CAPZ PREPARATION BY BACTERIAL EXPRESSION (LYSIS BY DETERGENT, NOT SONICATION.)
Soeno, Y. et al., J Mus. Res. Cell Motility 19:639-646

Notes before starting:

*Bacterial strain BL21(DE) pLys S transformed with pET3d-βI'/βII→ our pBJ 994 Obtained from T. Obinata. Do not carry this strain on a plate or in liquid culture. Instead, for every protein prep, do a fresh transformation of the expression plasmid that you know is good, from a restriction digest, into competent cells. Isolate transformants on a plate and then take them directly into the liquid culture.

*See list of solutions appended for recipes.

Day 1
1. Set up 100 ml overnight culture in LB containing 50mg/ml ampicillin. Grow at 37^0 C.

Day 2
2. Grow the overnight in 1 L of LB-amp. Induce expression with 10 ml of 100mM IPTG when the A_{600}=0.8. Take 1ml samples at pre-induction,1, 2, and 3 hours post-induction to monitor protein expression (also record the A_{600} for these samples).

3. After 3 hours of induction, harvest the cells by centrifugation at 4000rpm for 15'. **ALL STEPS AFTER THIS SHOULD BE DONE ON ICE OR IN THE COLD**

4. Wash cells once with 100ml of wash buffer. Harvest cells by centrifugation at 6000rpm for 15' in GSA rotor in Sorvall.

5. Resuspend cells in 100ml of sucrose buffer and incubate on ice for 30'. Harvest the cells as in step 4.

6. Resuspend the pellet in 100ml of lysis buffer. Use loose douncer to break up the cell clumps. Take a 1ml sample for SDS gel.

7. Ultracentrifuge the lysate (T145 rotor) at 35,000rpm for 1 hour. Record the volume of the supernatant and save a small sample for gel analysis. Resuspend the pellet to a volume equal to the supernatant (ie. "cell equivalent"), and save a sample from that as well.

SEE PREPARATION USING SONICATION FOR REMAINING STEPS OF THE PREP.

CAPZ PREPARATION BY BACTERIAL EXPRESSION (WITH SONICATION SO NO DETERGENT IS USED DURING LYSIS)
Soeno, Y. et al., J Mus. Res. Cell Motility 19:639-646

Notes before starting:

*Bacterial strain BL21(DE) pLys S transformed with pET3d-alpha1/beta1; this is our pBJ 994 Obtained from T. Obinata. Do not carry this strain on a plate or in liquid culture. Instead, for every protein prep, do a fresh transformation of the expression plasmid that you know is good, from a restriction digest, into competent cells. Isolate transformants on a plate and then take them directly into the liquid culture.

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3. After 3 hours of induction, harvest the cells by centrifugation at 4000rpm. **ALL STEPS AFTER THIS SHOULD BE DONE ON ICE OR IN THE COLD**

4. Wash cells once with 100ml of wash buffer. Harvest cells by centrifugation at 6000rpm for 15' in GSA rotor in Sorvall.

5. Resuspend cells in 100ml of sonication buffer. *Use the microprobe to sonicate the cells at setting 5 (~20% of maximum output). Sonicate ~12 times for 10s each. Watch for a subtle color change (sample gets darker). Save a sample for gel. **To break open the cells, can try freeze-thaw in liquid nitrogen before sonication or adding lysozyme. To solublize the protein, can try adding Triton to ~1%, after but not before sonication. **

7. Ultracentrifuge the lysate (Ti45 rotor) at 35,000rpm for 1 hour. Record the volume of the supernatant and save a small sample for gel analysis. Resuspend the pellet to a volume equal to the supernatant (ie. "cell equivalent"), and save a sample from that as well.

8. Precipitate the CapZ from the high-speed supernatant with a 50-70% $AmSO_4$ cut. Add the ammonium sulfate gradually and stir gently but continuously after addition of salt at each cut to dissolve the salt. The amount of ammonium sulfate depends on the volume. See Table on page 372 of Cooper, Terrance G. The Tools of Biochemistry. United States: John Wiley & Sons, 1977. pp 370-372. A blue tab marks the page

9. Centrifuge after each cut at 14,000rpm for 20' in GSA 600 rotor. Save pellets and a small amount of the supernatant from the 0-25%, and 25-50% cuts for gel.

10. Resuspend the 50-70% pellet (the good stuff!) in 40ml of HA Buffer A. The 50-70% supernatant should also be saved for analysis, if desired.

11. Dialyze the 50-70% resuspended pellet in HA buffer A at 4˚C. Change the buffer and mix the contents of the bag by hand at least once before you go home.

Day 3

12. Clarify the dialyzed sample by a brief spin and apply it to a 25x10cm BioRad HA (HT) resin column (approximately 40-50 ml bed). Wash the column with HA Buffer A until A_{280} <0.05.

13. Elute the protein with a 10-250mM K-phosphate gradient, Total volume=300ml (150ml of each buffer A and B). Collect 60 drop fractions using small round fraction collector. Capping protein elutes in early part of first big peak (see HA profile).

14. Analyze the fractions on a 12.5% polyacrylamide gel. Alpha and beta subunits are ~36 and 32 kD, respectively. Proteolysis of the beta subunit by removal of the beta tentace is a common problem, revealed as second band just below the primary one. Pool the desired fractions and dialyze overnight in MonoQ Dialysis Buffer (Buffer A) at 4ºC.

