I. Sample Preparation and Immunoprecipitation

A. Materials Required

- 1X PBS
- Magnetic Myc Immunoprecipitation Kit (Cat. No. 635698)
  - Anti-Myc Magnetic Beads (Cat. No. 635699) (Not sold separately)
  - Magnetic Beads Immunoprecipitation Buffer Set (Cat. No. 635696)
  - ProteoGuard™ EDTA-Free Protease Inhibitor Cocktail (Cat. No. 635673)
- Magnetic Stand (Cat. No. 631964)

NOTE: At the time of your experiment, add 10 µl of 100X ProteoGuard EDTA-Free Protease Inhibitor Cocktail per ml of lysis buffer prior to lysing cells to yield a 1X final concentration of inhibitors.

B. Protocol: Preparation of Mammalian Cell Lysate

1. Wash cells:
   - Adherent cells: Wash a 10 cm dish containing adherent cells twice with 5 ml of 1X PBS. Remove all residual PBS.
   - Suspension cells: Transfer 1 x 10^7 cells to a 15 ml conical tube. Wash twice with 10 ml 1X PBS. Remove all residual PBS.
2. Add 1 ml Lysis Buffer to the plate or tube. Incubate for 15–30 min at 4°C on a rocking platform. Collect cell lysate in a 1.5 ml tube.
   Alternatively, you may disrupt cells by repeatedly passing the sample through a 21-gauge needle.
3. Clear the lysate by centrifuging at 12,000 rpm for 30 min at 4°C. Use supernatant for immunoprecipitation and Western blotting.

C. Protocol: Immunoprecipitation

1. Transfer 20 µl of Anti-Myc Magnetic Beads to a separate microcentrifuge tube, and wash as follows:
   - Add 1 ml lysis buffer to the beads, place on the magnetic stand, and allow the magnet to pull the beads to the wall of the tube.
   - Remove and discard the supernatant.
2. Add 1 ml of cleared lysate (from Section I.B) to the bead pellet. Take care not to transfer any precipitated material, as this can cause nonspecific background. If necessary, re-clarify the lysate by centrifugation before adding to the beads.
3. Incubate for 1 hr to overnight at 4°C on a rotating apparatus.
4. Place the microcentrifuge tube on the Magnetic Stand and allow the magnet to collect the immunoprecipitate.
5. Remove as much supernatant as possible without disturbing the bead pellet.
6. Wash three times with 1 ml Wash Buffer.
7. Discard supernatant from final wash and re-suspend pellet in 20 µl IP elution buffer. Vortex briefly, spin down, and let stand for 5 min.
8. Elute sample:

Low pH elution:

a. Remove the supernatant using the magnetic stand.
b. Add 3 µl 1N NaOH to neutralize, followed by 7 µl 5X SDS sample buffer.
c. The sample buffer should be blue. If it is not, add 1N NaOH, 1 µl at a time, until the sample buffer turns blue.

Alternative elution (1X SDS):

Add 20 µl of 1X SDS sample buffer to the beads from step 7.

9. Boil sample for 5 min and spin down tube.
10. Load 10–15 µl of supernatant on an SDS/polyacrylamide gel.
11. Proceed with Western blotting.

NOTE: If you observe background in the Western blot, repeat the immunoprecipitation protocol with the following modifications.

- Step I.C.1: Wash twice with lysis buffer.
- Step I.C.6: Supplement the wash buffer with higher salt (up to 1 M NaCl) or detergents such as Tween-20 or Triton X-100. Acceptable concentrations will need to be determined empirically.

II. Western Blotting

A. Materials Required

- 1X PBS (Alternatively, you may substitute Tris-based buffers.)
- Wash buffer (PBS or TBS containing 0.2% Tween-20)

  NOTE: The wash buffer used for Western blotting is *not* the same wash buffer supplied in the Magnetic Beads Immunoprecipitation Buffer Set.
- Blocking buffer (5% nonfat dry milk in wash buffer)
- Primary antibody
- Horseradish peroxidase (HRP)-conjugated secondary antibody
- HRP chemiluminescent detection system

B. Protocol: Western Blotting of Immunoprecipitated Protein

NOTE: Optimal dilutions and incubation times vary with individual systems and must be determined empirically.

1. Transfer proteins from the gel (Section I.C.10) to a nitrocellulose membrane using standard techniques.
2. Add 10 ml blocking buffer and incubate the membrane for 1 hr at room temperature with gentle rocking. Alternatively, block the membrane overnight at 4°C with rocking.
3. Dilute the primary antibody in 10 ml of blocking buffer according to the specifications on the Certificate of Analysis (or dilute to a concentration of ~1 µg/ml).
4. Incubate the membrane with the diluted antibody for 1 hr at room temperature with shaking. Alternatively, incubate at 4°C overnight.
5. Wash the membrane three times with wash buffer (5 min per wash).
6. Dilute the secondary antibody conjugate 1:1,000–50,000 in blocking buffer. If you are using a HRP-conjugated primary antibody, refer to the suggested dilution on the Certificate of Analysis.
7. Incubate the membrane with the diluted antibody for 1 hr at room temperature with shaking.
8. Wash the membrane three times with wash buffer (5 min per wash).
9. Proceed with chemiluminescent detection according to the manufacturer's instructions.

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