Gst-Pak Binding assay

I. Make 32D lysates - Important: do the procedure quickly and follow the times noted

$3x10^6$ cells per assay

1) Wash exponentially growing cells 3x in HBSS
2) Starve cells in SFM at 5-10 x 10^6 per ml for 2 hours 30 min in 50 ml conicals
3) Add HEPES buffered Krebs Ringer buffer (KRB-H) and spin out cells
4) Repeat wash x 1 with KRB-H
5) Resuspend cells at 2.5 x 10^7/ml in KRB-H -> 120 μl of cell suspension per sample
6) Do pretreatment and stimulation
7) Prealiquot equal volume (120 μl) of 2X Rac LB supplemented with the usual inhibitors + **10 μg of GST-Crib-Pak** recombinant protein and keep on ice
8) At the indicated times, transfer cell suspension to tube with lysis buffer
9) Lyse for 10 min on ice
10) Spin out debris for 10 min, 4 deg.
11) Have ready tubes containing 10 μl of GSH sepharose (prewashed) in 90 μl of binding buffer.
12) Transfer supernatant to new tubes.
13) Transfer 180 μl of lysate to pulldown tubes. Bind for 1 hr. Save remaining lysate for protein determination (may be a problem if BSA in KRB-H) and TCL.

Krebs Ringer Buffer, HEPES buffered

1X	stock	MW	500 ml
20 mM HEPES	powder	238.3	2.383 g
107 mM NaCl	powder	58.44	3.127 g
5 mM KCl	powder	74.56	0.1864 g
3 mM $CaCl_2$	powder	147.02	0.2205 g
1 mM $MgSO_4$.(anhydr)	powder	120.37	0.0602 g
7 mM $NaHCO_3$	powder	84.01	0.2940 g
10 mM glucose	powder	180.16	0.901 g
[0.1% BSA	**powder**		**0.5 g]**
(this may interfere with protein determination)			
dH_20			450 ml

pH to 7.4
make up to 500 ml
filter sterilize

Rac Lysis buffer

2X	stock	200 ml	
50 mM Tris pH 7.4	1 M	10	ml
2% NP40 (w/v)	neat	4	gm
0.3 M NaCl	5 M	12	ml
20% glycerol	neat	40	ml
20 mM $MgCl_2$	1 M	4	ml
[0.5% DOC		**1**	**gm]**
(new UBI protocol does not have DOC)			
dH_20		110	ml

check pH (7.4 to 7.5), bring volume up to 200 ml
[UBI Rac lysis buffer = Ras lysis buffer, somewhat different]

Binding buffer (assumes a 2:1 lysate to binding buffer ratio)

1X	stock	10 ml
25 mM Tris pH 7.5	1M	0.25 ml
40 mM MgCl2	1M	0.4 ml
1 mM DTT	1M	10 µl
dH_20		9.35 ml

II. Making GST-Pak-Crib extracts

Buffers

HBS-E (50 ml)

1X	stock	amount
20 mM Hepes	0.5 M	2 ml
0.15 M NaCl	5M	1.5 ml
2 mM EDTA	0.25 M	0.4 ml
dH20		46.1 ml

HBS-E with 0.5 % NP40 (50 ml)

1X	stock	amount
20 mM Hepes	0.5 M	2 ml
0.15 M NaCl	5M	1.5 ml
2 mM EDTA	0.25 M	0.4 ml
0.5% NP40	10%	2.5 ml
dH20		43.6 ml

SDS-Urea sample buffer 10 ml

Na phosphate	0.01 M	1.0 ml of 0.1M stock
β-ME	1%	0.1 ml of neat
SDS	1%	1.0 ml of 10% stock
Urea	8M	4.804 gm
dH_20		to 10 ml

Elution buffer

10 mM reduced glutathione in 50 mM Tris-HCl (pH 8), freshly prepared

glutathione (reduced) MW 307.3

10 mM is 3.073 g/L or 3.073 mg/ml

Make a stock of 100 mM: 30.73 mg/ml, dilute 1:10 into 50 mM Tris pH 8

Protein storage buffer

	1X	4X	stock	5 ml
Tris	25 mM pH 7.5	25 mM*	1M	0.125 ml
glycerol	5% w/v	20 %	neat	1 g
NaCl	100 mM	400 mM	5 M	0.4 ml
DTT	0.2 mM	0.8 mM	1 M	4 μl
MgCl2	1 mM	4 mM	1 M	20 μl
DH20				3.475 ml

Growth and Induction

1. Streak out plate from glycerol stock
2. Inoculate 40 ml of LB/amp in a 250 ml flask, shake overnight
3. Transfer 40 ml of overnight culture into Fernbach flask containing 400 ml of LB/amp (1:10 fold dilution)
4. Grow with shaking at 37 deg until OD between 0.6-0.7
5. Add IPTG to final of 0.1 mM, a 1:1000 fold dilution from a fresh IPTG stock of 100 mM (23.83 mg/ml, filter sterilize).
6. Grow for 2-3 hrs with shaking.
7. Harvest bacteria by transferring to 200 ml bottles (x2). Spin at 6000 rpm for 20 min at 4 deg.
8. Decant.
9. Add 5 ml of PBS, resuspend and aliquot 1ml (equivalent to 80 ml of culture) to Sarstedt tubes. Add 4 more ml of PBS per tube, spin at 8000 rpm for 10 min.
10. Decant and freeze pellet at –80 deg.

