

## MEI Protocol

### PAK1 kinase assay

(All centrifuges and washes have to be carried out at four degree)

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1. C2C12 Cells were serum starved for 2 hours.
2. Add agrin at final concentration of 10 ng/ml for designated time. Return culture to incubator.
3. Wash cells with cold PBS twice.
4. Lyse cells in ice cold 0.8 ml HO lysis buffer with inhibitors. Incubate on ice for ten minutes.
5. Clean cell lysate by high speed centrifuge at 12,000 rpm for ten minutes at four degree, collect supernatant.
6. Wash protein-A agarose beads (50  $\mu$ l slurry per reaction) three times with 0.5 ml HO buffer and resuspend to original volume with HO buffer.
7. Add 2  $\mu$ l anti-PAK antibody to beads slurry, diluted with 500  $\mu$ l HO buffer, rotate for 1 hour at four degree.
8. Beads were washed twice with 500  $\mu$ l HO buffer and pelleted.
9. 500  $\mu$ l of cleared lysate supernatant (from step 5 above) was added to pelleted beads. The mixture was then incubated for two hours at four degree.
10. Remove supernatant and wash beads twice with 500  $\mu$ l HO buffer and subsequently twice with wash buffer.
11. Bead-antibody-PAK comple was then resuspend in 20  $\mu$ l MBP (myelin basic protein, 0.5 mg/ml) and 20  $\mu$ l 3 X hot mix. Incubate at 30 degree for 30 minutes.
12. Stop reaction by add 20  $\mu$ l 4X gel loading buffer. Boil mixture for three minutes and spin the sample 5 minutes at 12,000 rpm at room temperature. Run supernatant on 15% SDS-PAGE to visualize MBP phosphorylation.
13. Stain SDS-PAGE gel with coomassie brilliant blue R250 as described in Molecular Cloning.
14. Dry gel in Bio-Rad gel dryer at 65 degree for one to two hours. (the gel may crack at this step, so try to expose a film before this step even though the bands are more diffused)
15. Carry out autoradiography with intensifying screen.

Buffer recipe

### **1. HO Lysis Buffer (pH 7.5)**

50mM Hepes, 100 mM NaCl, 2 mM EDTA

Inhibitors: pepstain 1 $\mu$ M, leupeptin 1 $\mu$ g/ml, PMSF 2mM, Sodium Vanadate 0.2 mM, Aprotinin 2  $\mu$ g/ml, PNPP 40 mM.

### **2. 3 X Hot Mix**

10 $\mu$ Ci/reaction gamma ATP

150  $\mu$ M ATP

50 mM Hepes (pH 7.5)

10 mM MgCl<sub>2</sub>

2 mM MnCl<sub>2</sub>

### **3. Wash Buffer**

50 mM HEPES (pH7.5), 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>

### **4. 4 X Loading Buffer**

10 ml

1 ml 2M Tris.HCl, pH 6.8

2 ml 20% SDS

4 ml glycerol

3 ml H<sub>2</sub>O

308 mg DTT (preferred add fresh)

“some” bromophenol blue