Labeling Cells and Analyzing Inositol Phospholipids

For ^{32}P labeling

Reagents Required:

Phosphate-free DME containing 50 mM HEPES, pH 7.0 plus 1 mg/ml BSA
Carrier Free $^{32}PO_4$
Stop Solution – Methanol/conc. HCl (10:1)

1. Put cells into phosphate-free DME/0.1% BSA containing 50 mM HEPES, pH 7.0 for at least 1 hour before use. Typically use cells grown in 6-well dishes.
2. If necessary, treat cells with appropriate reagents prior to lipid assays
3. To assay, remove media and add 25 μCi/ml $^{32}PO_4$ in phosphate-free DME/0.1% BSA/HEPES. For 6 well dishes, it is only necessary to use 1 ml of media/well.
4. Incubate 2 min at 37° C.
5. Add EGF (25-50 nM) to start stimulation. Continue stimulation for the desired time. (The best stimulation with EGF is usually seen at 2 to 5 minutes.)
6. Stop by aspirating medium and adding 1 ml stop solution.

For [^{3}H]-inositol labeling

Reagents Required:

Inositol-free DME containing 5% FCS
[^{3}H]-myo-inositol
Stop Solution (as above)

1. Grow cells to about 50% confluence in 6-well dishes. Remove media and transfer into inositol-free DEM/5% FCS containing 0.75 μCi/ml [^{3}H]-inositol. Use 2 ml/well for 6-well dishes.
2. Allow cells to grow in labeling medium for 2 days
3. Treat as needed in inositol-free DME/0.1% BSA
4. Stimulate with EGF (25 – 50 nM) for desired time.
5. Stop by aspirating medium and adding 1 ml stop solution

Extraction of Inositol Phospholipids

Reagents Required:

Chloroform

Backwash 1 (Methanol/1 M HCl 1:1)

1. Scrape precipitated monolayer into the stop solution. Transfer to 16 x 125 mm glass tubes.
2. Wash wells with 0.5 ml stop solution and add to glass tube
3. Add 1.5 ml water
4. Add 3 ml chloroform and vortex 30 sec. Remove top layer
5. Add 2 ml Backwash 1 and vortex 20 sec. Remove top layer
6. Transfer chloroform extract to 12 x 75 mm glass tubes by pouring
7. Evaporate to dryness in a SpeedVac (takes ~ 1 hr)

Separation and Quantitation of Inositol Phospholipids

Reagents Required:

Silica-coated thin layer plates

Dipping Solution: 5 g potassium oxalate, 2 mM EDTA, 250 ml ethanol, 250 ml water

Spotting Solution: Chloroform/Methanol/10 mM HCl (20/10/1)

En^{3}Hance (for tritium plates only)

Tank Buffer: For one tank –

141 ml chloroform, 110 ml methanol, 23 ml water, 10 ml NH_4OH

1. Dip thin layer plates in dipping solution. Dry at 60° C for at least 60 min. Let plates cool before use.
2. Using a ruler, draw a line parallel to the bottom of the plate and about 3 cm from the bottom. Mark off even lengths on this origin line about 1.5 to 2.5 cm apart. Your sample will be spotted between these tic marks. Be sure to leave empty at least 1 cm at the edge of each side since there can be significant 'smiling' of samples. Six to eight samples fit comfortably on a 20 cm plate.
3. Add 50 μl spotting solution to each sample tube. Triturate to dissolve the sample. Spot by making a streak between the tic marks. The sample will evaporate quickly and additional solution can be streaked on top of the first application.
4. Add 10μl spotting solution to the tube just spotted to wash out the remaining sample. Triturate to wash down the tube. Apply to TLC plate on top of the original application.
5. If necessary, spot hot or cold standards in outside lanes (You can use the edges for this.)
6. Place TLC plates in a carrier and place in equilibrated TLC tanks. Let run for about 2 hr or until the solvent front is about 1 in from the top of the plate.

7. Remove plates and dry flat. Mark with radioactive markers as necessary to identify orientation and plate number.
8. If using [^{3}H], spray lightly with En^{3}Hance and wrap in Saran wrap (don't use cheap stuff; it dissolves.) For ^{32}P, just wrap in Saran wrap.
9. Expose to film. Use XAR for ^{3}H; use XK-1 for ^{32}P. It takes up to 3 days to expose a film for ^{3}H. For ^{32}P a few hours or overnight is usually sufficient.