Application Note – Phospho-Western
Abwiz Bio

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Phospho-Western: Western blot screening using Abwiz Bio's phosphorylation-specific IgG antibodies

This note provides a detailed protocol for Western blot-based screening of a given target's phosphorylation level using one of Abwiz Bio's many phosphorylation-specific rabbit monoclonal antibodies. This protocol has been rigorously optimized and is used by our own scientists for product validation. If you have any questions about this protocol or need help troubleshooting your own experiments, our Ph.D.-level support team is standing by to help you publish great results!

Materials

- Cells, cultured to >90% viability and 80-90% confluency
- T75 cell culture flask (or appropriate cell culture vessel)
- 1X PBS
- 15 mL Falcon tubes
- 1.5 mL microcentrifuge tubes
- 1X RIPA buffer containing protease inhibitors
- BCA assay kit
- SDS-PAGE loading buffer containing reducing agent
- Nitrocellulose membrane
- TBS
- TBST (TBS + 0.1% Tween-20)
- 5% BSA/TBS solution as blocking solution
- 3% BSA/TBST/0.1% SDS solution
- Phospho-specific IgG antibody (choose from Abwiz Bio's large collection of validated mAbs)
- anti-rabbit IgG secondary antibody
- Detection system

Cell lysate preparation

The cell treatment procedure will vary depending on the cell line and the desired treatment(s) to be used. This representative protocol is for Daudi (suspension) cells.

1. **Cell culturing**. Culture cells to 80-90% confluency (>90% viability). If you are treating cells with growth factors or cytokines, serum-starve cells for 16 hours in an appropriate media without FBS the day before. Otherwise, use complete media containing 10% FBS.
2. **Cell treatment**. Transfer 1 x 10^7 cells each to two 15 mL Falcon tubes. Leave one aliquot untreated and to the second, add IL-4 to a final concentration of 100 ng/mL. Incubate for 15 min at 37°C in a CO_2 incubator.
   a. Treatment conditions will vary based on the phospho target and cell type and may require optimization.
3. **Wash cells**. Incubate cells on ice for 5 min. Pellet cells 2,000 x g for 3 min. Aspirate the supernatant and resuspend the cells in 7 mL of ice-cold 1X PBS. Centrifuge (2000 rpm, 4°C, 5 min) and remove the supernatant.

4. **Lyse cells**. Add 500 µL 1X RIPA buffer containing protease inhibitor for $1x10^7$ cells to each tube and vortex to mix. Transfer each cell lysate to a 1.5 mL microcentrifuge tube and incubate on ice with occasional mixing for 20 min. Sonicate lysate for 10-15 seconds and centrifuge 13,000 x g for 15 min at 4°C to remove cell debris.
5. **Measure total protein content**. Use a BCA protein assay to determine the concentration of total protein content in each cell lysate.
6. **Prepare lysate for loading**. Add SDS-PAGE sample buffer containing reducing agent to each lysate, normalizing the total protein concentration to 3 mg/mL.
7. **SDS-PAGE**. Add 20-30 µg of cell lysate per well along with protein markers. Run gels at 120V for 90 min.
8. **Western blot transfer**. Transfer proteins from the SDS-PAGE gel to a nitrocellulose membrane.

Membrane blocking and antibody incubations
This is a generic procedure intended to be a guide. Your protocol will vary based on the detection system available.

1. **Membrane blocking**. After transfer, wash membrane in TBS with shaking for 5 min at room temperature. Cover membrane with 5% BSA/TBS blocking solution and incubate at room temperature with shaking for 1 hour.
2. **Primary antibody incubation**. Wash membrane in TBS with shaking for 5 min at room temperature. Prepare primary solutions by diluting antibodies in 3% BSA/TBST. Add antibody solution to the membrane and incubate at 4°C overnight with shaking.
    a. The recommended dilution for each of Abwiz Bio's phospho-specific IgG products is posted in the figure legend on the product page. Your experiment may require optimization by titration at multiple concentrations.
3. **Wash**. Wash membranes with TBST a total of three times for 5 min with shaking.
4. **Secondary antibody incubation**. Prepare the secondary solution using an anti-rabbit IgG secondary antibody at the dilution recommended by the manufacturer in 3% BSA-TBST. It may be necessary to titrate the secondary antibody to determine the optimal concentration. Incubate at room temperature for 1 hour.
    a. All Abwiz Bio phospho-specific rabbit mAbs have been tested using LI-COR IR-based detection with IRDye® 800CW Goat anti-rabbit IgG (LI-COR 925-32211) secondary antibody at 1:10,000 dilution.
5. **Wash**. Wash membranes with TBST a total of four times for 5 min with shaking. Perform a final wash in TBS for 5 min with shaking.
6. **Develop**. Develop and image as required using your detection system. For LI-COR detection, allow membrane to fully dry prior to imaging to improve signal.

Expected results

Below is a representative result from a titration study using Phospho-Stat6 (Y694) rabbit mAb clone Stat6Y694-G12, Cat. #1146. The antibody was titrated from 0.5 µg/mL to 0.01 µg/mL using Daudi cells, untreated or treated using 100 ng/mL IL-4.