PURIFICATION OF HSP70 PROTEINS
Column Guidelines

The purification protocol to be used for each of the Hsp70 proteins depends on whether that protein has the ATP binding domain. If the protein does have the ATP binding domain then that protein is purified utilizing the DEAE, ATP agarose and Resource Q columns. If the protein does not contain the ATP binding domain then it is probably expressed as a GST fusion protein and in such a case it is purified using the DEAE, Glutathione, and Resource Q. columns. Outlined below are the basic protocols used to purify all Hsp70 proteins.

DEAE Sepharose Fast Flow Column:

1. If the volume of the crude extract is larger than 5 ml use the 200 ml DEAE column, otherwise use the 30 ml DEAE column. In either case the sample is passed over the column at 4° C using the following FPLC protocol:
   I. the sample is loaded onto the column at a flow rate of 1 ml/min
   II. the column is flushed with 20 mM Tris pH 6.9 (at r.t.), 1 mM EDTA and 0 mM NaCl (TEN_0) until the chart recorder reaches base line- the flow thru is collected in a separate container
   III. a salt gradient from TEN_0 to $TEN_{0.35}$ (350 mM NaCl) is passed over the column in a total of 10 column volumes and at 10 ml/min
      a. fractions are collected during the gradient period (25 ml for the 200 ml DEAE column)
   V. a salt jump to $TEN_{1.0}$ is then done for 2 column volumes at 10 ml/min
      a. fractions collection continues through half the high salt wash
   VI. the column is equilibrated with TEN_0 for 2 column washes at 10 ml/min

2. Aliquots (12 µl) are taken of fractions 1, 5, 10, 12, 15, 17, 20, 22, 25, 27, 30, 35, 40 and 45
   A. The aliquots are resolved on a 10% SDS-PAGE gel to determine the location of the hsp70 protein peak
   B. if the strain utilized contains dnaK, take caution not to pool fractions containing dnaK (elutes from the column around 220 mM NaCl)

ATP Agarose Column:

1. Using a peristaltic pump the pooled samples are recirculated over a 20 ml ATP agarose column, equilibrated with 20 mM Tris pH 6.9 (r.t.), 5 mM $MgCl_2$ and 100 mM NaCl ($TMgN_{0.100}$) at 4° C,

for a total of 3-5 passes at 1-2 ml/min

A. a 12 µl aliquot is removed after each pass

2. The column is washed with 50-100 ml of $TMgN_{2.00}$ at 1-2ml/min and then equilibrated with 50-100 ml $TMgN_{0.100}$

3. 10 ml of $TMgN_{0.100}$ + 10% glycerol + 50 mM ATP is loaded onto the column at 1 ml/min and flushed through with $TMgN_{0.100}$

A. 1.5 ml fractions are collected for a total of 30-40 fractions

4. 12 µl aliquots are taken from each sample and resolved on a 10% SDS-PAGE

A. The fractions containing the hsp70 protein peak are pooled

5. The pooled fractions are concentrated down to ~10ml using a centriprep-10 and then dialyzed for ~8h at 4°C against $TEN_{0.100}$

Resource Q Column and Dialysis:

1. The dialyzed sample is passed over a 6 ml Resource Q column (equivalent to a Mono Q column) and a $TEN_{0.0}$ to $TEN_{0.4}$ salt gradient is done over 5 column volumes, a $TEN_{1.0}$ wash is done for 2 column volumes and then the column is equilibrated with 2 column volumes of $TEN_{0.0}$

A. 1.5 ml fractions are collected from loading the sample to half through the $TEN_{1.0}$ salt wash

B. 12µl aliquots of every other fraction are resolved on a 10% SDS-PAGE to determine the location of the hsp70 protein, these fractions are pooled and concentrated with a centricon-10

2. The concentrated sample is loaded into a dialysis tube (Spectra/Por 2 MW12,000-14,000) and dialyzed against $TEN_{0.100}$ (the buffer is changed every 12h)

A. A 50 ml sample is taken before dialysis begins and at every change of buffer. These samples are utilized to monitor the 260/280 ratio (i.e. the presence of nucleotide).

--the nucleotide should be completely removed by 3 days

B. the concentration of the dialyzed sample is then determined by either the BCA method, the extinction coefficient or both

Glutathione Agarose Column and Cleavage of GST Fusion Protein

1. Using a peristaltic pump the pooled samples are recirculated over a 20 ml glutathione agarose column, equilibrated $TEN_{0.100}$ at 4° C, for a total of 3-5 passes at 1-2 ml/min

A. A 12 µl aliquot is removed after each pass

2. The column is washed with 50-100 mls of $TEN_{3.00}$ at 1-2ml/min and then equilibrated with 50-100 ml of $TEN_{0.100}$

3. 10 ml of $TEN_{0.100}$ + 10% glycerol + 100 mM glutathione is passed over the column at 1 ml/min

A. 1.5 ml fractions are collected for a total of 30-40 fractions

4. 12 µl aliquots are taken from each sample and resolved on a 10% SDS-PAGE

A. the fractions containing the fusion protein are pooled, concentrated to ~1ml, desalted over a G-25 column and dialyzed for 4h at 4°C against $TEN_{0.100}$

B. the protein concentration of the dialyzed sample is determined using the BCA method

5. The GST fusion protein is now cleaved by the addition of $CaCl_2$ to a final concentration between 2-5 mM (individually determined for each protein) and thrombin to 1-2 mg/mg of fusion protein. The cleavage reaction is incubated at 25° C for the required length of time (determined by sampling the cleavage reaction over time)

6. The cleaved sample is re-passed over the glutathione column to remove the cleaved GST fragment and uncleaved fusion protein. The sample is flushed through the column with $TEN_{0.100}$ and 20 1.5 ml fractions are collected. The location of protein is determined utilizing the BioRad protein assay, the fractions are pooled and concentrated to ~10ml.

7. The sample is then resolved over the Resource Q column as described above except there is no need to extensively dialyze the protein.