Production of Empty and Peptide-filled H-2K^d / mβ_2m

Cell Line:

2B is the name Peggy gave to the K^d / mβ_2m producing CHO cell line. This is the mouse MHC class I heavy chain complexed with the mouse light chain. These cells must be grown is serum-free conditions, or the mouse light chain will exchange for bovine light chain present in bovine serum. I have only used harvests produced on the Cell Pharm by David. To the harvest I add azide to a final concentration of 0.05% 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 M1/42 column located in the coldroom. This column recognizes mouse heavy chains when they are complexed with mouse β_2m only. Currently, there is one such column, made by Peggy. 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. After elution, the pH of the column is neutralized with 5-10 column volumes of 50 mM Tris pH 7.4, 0.05% azide, and 1 mM EDTA. The column is then ready to have another harvest run over it. Flowthroughs from each harvest should be saved and re-run over the columns to ensure all the protein has been removed. Typically, Cell Pharm harvests will produce 2-3 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 (determined by reading the A_{280} of the individual fractions) 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 as much K^d / $m\beta_2m$ as possible for the process. Pool the samples 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 $m\beta_2m$ (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 / $m\beta_2m$ 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 / $m\beta_2m$ by BCA assay. Protein should then be stored at 4°C.