



Immunoprecipitation-Kinase Assay Protocol

Solutions and Reagents

Note: Prepare solutions with Milli-Q or equivalently purified water.

•**1X Phosphate Buffered Saline (PBS)**

•**Cell Lysis Buffer (1X) :**

25 mM Hepes (pH 8.0)
250 mM NaCl
5 mM EDTA
1 mM EGTA
0.2 % NP-40
0.5 mM DTT
2.5 mM Sodium pyrophosphate
1 mM beta-Glycerolphosphate
1 mM Na₃VO₄
1 ug/ml Leupeptin
1 µg/ml Leupeptin
1 µg/ml Pepstatin
0.3 µM Aprotinin
15 µM E-64

•**Transfer Buffer:** 25 mM Tris base, 192 mM glycine, 20% methanol (pH 8.5)

•**Protein A Sepharose:** Wash Protein A Sepharose twice with Cell Lysis Buffer. Resuspend the resin in 1 volume of Cell Lysis Buffer (50 % bead slurry). This can be stored for 2 weeks at 4°C.

•**1X Kinase Buffer:**

25 mM Tris (pH 7.5)
5 mM beta-Glycerolphosphate
2 mM DTT
0.1 mM Na₃VO₄
10 mM MgCl₂

Note: Please remember that this is general kinase buffer. Please use appropriate kinase buffer for each kinase.

•**3X SDS Sample Buffer:** 187.5 mM Tris-HCl (pH 6.8 at 25°C), 6 % w/v SDS, 30 % glycerol, 150 mM DTT, 0.03 % w/v bromophenol blue

Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under non-denaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 ml 1X ice-cold cell lysis buffer to each plate (10 cm²) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate four times for 5 seconds each on ice.
6. Microcentrifuge for 10 minutes at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, lysate can be stored at –80°C.

Pre-clearance

1. Add 30-50 µl of ProteinA Sepharose (50% suspension) into 250 µl of the extract (Pre-clearance step).
2. Rotate the tube for 1 hour at 4°C.
3. Microcentrifuge at 15,000 rpm for 10 minutes at 4°C and transfer the supernatant to a new tube.

Immunoprecipitation

1. Take 200 µl of cell lysate and add primary antibody; incubate with gentle rocking overnight at 4°C.
2. Add protein A Sepharose (20 µl of 50% bead slurry). Incubate with gentle rocking for 1–3 hours at 4°C.
3. Microcentrifuge for 30 seconds at 4°C. Wash pellet two times with 500 µl of 1X cell lysis buffer. Keep on ice during washes.
4. Wash pellet twice with 500 µl 1X kinase buffer. Keep on ice.

Kinase Assay (by Western blotting using anti phospho-specific antibody)

1. Suspend pellet in 20 µl of 1X kinase buffer supplemented with 200 uM ATP and substrates.
2. Incubate 30-60 minutes at 30°C.
3. Terminate reaction with 10 ul 3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.
4. Heat the sample to 95–100°C for 2–5 minutes.
5. Load the sample (15 µl) on SDS-PAGE gel (12–15 %).
6. Analyze sample by Western blotting using anti phospho-specific antibody

Kinase Assay (by autoradiography)

1. Suspend pellet in 20 μ l of 1X kinase buffer supplemented with 5-10 μ Ci γ ³²P ATP (50 μ M) and substrates.
2. Incubate 30-60 minutes at 30°C.
3. Terminate reaction with 10 μ l of 3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.
4. Heat the sample to 95–100°C for 2–5 minutes.
5. Load the sample (15 μ l) on SDS-PAGE gel (12–15 %).
6. Dry the gel.
7. Analyze by autoradiograph.