5 tips for...

Phosphorylated Western Blot

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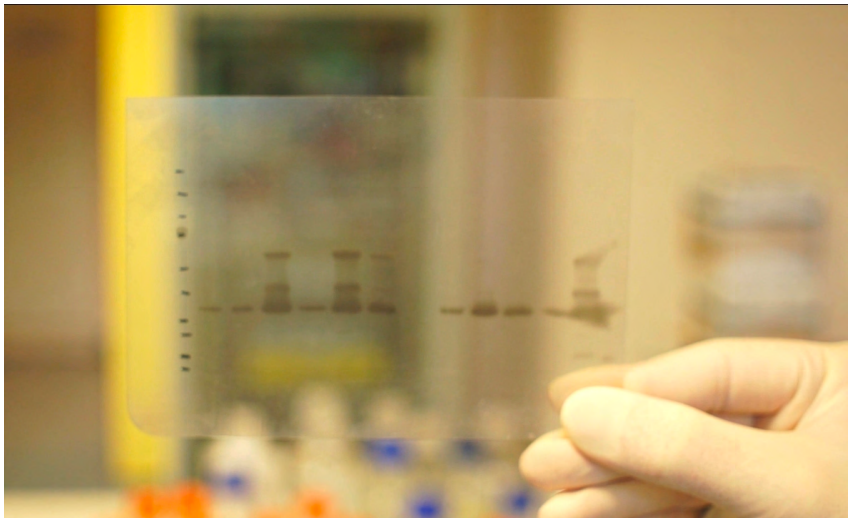
Innovative Lab Services & Reagents

Introduction

When preparing a protocol for Western blotting phosphorylated proteins, a few essential points should be taken into consideration to obtain accurate immunoblotting results.

Your regular WB protocol, such as the WB protocol edited by tebu-bio's antibody users[1], can be fine-tuned according to this "key" post-translational protein modification.

We'd like to suggest 5 tech tips here, which are sure to support you in designing your phosphorylated protein Western blot.



[1] Western Blot (WB) - tebu-bio Antibody Users' Recommended Protocol

1 - Check phospho-specificity of primary antibodies used in Western blot

Might seem a little crazy, but this deserves a few lines in order to avoid having to troubleshoot because after performing your WB you see... no signal at all!

The selected primary antibody must be:

- validated for WB
- recognize the phosphorylated target (phospho-antibody)

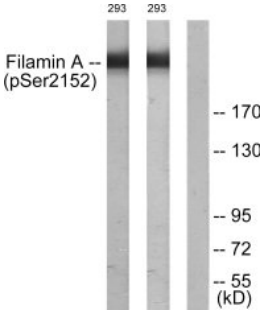
You might also select its "companion" antibody, that's to say an antibody recognizing the unmodified total protein of interest (a kind of control which in parallel gives you a raw estimation of the phosphorylation level of the protein target in your sample).

See Tip #5 to effectively strip and re-probe the blot to do so.

A few examples for selecting "phospho-antibodies":

Anti p-Filamin A (Phospho-Ser2152) antibody and its counterpart for total filamin A protein

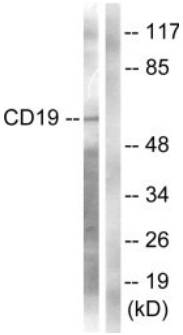
The anti p-Filamin A (Phospho-Ser2152) is produced against a synthesized peptide derived from Human Filamin A around the phosphorylation site of Ser2152. Filamin A (Phospho-Ser2152) Antibody will only detect endogenous levels of Filamin A when phosphorylated at Ser2152. On the opposite, the total Filamin A Antibody detects endogenous levels of total Filamin A protein.



Anti p-CD19 (Phospho-Tyr531) antibody and its counterpart against total B-lymphocyte surface antigen B4 (CD19) protein

The anti p-CD19 is against a synthesized peptide derived from human CD19 around the phosphorylation site of Tyr531 CD19 (Phospho-Tyr531).

This Antibody will detect endogenous levels of CD19 by WB only when phosphorylated at Tyr531. On the opposite, the CD19 Antibody detects endogenous levels of total CD19 protein.



2 – Pamper your phospho-proteins in your samples

Whether you use home-made or commercial lysis buffers, try to work in a controlled environment to reduce as much as possible protein lysis and dephosphorylation.

For this, it's preferable to leave and handle your samples on ice, and use extemporaneously made fresh reagents.

You might also use optimized lysis buffers which are "non-toxic" for phospho-proteins, without using harsh detergents or oxidizers.

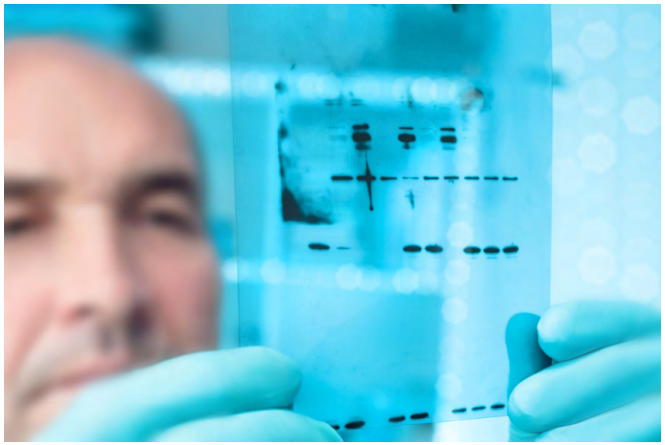
Click here to learn more about how to enhance phospho-protein preservation during lysis steps.

