10 – Protein Kinase Assay with an Immunocomplex

Preparation of Cell Lysate

1) Rapidly rinse control or mitogen-treated cells grown to confluence on 10cm diameter culture dishes with phosphate-buffered saline
(PBS) pH 7.4.
2) Lyse the cells with 1.0ml cold lysis buffer (10mM Tris pH 7.4, 1.0% Triton X-100, 0.5% Nonidet P-40, 150mM NaCl, 20mM sodi-
um fluoride, 0.2mM sodium ortho-vanadate, 1.0mM EDTA, 1.0mM EGTA, 0.2mM PMSF) in the culture dish for 30 minutes at 4°C
with constant agitation.
3) Scrape the cells off the dish and pass the lysate several times through a 26 gauge needle to disperse any large aggregates.
4) Centrifuge for 30 minutes (16,000 x g, 4°C). The supernatant is the "total cell lysate".

Immunoprecipitation of the Protein Kinase

1) Incubate the cell lysate (0.2-1.0mg protein) with 2-5µg soluble antibody.
2) Immunoprecipitate for 1 hour at 4°C with end-over-end rotation.
3) If a soluble monoclonal antibody is used, add an equal amount of rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories)
and incubate the complexes for an additional 30 minutes at 4°C.
4) Wash the desired amount of protein A:agarose in 1X immunoprecipitation (IP) buffer (1% Triton X-100, 150mM NaCl, 10mM Tris
pH 7.4, 1mM EDTA, 1mM EGTApH 8.0, 0.2mM sodium ortho-vanadate, 0.2mM PMSF, 0.5% NP-40), followed by a 4 minute
centrifugation (16,000 x g, 4°C). Bring the protein A:agarose to a 50% suspension in 1X IP buffer.
5) Incubate the immune complexes from step 2 or 3 for 30 minutes at 4°C with 10µl of the 50% protein A suspension.
6) Wash the complexes by resuspension in IP buffer, followed by a 4 minute centrifugation (16,000 x g, 4°C). Repeat the wash at least
twice.
7) Collect the complexes by centrifugation for 3 minutes (16,000 x g, 4°C).

Kinase Assay

1) Wash the immunocomplexes three times at 4°C with kinase buffer (10mM Tris pH 7.4, 150mM NaCl, 10mM $MgCl_2$, 0.5mM DTT).
2) Remove the supernatant by aspiration. Incubate the pellets for 15 minutes at 37°C with 40µl of the kinase buffer that contains 25µM
ATP, 2.5 µCi [^{32} Pg]ATP, and the appropriate protein substrate* at 1.0 mg/ml.
3) Add 15µl of boiling 5X concentrated electrophoresis sample buffer (625mM Tris pH 6.8, 10% SDS, 25% glycerol, 0.015% bro-
mophenol blue, 5% b-mercaptoethanol) to terminate the reaction. Boil for an additional 5 minutes.
4) Centrifuge the samples and electrophorese the soluble fractions.
5) Fix and stain the gel (0.25% Coomassie blue in 45% methanol, 10% acetic acid), destain (40% methanol, 10% acetic acid), dry, and
expose to X-ray film. Kinase activity will be indicated by a band of phosphorylated protein substrate.

***Protein Substrates commonly used for specific kinases**

Enzyme	Substrate
$p34^{cdc2}$ kinase	Histone H1 (Sigma)
EGF receptor	Annexin I, Angiotensin (Sigma)
src kinase	Annexin II
MEK	ERK1 & ERK2
ERK	Myelin Basic Protein