Lysis

11. Thaw the equivalent of _____(80) ml of bacterial pellet
12. Lyse pellet in _____ (8) ml (10% of culture volume) of HBS-E.
13. Supplement lysis buffer with protease inhibitors (aprotinin, leupeptin, pepstatin, PMSF, DTT, benzamidine, usual concentrations). Add lysozyme to final of 0.1 mg/ml.
14. Incubate on ice for 15 min
15. Add _____ (0.42) ml of 10% NP40 to final of 0.5% . Incubate at 4 deg for 30 min (rock end-over-end).
16. (A) Divide into 2 x 4.5 ml aliquots for sonication with microtip. Volume limit for doublestep microtip is 5 ml. If volume is > 15-20 ml, transfer to sawed off clear conical and change to 1/2" disrupter.
    - Microtip: setting of 4, output 20.
    - Sonicate with 6 x 1 min bursts, 2 min of cooling in between. Make sure tip is at least 5 mm away from the bottom (make mark on the mcirotip); too close will drill a hole in the tube, too high lysate will foam and proteins will denature.
17. (B) 1/2" horn disrupter: setting of 5, output 40
    - Same sonication protocol as above.
18. Transfer to Oakridge tubes.
19. Centrifuge at 11500 rpm (Superspeed) or at 15000 rpm (Beckman) for 20 min at 4 deg (SS34 rotor for Sorvall or JA20 for Beckman)
20. Transfer supernatant (8.42 ml) to 15 ml conical. Keep on ice. Remove 20 μl aliquot (**Test #1**).
21. To test for efficiency of extraction, resuspend remaining pellet in 1 ml of HBS-E/NP40. Remove 20 ul to test for efficiency of extraction -keep on ice (**Test #2**).

Bulk purification with GSH sepharose followed by elution and storage

1. Prepare GSH beads (Pharmacia) - capacity is 5 mg of GST per 1 ml of bed volume
   [dispense 1.33 ml of original slurry per ml of bed volume required].
   a. For an extract estimated to have 0.1 mg/ml GST fusion protein, assuming 8 ml of extract, I calculate I need (8 ml x 0.1 mg/ml)/5*1.33 ml of beads = 0.22 ml of original slurry.
   b. in one conical, add 0.4 ml of original slurry (0.3 ml of bed volume), try to get as much of the beads as possible (sticks to sides)
   c. wash 2x with HBS-E/0.5% NP40 (spin in tabletop centrifuge, 4 deg at top 500 x g for 5min), aspirate off wash
2. Add all of the extract to beads, incubate for 1 hr at 4 deg, rocking end-over-end
3. Spin down and transfer supernatant to new tube (just in case)
4. wash GSH beads, 3 x 5 ml in HBS-E/Triton supplemented with benzamidine and PMSF. Aspirate wash.
5. Wash beads 1 x 5 ml in PBS/0.1% Triton. Aspirate wash, remove as completely as possible.
6. To the beads in the conical, elute with reduced glutathione: 1 ml of elution buffer per ml of bed volume (0.2 ml)
7. Mix gently to rsuspend beads. Incubate at RT for 10 min to elute the bound material from beads.
8. Spin at 500 x g for 5 min to sediment beads. Remove and save supernatant (containing the recombinant protein) to a new tube.
9. Repeat elution and spin steps twice more; total volume = 0.6 ml
10. Pool the 3 eluates.
11. Save the beads (label the construct) - can be regenerated. Store at 4 deg. Take an aliquot (**Test #8**)
12. Load different amounts of eluate onto gel, use BSA as standard
13. Add protein storage buffer - exact stock to depend on concentration of eluted material. Store in aliquots at -80 deg.

Rac RBD binding assay and Western blot

Binding

Continue from step 14 of pg 1

1) Collect agarose beads by pulsing in microfuge (30 seconds). Remove supernatant.
2) Wash beads 3 x with Rac LB. Remove last wash with a bent needle setup.
3) Resuspend beads in 25 μl of 2X Laemmli buffer (made in Rac LB)
4) Boil x 5 minutes. Remember total cell lysate control.
5) [Collect samples as for Ips (poke hole in cap with 21 G needle, then heat up 25 G needle and poke hole in the bottom of tube). Set tube on top of collection microfuge tube and the whole thing in a 50 ml collection conical. Spin for 1 min.]

According to UBI, best to load slurry because Pak may rebind Rac after boiling.

6) Freeze samples at –80 deg or load immediately on to gel.
7) Run 11% 0.75 mm gel overnight

Western Blot

8) Transfer 2 hrs
9) Dry blot, rewet and wash in 1X PBS x 15 min
10) Block 2 hr in 3% NFDM/PBS
11) wash x 1 with PBS
12) Incubate with Rac Ab at 1 μg/ml (1:1000 dilution of stock) in 3% NFDM/PBS. Rock overnight at 4 deg.
    _____ ml ; _____ μl of antibody
13) washes PBS-T (0.1% Tween) x 4, 10 min each
14) Incubate wtih GAMγ-HRP (Zymed) at 1:5000 in 3% NFDM/PBS for 1.5 hr
    _____ ml; _____ μl of antibody
15) washes PBS-T x 4, 10 min each
16) wash PBS x 1, 10 min
17) ECL (undiluted)