

## Preparation of GST-Rb for Kinase Assays

Tween Lysis Buffer (200ml)		(500ml)
	167ml water	4.17.5 ml
50mM HEPES pH8.0	20ml 0.5M HEPES pH8.0	50ml
1mM EDTA pH8.0	400µl 0.5M EDTA	1ml
2.5mM EGTA pH8.0	2ml 0.250M EGTA	5ml
150mM NaCl	6ml 5M NaCl	15ml
1mM DTT	200µl 1M DTT	500µl
0.1 % Tween 20	200µl Tween 20	500µl
1mM NaF	200µl 1M NaF	500µl
0.1mM NaVO <sub>4</sub>	200µl 100mM NaVO <sub>4</sub>	500µl

prior to use add inhibitors, 10µl/ml of 10mg/ml PMSF in isopropanol and 0.2µl/ml of 10mg/ml aprotinin,

### NETN Buffer (500ml)

20mM Tris-HCl (pH8.0)	10ml of 1M Tris-HCl pH8.0
100mM NaCl	10ml 5M NaCl
1mM EDTA	2ml 0.25M EDTA
0.5% NP-40	2.5ml 100% NP40
	475.5ml water

### wash beads before use

0.5% milk: NETN buffer

add an equal volume and wash three times

- set up overnight culture of Rb-C-terminus (~75kD) in 50ml LB+Amp
- next day take one tenth (40ml in 360ml LB+Amp) and incubate at 37°C for 2 hours
- add 0.2mM IPTG to the culture (prepare fresh IPTG 1M=0.2384g in 1ml water and add 80µl to 400ml culture) incubate at 37°C for 3 hours
- spin down the cells in big bottles at 4°C (4000rpm/5K) for 15min
- pour off the supernatant, and resuspend the cells in 40ml Tween lysis buffer, transfer to 30ml corex tubes

- sonicate the cells 3 times, each time for 15 seconds at ~23
- spin the cells at 12000rpm for 10 minutes
- take off the supernatant and put into falcon tubes (~10ml) add 250 $\mu$ l sepharose-GST (previously washed beads) to each tube and incubate 1 hour-24 hours at 4°C
- either,
- spin down the beads 2000rpm for 5 min, and wash 5x with NETN buffer
- freeze half and put the other half in 2mM glutathione to elute the protein, 2 hours at 4°C
- take off the supernatant and put in the freezer (keep the beads)
- run 10% gel to test the protein, run gel then stain with comassie blue for ~20 minutes, destain overnight and then dry
- or,
- pellet the beads at 2000rpm for 5 min. Wash the beads 4 times with 5ml Tween lysis buffer. Combine the beads in 1 tube and elute twice with 2.5ml 2mM glutathione in Tween lysis buffer (10ml Tween lysis buffer + 6mg glutathione) by rocking for 2 hours, total eluate volume is 5ml.
- transfer the eluate to dialysis tubing and dialyze for 2 hours with stirring at 4°C. Dialysis buffer 750ml PBSA/0.1% Tween/ 1mM DTT/ 50% glycerol. Volume in bag will be reduced after dialysis. Change to fresh dialysis buffer 750ml and dialyze overnight.
- Remove peptide solution from tubing and estimate concentration by running 2, 5 and 10 $\mu$ l on a gel against BSA standards and staining gel with Comassie Blue.