

### **Southern Blotting utilizing [ $\alpha$ <sup>32</sup>P]dCTP:**

Each experiment will utilize one probe, and will be performed 5 times a year.

Total activity per experiment = 50 $\mu$ Ci

#### Protocol

1. A <sup>32</sup>P-labeled probe will be prepared using a random priming DNA labeling kit (Roche, catalog # 11004760001) according to the manufacturer's instructions. 50 $\mu$ Ci [ $\alpha$  <sup>32</sup>P]dCTP will be utilized per probe.
2. 10  $\mu$ g of genomic DNA will be digested overnight, electrophoresed on an agarose gel and subjected to Southern blotting on Hybond-N +nylon membrane. The DNA blot will be placed in pre-hybridized for 3 hours at 68°C in rotating hybridization oven.
3. The <sup>32</sup>P-labeled probe will be denatured by heating at 100°C for 10 min. and then placing on ice.
4. The solution from the blots will be discarded and replaced with fresh pre-hybridization solution, and the denatured probe added to this solution. The blot will be rotated overnight at 68°C in the hybridization oven.
5. The next day, the hybridization solution will be discarded and the blot will be washed 4 times in wash buffers of various stringencies.
6. After the final wash, the blot will be wrapped in plastic wrap and exposed to radiographic film.
7. At the end of the experiment the blot will either be discarded in solid radioactive waste, or the probe will be stripped using alkaline stripping solutions and the blot stored for re-use.

All liquid waste (discarded hybridization solution, stringency washed, and stripping washes) will be collected in appropriately labeled containers and stored behind Plexiglass shielding prior to pickup.

All solid waste (ependorf tubes, used blot if applicable) will be discarded in yellow radioactive disposal bags and stored in Plexiglass bins prior to pickup.

Pipettes and pipette tips will be placed in cardboard boxes and then placed with solid waste to avoid puncturing of yellow waste bags.

**Protein Kinase Assay utilizing [ $\gamma$ - $^{32}$ P]ATP:**

Each experiment will utilize 6 cultures, and will be performed twice a year.

Amount of activity per culture = 100 $\mu$ Ci

Total activity per experiment = 600 $\mu$ Ci

Protocol

8. Cells lysates will be prepared in lysis buffer.
9. 250 $\mu$ g of cell lysate will be incubated with either anti-cdc2 antibody or normal rabbit serum overnight at 4°C.
10. 40 $\mu$ l of protein-A/G PLUS agarose slurry will be added, and incubated for 3 hours to immunoprecipitate cdc2 protein.
11. The immunoprecipitated cdc2 will be washed three times in the lysis buffer, and the precipitated beads incubated with 10 $\mu$ l of reaction buffer and 10  $\mu$ l of a non-cdc2 kinase inhibitor mixture.
12. The reaction will be started by adding 9  $\mu$ l of 75 mM magnesium chloride/500  $\mu$ M ATP containing 1 $\mu$ l of 10mCi/ml  $^{32}$ P-ATP and incubated for 10 min at 30°C. (Total activity/sample = 100 $\mu$ Ci).
13. 25 $\mu$ l of the reaction mixture will be spotted onto P81 phosphocellulose paper and washed three times with 0.75% phosphoric acid and one time with acetone.
14. The paper will be drained, scintillation cocktail added, and CPM measured using a scintillation counter.

All washes and any remaining reaction mixture will be discarded as liquid waste in appropriately labeled containers and stored behind Plexiglass shielding prior to pickup.

Scintillation vials will be discarded as solid waste in appropriately labeled yellow radioactive disposal bags and stored in a Plexiglass container prior to pickup.

Pipettes and pipette tips will be placed in cardboard boxes and then placed with solid waste to avoid puncturing of yellow waste bags.

### **Pulse-chase experiments utilizing $^{35}\text{S}$ -methionine:**

Experiments will be performed 4 times a year.

Each experiment will utilize 3 cell lines and 2 plates/cell line.

Amount of activity/plate = 200 $\mu\text{Ci}$

Amount of total activity/experiment = 1.2mCi

#### Protocol

1. RWPE-Pim-1/Neo/K67M cells will be grown in 100-mm tissue culture dishes until 80% to 90% confluent.
2. Cells will then be starved in cysteine/methionine-free DMEM for 10 min.
3. Each plate of cells will be "pulsed" for 15 minutes by incubating (37°C, 5%  $\text{CO}_2$ ) in 2ml. cysteine/methionine-free DMEM supplemented with 200 $\mu\text{l}$  of 1mCi/ml  $^{35}\text{S}$ -methionine. (total activity/plate = 200 $\mu\text{Ci}$ ). Plates will be sealed with parafilm.
4. At this point, one set of pulse-labeled cells (1 plate/cell-line, 3 plates total) will be lysed.
5. The remaining set will be then "chased" by incubation in complete DMEM for an additional 15 min prior to lysis.
6. Using a rabbit anti-cyclin B1 antibody, cyclin B1 protein will be immunoprecipitated from all the cell lysates.
7. The eluted protein will then be analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel will be dried and then exposed to radiographic film.

Though the plates will be sealed during incubation to prevent any release of volatile compounds, filters impregnated with activated carbon will still be attached to the covers of tissue culture dishes as an added precautionary measure.

All  $^{35}\text{S}$ -containing liquid waste (cell-culture media, immunoprecipitation washes and SDS-PAGE buffer) will be collected in appropriately labeled containers and stored behind Plexiglass shielding prior to pickup.

Solid  $^{35}\text{S}$  waste (cell-culture plates, immunoprecipitation columns) will be collected in appropriately labeled yellow radioactive waste disposal bags and stored in plexiglass bins prior to pickup.

Pipettes and pipette tips will be placed in cardboard boxes and then placed with solid waste to avoid puncturing of yellow waste bags.