

Hsp70 PURIFICATION
Brian Freeman's Protocol

Materials/Reagents:

LB/amp media (50mL and 1L with each having 100mg/L amp.)
PMS119-Hsp70 plasmid
BL21/DE3 competent cells
LB/amp plates (2)
IPTG (1mL of 1M)
Lysozyme
Leupeptin and Pepstatin A
Dialysis tubing
TEN₁₀₀ buffer (20mM tris pH 7.4, 0.1mM EDTA, 100mM NaCl)
ATP agarose column
TMN buffer (Tris 20mM pH7.4, MgCl₂ 5mM, NaCl 100mM) around 500mL-1L
ATP
Glycerol
Millipore concentrators.

Procedure:

Overexpression

- 1) (day 1) Transform 100ng of the PMS119-Hsp70 plasmid into BL21/DE3 competent cells and plate on an LB/amp plate.
- 2) (day 2) Pick a single colony of the transformation and streak several times onto another LB/amp plate.
- 3) (day 3) Pick a single colony off of the streaked plate and place into 50mL LB/amp and incubate overnight at 37°C.
- 4) (day 4) Take the overnight culture and inoculate it to a 40X dilution in 1L LB/amp.
- 5) Grow at 37°C in a shaker until the O.D.₅₉₅ is 0.5-0.9 (around 2 hours).
- 6) Take a 1mL sample and save as an uninduced sample.
- 7) Induce the cells with a final concentration of 1mM IPTG.
- 8) Grow at 30°C in a shaker for 4-6 hours.
- 9) Take a 1mL sample (induced).
- 10) Harvest cells by spinning them down at 5,000 rpm for 20 minutes.
- 11) Remove the supernatant. Pellet can be frozen at -80°C for use later.

Test for overexpression

- 1) Take the uninduced and induced sample and spin at max rpm for 1 minute.
- 2) Aspirate the supernatant.
- 3) Resuspend the pellets in 1x SDS sample buffer (100µL for the uninduced and 200µL for the induced)

- 4) Load 10 μ L each on a SDS-PAGE gel.
- 5) Look for overexpression at the predicted size. There should be a much larger band in the overexpression lane.

Cell lysis

- 1) Resuspend cells in Ten₁₀₀ buffer at 3ml per 1g of cells.
- 2) Add lysozyme to 0.5mg/mL concentration.
- 3) Add leupeptin and pepstatin A to concentrations of 1 μ g/ μ L.
- 4) Put on a nutator in the cold room for 30 minutes.
- 5) Freeze and thaw 4X in a methanol bath and 37°C water bath.
- 6) Sonicate 30X in 3X cycles (using 30-40% duty cycle and 3-4 output control) or until the viscosity is greatly reduced.
- 7) Centrifuge at 32000rcf for 60 minutes
- 8) Take the supernatant to load onto a column.

Chromatography

- 1) First separate the lysate over a DEAE column using a salt gradient from 0mM NaCl to 500mM NaCl over 1000mL of elution.
- 2) Run gels with uninduced and induced samples along with 16 μ L of each odd fraction.
- 3) Stain by coomassie and identify fractions containing Hsp70.
- 4) Pool fractions.
- 5) Pour ATP beads into an empty column.
- 6) Put the plunger part-way in and run water through it until the beads settle.
- 7) Push the plunger all the way down to just above the bead line.
- 8) Wash by running 5x of the bed volume 20% ethanol.
- 9) Wash 5x volume ddwater.
- 10) Wash 5x volume TMN buffer.
- 11) Recirculate the pooled fractions overnight (meaning the pump pushes the pooled fractions through the column and back into the container containing the pooled fractions. The fractions thus are continuously going through the column.)
- 12) Wash the column with 30ml TMN and collect with the recirculation
- 13) Wash with 5x volume TMN and discard
- 14) Elute with 10 ml elution buffer (25mM ATP, 10% glycerol, 90% TMN) and collect
- 15) Wash with 50mL TMN and collect.
- 16) Concentrate down to 10mL using the Millipore concentrators (MW 30,000).
- 17) Do dialysis overnight in TEN₁₀₀ for two nights (one buffer change, 2L each).
- 18) Recirculate the previous sample solution that you recirculated and perform the procedure again

- 19) Resolve the samples after dialysis over the Resource Q column using a salt gradient of 0mM NaCl to 500mM NaCl and elution volume of 350mL.
- 20) Run odd samples on a gel as described before and identify fractions that contain Hsp70.
- 21) Concentrate the protein using Millipore concentrators.

Troubleshooting/Critical Parameters

If overexpression is not achieved, try lowering the overexpression temperature. Also, the overnight and initial dilutions can be performed at lower temperatures. IPTG concentration can also be increased. The indicated parameters should work. See protocols for FPLC use and programming for column help.

References:

Freeman, BC., Myers, MP., Schumacher, R., and Morimoto, RI. Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with Hdj1. *EMBO*. **14**:2281-2292 (1995).

Submitted by:

Brandon Toyama