

In Vitro EGF Receptor Autophosphorylation

Buffers Needed:

Lysis Buffer: 25 mM HEPES, pH 7.2, 1 mM PMSF, 1 mM EDTA, 2 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ pepstatin and 1 $\mu\text{g/ml}$ α 2-macroglobulin.

Assay Buffer: 70 mM β -glycerophosphate, pH 7.2, 200 mM NaCl, 10% glycerol plus protease inhibitors as above.

Reaction Mix: 1.25 mM ATP, 60 mM MgCl_2 , 10 mM MnCl_2 , 500 μM sodium orthovanadate, 100 mM p-nitrophenylphosphate, 100 mM β -glycerophosphate, pH 7.2.

1 M p-Nitrophenylphosphate

100 mM Sodium orthovanadate

20 mM ATP

1 M MgCl_2

1 M MnCl_2

1 M β -glycerophosphate, pH 7.2

Procedure:

Cell lysis:

All procedures are carried out on ice.

A 100 mm dish of cells is sufficient for 40 to 50 assays.

1. Cells are washed in phosphate buffered saline to remove serum proteins.
2. Approximately 3 to 5 ml lysis buffer is added to the plate and the cells are scraped into the lysis buffer.
3. The mixture is transferred to a Dounce homogenizer and the cells are lysed with 20 strokes of homogenization. For some cells that are difficult to break (e.g. A431 cells), it may be necessary to pass the cells through a 23 gauge needle 20 times to achieve adequate lysis.
4. The lysate is spun for 15 min at 17,000 rpm in a Sorvall RC5 centrifuge to pellet the membranes.
5. The supernatant is discarded and the membrane pellet is resuspended by homogenization in assay buffer. Use \sim 0.5 ml buffer for membranes from 1 –100 mm plate of cells. This can be adjusted as needed according to confluence of cells and assay requirements

Assay:

Assays are done in a final volume of 50 μl .

30 μl membranes (containing \sim 15 μg protein)

10 μl EGF or buffer (usually 25 nM final EGF. Make stock of 1.25 μM EGF)

10 μl Reaction Mix

Chart for Making Reaction Mix:

Reagent	[Stock]	[In Mix]	[Final in assay]	Volume added for 1 ml reaction mix
ATP	20 mM	1.25 mM	250 μ M	62.5 μ l
MgCl ₂	1 M	60 mM	12 mM	60 μ l
MnCl ₂	1 M	10 mM	2 mM	10 μ l
VO ₄	100 mM	500 μ M	100 μ M	5 μ l
PNPP	1 M	100 mM	20 mM	100 μ l
β -glycerophosphate	1 M	100 mM	20 mM	100 μ l
H ₂ O				712.5 μ l

Note: Add MnCl₂ last and vortex immediately. This avoids oxidation and precipitation.

1. EGF is added to the membranes and incubated on ice for 20 min.
2. Assays are begun by the addition of an aliquot of reaction mix.
3. Tubes are quickly vortexed after this addition and placed in a 30° C water bath.
4. Incubations range from 5 to 15 min, depending on the amount of tyrosine kinase in the sample.
5. Incubations are stopped by the addition of 50 μ l SDS sample buffer
6. Samples are boiled and run on a 10% SDS gel
7. Gels are transferred to PVDF and EGF receptor autophosphorylation assessed by Western blotting with an anti-phosphotyrosine antibody.