Protocol-at-a-Glance

TALON® PMAC Magnetic Phospho Enrichment Kit
Protocol-at-a-Glance

(PT3905-2)

Please read the User Manual before using this Protocol-at-a-Glance. This abbreviated protocol is provided for your convenience, but is not intended for first-time users. For more detailed information, consult the Phosphoprotein Enrichment Kit User Manual (PT3731-1).

A. General Considerations

The TALON PMAC Magnetic Phospho Enrichment Kit (Cat. No. 635641) can be used for microscale purification of phosphorylated proteins from cell line or tissue samples. Buffers are provided in the TALON PMAC Magnetic Phospho Enrichment Kit for purification under native conditions.

1. Kit Components
   - 2 x 1 ml TALON Phospho Magnetic Beads (5% suspension)
   - 110 ml Extraction/Loading Buffer (same as Buffer A)
   - 110 ml Wash Buffer (Buffer A without detergent)
   - 80 ml Elution Buffer (250 mM sodium phosphate, 0.5 M NaCl, pH 7.2)
2. Additional Materials Required
   - Magnetic separator for 1.5 ml tubes
3. Optional Additional Materials
   - ProteoGuard™ EDTA-Free Protease Inhibitor Cocktail (Cat. Nos. 635672 and 635673)
   - Phosphatase inhibitor: sodium fluoride (10 mM)
   - 100 mM carbonate buffer, pH 9.3
   - 1.5 ml and 0.5 ml microfuge tubes

B. Standard Sample Preparation for the Magnetic Phospho Enrichment Kit

This protocol is recommended for extracting proteins from cell pellets. For extracting proteins from crude tissues, please see Section VI.B of the User Manual (PT3731-1).

1. Wash 25–50 mg of cells three times with 20 volumes of Phosphate Buffered Saline (PBS) by centrifuging at 500 x g for 5 min in a preweighed centrifuge tube.
   Note: We find that one 150 mm culture plate containing 80–90% confluent cells yields ~70 mg of cells. Smaller or larger cell pellets can be used.
2. After washing, centrifuge cells as in Step 1, decant the supernatant, and aspirate the residual liquid.
3. Centrifuge the tube again (for ~2 min), and aspirate any residual traces of liquid. Then reweigh the tube to determine the weight of the cell pellet.
4. Freeze your samples by placing them in liquid nitrogen or in an –80°C freezer.
5. Resuspend the cell pellet in 20 µl of Extraction/Loading Buffer for each mg of cells. (e.g., if your sample consists of 50 mg of cells, add 1 ml of Extraction/Loading Buffer.)
   Optional: Add 1 µl of 1 U/µl DNAse I solution.
   Notes:
   - If a smaller sample volume is desired, scale the amount of the pellet correspondingly—i.e., resuspend 12 mg of cells in 240 µl of Extraction/Loading Buffer. Scale all volumes correspondingly.
   - The phosphatase inhibitor NaF (10 mM) can be added during extraction and incubation (Section C.9). Sodium orthovanadate is not compatible with magnetic phosphoprotein enrichment and if used during incubation will result in no phosphoprotein binding to the beads, unless removed beforehand using a desalting column (see Section III).
6. Mix pellet by gently pipetting up and down approximately 20 times.
7. Incubate at 4°C for 10 min with additional mixing (inverting tube) approximately every minute. Transfer cell lysate to a microcentrifuge tube.
8. Centrifuge the cell extract at 10,000 x g for 20 min at 4°C to pellet any insoluble material.
9. Transfer the supernatant to a clean tube without disturbing the pellet. This is the clarified sample.
10. Reserve a small portion of the clarified sample at 4°C for protein assays and other analyses. Then proceed to Section C to perform phosphoprotein enrichment using Phospho Magnetic Beads.
    Note: We recommend using the Pierce BCA Protein Assay Reagent Kit [see Section III of the User Manual (PT3731-1)] for protein quantification. The detergents in the Extraction/Loading Buffer may interfere with the Bradford assay.

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C. Protein Purification under Native Conditions using Phospho Magnetic Beads

General guidelines for working with magnetic beads are as follows:

- Use a pipette to mix buffer thoroughly with the beads.
- If needed, magnetic beads can be mixed using a vortexer.
- If there is a great deal of liquid/buffer adhering to the sides of the tube, centrifuge the tubes using a microfuge before placing them on a magnetic separator.
- Ensure that the beads are adhering to the sides to the magnet before removing the supernatant.

The protocol for magnetic bead-based purification in a single tube consists of the following steps:

1. Aliquot 200 µl of bead suspension into a 1.5 ml microfuge tube. Different amounts of beads may be used, depending on the initial protein concentration of the sample [see Table III of the Phosphoprotein Enrichment Kit User Manual (PT3731-1)].
2. Place the tube on a magnetic separator for 1 min and remove storage buffer.
3 Add 0.5 ml of deionized water to the beads.
4. Mix the liquid and the beads thoroughly using a pipette.
5. Place the tube on a magnetic separator and remove the supernatant.
6. To equilibrate the beads, add 0.5 ml of Extraction/Loading Buffer.
7. Repeat Steps 4 and 5.
8. Add the cell lysate (from Section B) to the beads.
   Note: If the cell lysate volume is less than 100 µl, add sufficient Extraction/Loading Buffer to bring the volume up to at least 100 µl (200 µl is optimal). This is necessary to ensure thorough mixing of beads with the cell lysate, for optimal binding.
9. Mix on a rotary shaker or end-to-end shaker for 30 min at room temperature.
   Notes:
   - If the protein is vulnerable to degradation at room temperature, incubate at 4°C for 1 hr. Protease inhibitors that do not contain EDTA can also be added during incubation.
   - The phosphatase inhibitor NaF (10 mM) can be added during extraction (Section B) and incubation. Sodium orthovanadate is not compatible with magnetic phosphoprotein enrichment and if used during incubation will result in no phosphoprotein binding to the beads, unless removed beforehand using a desalting column [see Section IIIof the User Manual (PT3731-1)].
10. Place on a magnetic separator and collect the supernatant, which contains nonadsorbed material.
11. Add 0.5 ml of Wash Buffer.
12. Mix thoroughly & let it stand for a minute before placing on a separator and collecting the first wash.
13. Repeat step 11 & 12 twice to collect the second and third washes.
14. To elute the protein, add 100 µl of Elution Buffer. (The volume of Elution Buffer can be varied depending on the amount of beads. 100 µl of Elution Buffer can be used for eluting from 200 µl of bead suspension. Most of the protein will elute in this fraction. Smaller volumes, such as 50 µl, can be used if a concentrated sample is needed. Volumes below 50 µl may be difficult to handle.)
15. Mix for 5 min and collect Eluate 1.
    Note: If necessary, proteins can be eluted in 100 mM carbonate buffer, pH 9.3.
16. Add another 100 µl of Elution Buffer
17. Mix for 5 min and collect Eluate 2.
18. If necessary, Steps 14 and 15 can be repeated twice to ensure that protein recovery is maximized. In a specific instance, when using 200 µl of bead suspension, 85% of the total protein was eluted in first 100 µl fraction and 15% in the second fraction.
19. Use spectrophotometric and SDS-PAGE analyses to determine which eluate fractions contain the bulk of the protein.
    Note: We recommend using the Pierce BCA Protein Assay Reagent Kit [see Section III of the User Manual (PT3731-1)] for protein quantification. The detergents in the Extraction/Loading Buffer may interfere with the Bradford assay.

This document has been reviewed and approved by the Clontech Quality Assurance Department.