Production of Empty and Peptide-filled H-2K^d/cβ_2m

Cell Line:

3D is the name Peggy gave to the K^d/cβ_2m producing CHO cell line. This is the mouse MHC class I heavy chain complexed with the chimpanzee light chain (the chimpanzee light chain is identical to the human light chain). These cells grow in αMEM, 5% DFCS, 100μM MSX, and penicillin/streptomycin. I typically thaw one vial of frozen cells, grow them to confluency, and split them several times to a total of 50 plates (10 ml of media/plate). Plates are harvested every other day. To the harvest I add azide to a final concentration of 0.05% azide and EDTA to 1mM final concentration, and then filter the harvest through a 0.45 μm nalgene filter. Harvests are stored in the coldroom until they can be purified.

Purification:

The harvests are run over the 34-1-2 columns located in the coldroom. Currently, there are three such columns. Two were made by Peggy, and one was made by Bong-Gyoon. One of Peggy's columns says "contaminated" on it. As far as I can tell from her notebooks, the "contamination" is due to the column picking up bovine Ig from serum in the harvests. If the harvest is run in series through a protein A column before it goes over this 34-1-2 column, the purified K^d/cβ_2m should be free of bovine Ig. After the harvest is run over the column, the column is washed with 5-10 column volumes 50 mM Tris pH 7.4, 0.05% azide, 1 mM EDTA. The column is then eluted with 50 mM diethylamine, and 1 ml fractions are collected. Each tube used for collecting fractions should contain 100 μl of 1 M Tris pH 7.4 to neutralize the diethylamine as it comes out of the column. This prevents degradation of the protein due to high base. The protein A column should be eluted separately from the 34-1-2 column to remove the Ig on it. I usually use 0.1 M glycine pH 2.7, but 0.1 M citric acid pH 3.0 can also be used. After elution, the pH of the columns is neutralized with 5-10 column volumes of 50 mM Tris pH 7.4, 0.05% azide, and 1 mM EDTA. The columns are then ready to have another harvest run over them. Flowthroughs from each harvest should be saved and re-run over the columns to ensure all the protein has been removed. Typically, healthy plates of cells will produce 10 mg/L quantities of protein.

Concentration and Characterization:

The best way to concentrate the protein is to use the vacuum dialysis apparatus with a 25 kDa MWCO bag. The fractions containing protein are put into the apparatus, vacuum is applied until the volume left in the bag is around 1 ml, the

buffer is poured out and replaced with fresh buffer (50 mM Tris pH 7.4, 0.05% NaN_3, and 1 mM EDTA [150 mM NaCl can also be helpful if you wish to put it in]), and the sample is allowed to dialyze overnight. The next day vacuum is again applied if necessary to obtain a final volume around 0.5 ml (I feel that this is the lower limit of recovery from the apparatus). Remove the protein and rinse the bag with 0.5 ml buffer to wash off any protein sticking to the membrane. Determine the concentration by BCA assay, and run the protein on a 13% SDS gel.

Denaturing and Renaturing Protein

It is best to start with 20 mg of K^d / $c\beta_2m$ for the process. Any excess β_2m that can be added to the sample will increase the final yield. Pool enough samples to have 20 mg and concentrate in an Amicon centricon (MWCO 10 kDa to avoid losing β_2m) to a volume of 200 µl. 2 ml of 6 M guanidine buffered to 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.05% azide should be added to the centricon and spun down to 200 µl. Wash the centricon with another 200 µl guanidine buffer. This sample is then run in 2 x 200 µl over the Superose 12 sizing column on the FPLC. The peaks containing K^d and $c\beta_2m$ (2 separate peaks) are pooled and put into dialysis tubing with a MWCO of 6-8 kDa and dialyzed overnight in 8 M Urea with Tris, NaCl, EDTA, and azide as above. If the protein is to be left empty, then the tubing is placed the next morning into the Tris / NaCl / EDTA / azide buffer without urea for 24 hours. The tubing is then placed into a fresh batch of buffer. I typically use 1 L of the denaturing urea buffer, and 2 L (twice) for the renaturing buffer. If the protein is to be renatured with a peptide, the steps are the same until after the protein is removed from the urea. After removal from urea buffer, the protein is transferred to dialysis tubing with a MWCO of 500 Da and peptide is added to a 30-fold molar excess. This new tubing is then put into the 2 batches of renaturing buffer in the same manner as the empty protein. After the second day of renaturation, the protein is removed from the dialysis tubing, tubing is washed with buffer, and the total sample is concentrated (usually with an Amicon centriplus, MWCO 10 kDa) to 1 ml. Transfer the protein to a centricon-10, wash the centriplus with 1 ml buffer (put this in the centricon), concentrate to 200 µl, and wash the centricon with 200 µl buffer. The sample is then run 2 x 200 µl over the Superdex 200 HR 10 / 30 sizing column. The fractions containing properly renatured K^d / $c\beta_2m$ are pooled and concentrated to 0.5-1 ml. If there is any remaining β_2m, save it for the next denaturation. Determine final concentration of K^d / $c\beta_2m$ by BCA assay. Protein should then be stored at 4°C.