Day 4

15. Apply the dialyzed sample to the large MonoQ column, which has been previously cleaned with a sawtooth gradient and equilibrated in buffer A. Elute protein with a 10-400mM KCl gradient over 300 min. Collect 5ml fractions (60 drops) at a rate of 1 ml/min.

17. Analyze the fractions on 12.5% polyacrylamide gel. Pool the appropriate fractions and dialyze overnight in MonoS dialysis Buffer, **pH 6.0**, at 4ºC.

Day 5

18. The next day, transfer the dialysis sample to 10 mM MES, pH 5.8, 1 mM DTT, 0.5mM EDTA for 1-2 hours before running on MonoS column. Watch out for ppt forming at low pH; don't dialyze too long at this pH!

19. Apply dialyzed sample to large MonoS column. Wash with MonoS buffer A ~2-3 column volumes till OD is at baseline. Elute with 0-350 mM NaCl over 60min in 10 mM MES, pH 5.8, 1 mM DTT, 0.5 mM EDTA. Pool appropriate fractions and dialyze against 10mM Tris-Cl, pH 8.0, 40 mM KCL, 0.5mM DTT.

20. There is sometimes problems getting all the CapZ to stick and it comes out in the flowthrough. I save this and reapply to the column.

21. Sample can be concentrated by batch elution on a MonoQ/DEAE cellulose resin or using Centricon-30.

CapZ prep solutions

What	How Much	Final [ ]
IPTG 100mM	.238g	1mM in 1L LB
	10 ml ddH2O	
DTT 1M	1.542g	
	10ml ddH2O	
PMSF 1M	1.742g	
	10ml DMSO	
add any other protease inhibitors you like!		
Wash Buffer		
NaCl	11.69g	0.14 M
EDTA , 500mM pH 8.0	16ml	40mM
Tris, 1M pH8.0	8ml	40mM
	200ml	
Sonication Buffer		
Tris, 1M pH8.0	10ml	50mM
EDTA, 500mM pH8.0	400υl	1mM
DTT, 1M	200μl	1mM
PMSF, 1M (add before use)	200μl	1mM
	200ml pH8.0	
Phosphate Dialysis Buffer		
KCl	149.12g	0.5M
K-phosphate	5.44g	10mM
DTT, 1M	4ml	1mM
NaN_3, 10%	4ml	0.01%
PMSF, 1M	4ml	1mM
	4L pH7.0	

What	How Much	Final [ ]
HA Column Buffer A		
KCl	37.28g	0.5M
K-phosphate	1.36g	10mM
DTT, 1M	1ml	1mM
NaN_3, 10%	1ml	0.01%
PMSF, 1M	1ml	1mM
	1L 0.2μm filter pH7.0	
HA column Buffer B		
KCl	18.64g	0.5M
K-phosphate	17.01g	250mM
DTT, 1M	500μl	1mM
NaN_3, 10%	500μl	0.01%
PMSF, 1M	500μl	1mM
	500ml 0.2μm filter pH7.0	
MonoQ Dialysis Buffer		
KCl	14.912g	10mM
Tris, 1M pH8.0	40ml	10mM
EDTA, 500mM pH8.0	4ml	0.5mM
DTT, 1M	4ml	1mM
PMSF, 1M (add before use)	4ml	1mM
NaN_3, 10%	4ml	0.01%
	4L	
MonoQ Buffer A		
KCl	0.746g	10mM
Tris, 1M pH8.0	10ml	10mM
EDTA, 500mM pH8.0	1ml	0.5mM
DTT, 1M	1ml	1mM
PMSF, 1M (add before use)	1ml	1mM
MonoQ Buffer B		
KCl	18.64g	0.5M
Tris, 1M pH8.0	5ml	10mM
EDTA, 500mM pH8.0	500μl	0.5mM
DTT, 1M	500μl	1mM
PMSF, 1M (add before use)	500μl	1mM
NaN_3, 10%	500μl	0.01%
	500ml 0.2mm filter pH8.0	

Alternative to Sonication:

What	How Much	Final [ ]
Sucrose Buffer		
Tris, 1M pH8.0	10ml	50mM
EDTA, 500mM pH8.0	4.8ml	12mM
Sucrose	20g	10%
	200ml	
Lysis Buffer		
Tris, 1M pH8.0	2ml	20mM
EDTA, 500mM pH8.0	2ml	10mM
TritonX-100	2g	2%
PMSF, 1M	100µl	1mM
	100ml	

What	How Much	Final [ ]
MonoS Dialysis Buffer		
MES (use Na+)	?	10mM
EDTA, 500mM pH8.0	4ml	0.5mM
NaN_3, 10%	4ml	0.01%
DTT, 1M	4ml	1mM
PMSF, 1M	0.4ml	1mM
	4L pH=6.0	
MonoS Buffer A		
MES (use Na+)	?	10mM
EDTA, 500mM pH8.0	1ml	0.5mM
NaN_3, 10%	1ml	0.01%
DTT, 1M	1ml	1mM
PMSF, 1M	0.1ml	1mM
	1L pH5.8	
MonoS Buffer B		
MES (use Na+)	?	10mM
EDTA, 500mM pH8.0	1ml	0.5mM
NaN_3, 10%	1ml	0.01%
DTT, 1M	1ml	1mM
PMSF, 1M	0.1ml	1mM
NaCl	29.22g	1M
	500ml pH5.8	