

## **RHO GAP ASSAY**

### **I. Radioactive labeling assay**

1. Cleavage of GST from GST-RhoA fusion protein:

GST fusion protein is cut with thrombin (from sigma, catalogue #T-7513, use PBS to resolve the thrombin into 100units/1ml stock solution, add 10% glycerin to the stock solution and store at -80°C) at room temperature for 1-3hrs in 50mM Tris.HCl pH8.0, 2.5mM CaCl<sub>2</sub>, 150mM NaCl, 0.1 mM DTT. Use 1 unit of thrombin /mg protein.

Inhibit thrombin by addition of equal units amount hirudin (From sigma, catalogue #H-0393, use PBS to resolve hirudin into 100units/1ml stock solution, add 10% glycerin to the stock solution and store at -80°C). Thrombin can also be removed by passage of the reaction mixture over a benzamidine-Sepharose column.

2. Loading the RhoA with GTP:

Using gamma -labeled <sup>32</sup>P-GTP, take 1ug of the cleaved RhoA protein, and dilute into 100ul of 25 mM MOPS pH7.6, 1mM EDTA, 0.1% BSA on ice. Add 5uCi of the hot GTP (3000mCi/ml, catalogue #35007, from ICN) and incubate on ice for 20min. Then add MgCl<sub>2</sub> to a final concentration of 10mM(2ul of 0.5M stock).

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Loading can be checked by filter-binding 2ul of the mix, wash with Quench buffer (10ml) and count the filter. Store the loaded RhoA on ice.

### 3. GAP assay:

Dilute 2ul(20ng) of the GTP-RhoA into 20ul of Reaction buffer [25mM MOPS pH7.6, 1mM DTT, 0.1mg/ml BSA, 5mM MgCl<sub>2</sub>, 2mM GTP], mix, then add 20ul of the GAP protein or immunoprecipitation complex from mammalian cells. Mix. Incubate at room temperature. At intervals(1min, 5min, 15min), remove 10ul aliquots, dilute into 1ml of ice-cold Quench buffer and filter-binding (as described below). Count the filters in 5ml of scintillation fluid. GAP activity is measured by the loss of bound <sup>32</sup>P counts.

[Quench buffer: 25mM MOPS pH7.6, 5mM Na<sub>2</sub>HPO<sub>4</sub>, 2mM Na<sub>2</sub>ATP, 5mM MgCl<sub>2</sub>, 150mM NaCl. Filter-binding: use Millipore 0.45um nitrocellulose filters (catalogue #HAWP02500, from Millipore) on a Hoefer filtration apparatus.

Prewet filters with the Quench buffer, then add the sample and wash under vacuum with about 10ml of ice-cold Quench.]

## II. Non-Radioactive Assay

1. lyse cells with 1ml modified RIPA buffer(including proteases inhibitors) for one 10cm dish, collect the cell lysate .
2. spin cell lysate at 12,000rpm for 10mins at 4°C, save the supernatant for pull-down assay immediately or store it at -80°C.

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3. use about 20ug GST-Rhotekin to pull down GTP-Rho in a total volume 1ml for 1hr at 4C, shake the tube gently with a Rotator.
4. Briefly wash the beads with modified RIPA buffer for 3 times.
5. Elute the beads with SDS loading buffer, then run SDS-PAGE gel, westblot for detecting RhoA .