Generation of 32P-GST Fusion Proteins (W. Kaelin)

1. Grow O/N culture of pGEX-2TK recombinant expressing fusion of interest (LB + Amp)
2. Next day dilute O/N culture 1:10 in fresh LB + Amp. Grow 1 hour with shaking (37 °C)(see also accompanying table)
3. Add IPTG to final concentration of 0.1 mM (1000X stock = 0.24 mg/10 ml, filter sterilize)
4. Grow additional 4 hours with shaking (37 °C)
5. Spin down bacteria 5K RPM x 5 min at 4 °C (from now on, keep things cold)
6. Resupend bacteria in 1/10 vol of NETN
7. Sonicate on ice (1-10 ml bacterial suspension/sarstedt tube) with three brief (5-10 sec) bursts
8. Spin down sonicate 10,000 x G for 5 min at 4 °C
9. Rock supernatant with glutathione sepharose (1:1 in NETN + 0.5% powdered milk*) for 15-30 min
10. Wash beads x 3 with NETN (transfer to eppendorf(s) after 2 washes)
11. Wash x 1 with 1 X HMK buffer without DTT
12. Resuspend beads in reaction mix. 30 ul of reaction mix =

    3 ul 10 X HMK Buffer with DTT
    1 ul HMK** (add 25 ul 40 mM DTT to vial to resuspend enzyme***)
    2 ul 32P-γ ATP 6000 Ci/mMole
    24 ul dd H2O

    ** Sigma # P-2645; 250 units/vial
    *** let sit 5-10 min at room temp to help dissolve. Can store at 4 °C for at least several days.
13. Incubate beads at 4 °C for 30 min. "Flick" tube occasionally to resupend beads.
14. Add 1 ml of HMK Stop buffer. Mix. Spin down beads. Aspirate supernatant with syringe and 23 G Needle and discard appropriately
15. Wash beads x 5 with NETN using above needle/syringe (Aspirate supernatant with syringe and 23 G Needle and discard appropriately).
16. Add 1 ml of freshly prepared 20 mM glutathione in 100 mM Tris pH 8, 120 mM NaCl per eppendorf tube.
17. Rock for 5-10 min at 4 °C .
18. Spin down beads. Carefully aspirate supernatant with P-1000. Save supernatant. Count 25 ul aliquot for ^{32}P.

* Resuspend beads several times in NETN+0.5% dry milk, each time letting beads fall by gravity and aspirating supernatant (this removes "fines"). Then resuspend 1:1 V/V in NETN+0.5% dry milk. Beads may be stored at 4 °C for 1 month (I usually wash beads with NETN+0.5% dry milk prior to use if they have been stored for a while)