1/4 or 1/5 as needed. Do not let cells overgrow. Count the number of passages that you have split the cells. Do not grow over passage number 30 because it may cause aging of cells resulting cell lysis. When you reach the desired volume at the concentration of 1 million/ml, infect the cells.

I generally grow up to 2 to 2.5 liters, which usually amounts to seventy 175cm² flasks and approx. 25 to 30ml / flask, or five 1 liter Erlenmeyer flasks and approx. 400ml to 500 ml in each flask

Note: You either can grow cells in T-175 plug seal flask or Corning Erlenmeyer flask. Growing cells in Erlenmeyer Flasks is faster and easier.

III. Titering the Virus by End Point Dilution Method

1. Plate 3000-5000 cells per well in flat bottom 96 well plate in 200ul of Express five SFM medium. Let the cells settle at 27°C for at least 30 minutes.
2. Make serial dilution of baculovirus stock. One should do 1/10 dilution in one line of 96 well U bottom plate, using 250ul per well. Dilutions are made in Express Five SFM medium.
3. Using a multichannel pipette, transfer a fixed amount (usually 20 ul) of different dilutions to the cells.
4. Culture at 27°C for 7 days. To avoid the evaporation from the edge rows, wrap the edges of plate with parafilm.
5. After 7 days, check the plate and determine which dilution of virus gives a 50% infection - half the wells at this virus concentration are infected). Then use a formula to compute the titer, which is expressed in pfu/ml (pfu-plaque forming units). The formula is found in most baculovirus manuals.

IV. Infecting the cells

It is very important to know the Exact titer of virus. Add required amount of virus. For viral stock preparation the cells should be infected at Multiplicity of Infection (MOI) of not more than 1 (1pfu to 1High Five cell) Higher MOI leads to production of viral mutations. For protein production, usual amount is MOI 5-10.

Example:

The titer of mCD1d baculovirus = 1X10⁸ pfu/ml

Add MOI=1 (to amplify the virus)= 20X10⁶ cells/ flask / 1X10⁸ pfu / ml=200 ul / flask

MOI= 5 to 10 (for protein production)= 1ml to 2ml For 20x10⁶ cells/flask

On Day 4 after infection, look at the infected cells. They should be detached, floating, swollen and forming sausages.

V. Recovering Supernatants

Take the medium from infected flasks, and transfer to 250ml blue cap Falcon Spinning bottles. They can be autoclaved and reused. Fill the bottles evenly and centrifuge the cells in Sorvall GSA rotor at 200rpm, 20 minutes, 4°C. Collect the supernatant and proceed for further purification.

VI. Purification Of Recombinant mCD1d

1. Dialysis and Concentration in 150mM Sodium Phosphate buffer (pH 7.4)
2. By Ni-NTA Agarose Chromatography, elute the protein mCD1d+b2m with 150mM Sodium phosphate +200mM Imidazole
3. Run Desalting column using 20mM Tris HCl pH8 buffer to get rid of Imidazole
4. Run Anion Exchange Chromatography using MonoQ column to eliminate the remaining contaminants.
5. Concentrate to 1mg/ml using YM 30 concentrator (Millipore)
6. BirA enzymatic biotinylation of mCD1d according to manufacturer's protocol (Avidity)
7. The free biotin is eliminated by S200 column (size exclusion Chromatography) using phosphate buffered saline (PBS), pH7.4. An average of 2 to 3 mgs of biotinylated protein could be obtained from 2 liter of supernatant
8. Production of a-Galcer CD1d tetramer.

In order to load CD1d molecule with a-Galcer, soluble biotinylated CD1d-b2m is incubated overnight at room temperature with three fold molar excess of a-Galcer, dissolved in 0.5% Tween -20 in PBS, 0.9% Sodium Chloride. Tetramers are generated by mixing of a-Galcer loaded CD1d monomers with 4 fold molar excess of PE - Conjugated streptavidin and incubating for 4 hours at room temperature. Store at 48°C.

Discussion

In this procedure, it is shown how to initiate, grow, infect Insect cells and how to titer the baculovirus using these cells. The most important aspects of this procedure are maintenance of cells and to know the exact titer of baculovirus. Once you have generated CD1 tetramers, they can be used as a powerful tool for analysis of glycolipid reactive T cells. Baculovirus systems are also used widely to produce recombinant proteins, including MHC class II tetramers so this procedure has a much wider applicability.

References

1. Benlagha, K., Weiss, A., Beavis, A., Teyton, L., and Bendelac, A. (2000) . In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J. Exp. Med.* 191, 1895-1903.
2. Kinjo, Y., Wu, D., Kim, G., Xing, G. W., Poles, M. A., Ho, D. D., Tsuji, M., Kawahara, K., Wong, C. H., and Kronenberg, M. (2005) Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* 434, 520-525
3. Naidenko, O. V., Maher, J. K., Ernst, W. A., Sakai, T., Modlin, R. L., and Kronenberg, M. (1999) . Binding and antigen presentation of ceramide-containing glycolipids by soluble mouse and human CD1d molecules . *J. Exp. Med.* 190, 1069-1080.
4. Porcelli, S. A. (1995). The CD1 family: A third lineage of antigen- presenting molecules. *Adv. Immunol.* 59, 1-98
5. Sidobre, S., and Kronenberg, M. (2002) . CD1 tetramers: A powerful tool for the analysis of glycolipid-reactive T cells. *J. Immunol.* 169, 1340-1348.