Denaturing purification of Insoluble Proteins

Buffer A	**500 ml**	**1 L**
6 M Guanidine hydrochloride	286.59 g	573.18 g
0.1 M NaH_2PO_4	6.9 g	13.8 g
0.01 M Tris	5 ml	10 ml (of 1 M Tris HCl pH 8.0)
adjust pH to 8.0 with NaOH		

Buffer B

8 M Urea	240.24 g	480.48 g
0.1 M NaH_2PO_4	6.9 g	13.8 g
0.01 M Tris	5 ml	10 ml (of 1 M Tris HCl pH 8.0)
adjust pH to 8.0 with NaOH		

Buffer C

8 M Urea	240.24 g	480.48 g
0.1 M NaH_2PO_4	6.9 g	13.8 g
0.01 M Tris	5 ml	10 ml (of 1 M Tris HCl pH 8.0)
adjust pH to **6.3** with HCl		

Buffer E

8 M Urea	240.24 g
0.1 M NaH_2PO_4	6.9 g
0.01 M Tris	5 ml (of 1 M Tris HCl pH 8.0)
adjust pH to **4.5** with HCl	

1. Resuspend the cells in Buffer A: 5 ml per gram weight.
2. Stir cells for 2 hours at room temperature.
3. Centrifuge lysate at 10,000 rpm for 30 minutes at 4 C, collect supernatant.
4. Take 1 ml of a 50% slurry of Ni-NTA resin, wash 3 times in Buffer A (50 ml), equilibrate in Buffer A (v/v).
5. Add 50% Ni-NTA resin to supernatant from step 3 and stir at room temperature for 1-2 hours.
6. Centrifuge at 1600 rpm for 5 minutes. Discard supernatant.
7. Wash beads 3 times with 10-50 ml of Buffer A (centrifuge at 1600 rpm for 5 minutes).
8. Wash beads 3 times with 10-5- ml of Buffer B.
9. Wash beads 3 times with 10-50 ml of Buffer C.
10. Elute protein with 500 µl of Buffer E (7 times).
11. Centrifuge the eluates for 20 seconds at 10,000 rpm to remove rest of beads.
12. Analyze samples on SDS-PAGE: 20 µl sample + 10 µl 3x Lämmli buffer.

Protein gel electrophoresis (Lämmli)

- solutions:

-Tris gel buffer:
375 mM Tris pH 8.8
0.1% SDS

-stacking gel (4%):
26.67 ml acrylamide-stock (30%)
0.2 g SDS (0.1%)
3 g Tris base
adjust pH to 6.8
add H_2O to 200 ml
store at 4 C

-10x running buffer:

250 mM	Tris	30.3 g/l Tris base
1920 mM	glycine	144.2 g/l
1%	SDS	10 g/l

pH should be ~8.3 (<u>do not</u> adjust pH!!!)

-3x Lämmli:
187.5 mM Tris pH 6.8
6% SDS
30% glycerol
15% β-mercaptoethanol
0.003% bromphenolblue
make 1 ml aliquots
heat 5 min at 100oC in heating block

-sample preparation:
x μl protein extract
5 μl 3x Lämmli
H_2O to 15 μl
-denature 5 min at 95-100 C (heating block)
-samples can be stored at –20 C (heat again before loading)

-gel preparation:

-for Hoefer Minigels (Mighty Small II SE250)
-clean 1 glass plate, 1 white plate and 2 spacers (0.75 mm) per gel
-assemble for pouring (white plate in the back, do not tighten screws too much, make sure spacers and plates are level at the bottom)
-prepare 10% APS (ammonium persulfate) in H_2O
-make a mark on glass plate to indicate the volume of the gel (~1 cm below the comb)
-acrylamide-solution: e.g. for two 10% gels:
3.3 ml acrylamide-stock (30%)
6.7 ml Tris gel buffer
200 μl 10% APS
6 μl TEMED
-mix well and pour gel up to mark
-add 200 μl n-butanol on top
-polymerize for ~1 h

-remove gel from pouring unit, remove n-butanol with H_2O
-remove drops between plates using a whatman paper
-stacking gel: for 2 gels:

4 ml	stacking gel (4%)
200 µl	10% APS
4 µl	TEMED

-mix well and fill gel up to the very top
-immediately add combs (stacking gel polymerize very fast)
-polymerize for 15 min

-gel run:
-prepare 250 ml 1x running buffer
-carefully remove combs and load samples
-add protein standard
-run gels for 50-60 min at 25-30 mA per gel (use water cooling and mix buffer) until bromphenolblue reaches bottom of gel and runs out
-the protein gels can be stained directly or used in a western blot procedure

Staining of protein gels with Coomassie

- solutions:

-Coomassie-solution 1:	0.025%	Coomassie Brilliant Blue R250
	25%	isopropanol
	10%	HAc
-Coomassie-solution 2:	0.0025%	Coomassie Brilliant Blue R250
	10%	isopropanol
	10%	HAc
-destaining solution:	10%	isopropanol
	10%	HAc

-shake protein gel 20-30 min in Coomassie-solution 1
-partially destain in Coomassie-solution 2 (gels can be left in this solution almost indefinitely)
-destain 2-3 h in destaining solution (add a rolled kim wipe to absorb the coomassie)
-fix for ~1 h in 10% HAc
-sandwich gel between wet cellophane foil, put sandwich on a whatman filter and cover with saran wrap
-dry for 1 h on gel drier at 80 C
-make sure gels do not curl