3 – Use blotting buffers compatible with phosphorylation studies by Western Blot

Classically, Blotto buffers and other classical home-made blocking solutions containing milk (ex. Western-blot grade Non-Fat Milk Powder) are not suited to phospho-protein WB.

This is due to the fact that phospho-antibodies might interact with proteins contained in the milk, bringing high signal background and experimental bias.

You might therefore prefer to use Bovine Serum Albumin-based blocking buffers (ex. BSA Biotech Quality) when performing Western Blot of phosphorylated proteins.



4 – Enrich your samples to detect low abundance proteins

In your cell lysates, phospho-protein represents a small portion of the total amount of cellular proteins. Their detection by using a phospho-antibody is sometimes below the detection limits of the assay. This is also true for total target proteins expressed at very low levels in tissues or cellular models.

To detect low abundant proteins, immunoprecipitation (IP) with an appropriate antibody before running WB is recommended.

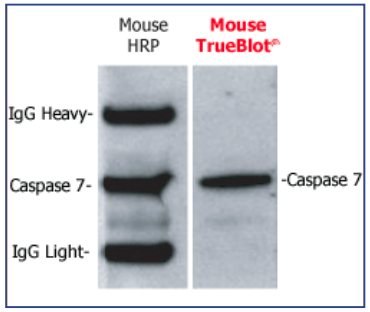
IP allows a higher amount of the protein of interest to be loaded on the sample well of the PAGE gel. While IP will strongly amplify the detection level of the target protein, it might also bring an artifact in the detection step of the WB protocol.

Sometimes, for proteins with a molecular weight around ~55 kDa and ~23 kDa, the target protein can migrate together with the ~55 kDa heavy and ~23 kDa light chain of the IP antibody. In this case, the secondary antibody used for WB detection would simultaneously label the primary used to detect the protein and the IP antibody chains.

To overcome this issue, specific sets of secondary antibodies have been designed to increase sensitivity with less background noise in IP Western blot.

The use of enhanced chemiluminescence TMB substrate reaching lower detection levels can also be used to detect low abundance of proteins of interest.

Optimised IP-WB results with Rockland's TrueBlot® technology.



Typical IP-WB results with Mouse TrueBlot®

5 – Stripping and re-probing your Western Blot membrane for using another antibody

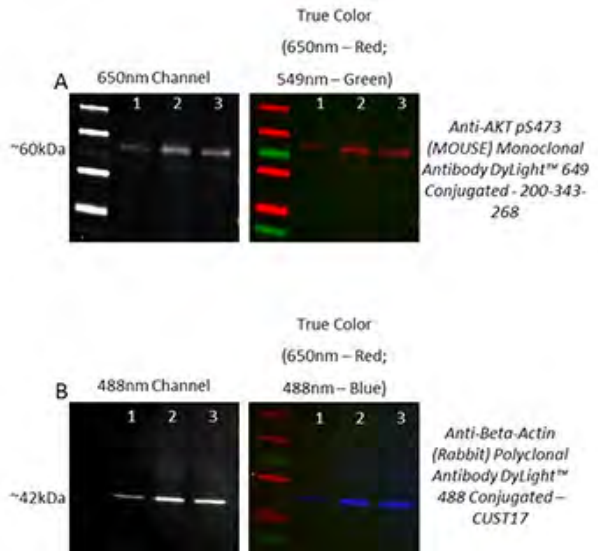
When you don't have access to multiple staining of WB (in Fluorescence), a same WB membrane can be tested for analysing several proteins using different antibodies. In the case of Phospho-proteins, I recommend to test the phosphorylation first, since it is the most fragile part of the assay, and then the total protein.

Stripping and re-probing is always possible even if it:

- might generate loss of signal
- requires experimental optimization for each and every antigen-antibody interaction

For these reasons, repeated stripping and re-probing isn't recommended. Usually WB stripping buffers combine low pH, detergents, and/or reducing heat conditions to completely remove residual antibodies.

Revitablot™ is ready-to-use buffer which efficiently and rapidly (5-20 minutes at room temperature) strips primary and secondary antibodies including HRP and fluorescence labeled, from blots (PVDF and nitrocellulose) that can be re-used for another analysis.



WB comparison of Revitablot™ Stripping Buffer using Fluorescent Antibodies.
A - blot probed with Mouse anti-AKT pS473 and reprobed with Revitablot™ (5 minutes at room temperature), probed with Rabbit anti-beta actin DyLight 488.
B - Imaging: 488nm and 650nm.
Source: Rockland Immunochemicals Inc.

About the author...

Philippe Fixe has a PhD in Molecular and Cellular Biology, and studied Marketing, Commercial development & Human Resources at the ESSEC Business School in Paris.
He is currently manager of the e-Commerce team at tebu-bio, curator of the blog Being bio-reactive and owner and administrator of the LinkedIn group «Stem Cell & Cell Reprogramming» (S2CR)

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