Detection of phosphorylation on large proteins by western blotting using Phos-tag containing gel

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Method Article

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Abstract

Introduction

Detection of phosphorylation by western blotting is an important procedure to elucidate molecular mechanisms in signal transduction pathways involving kinases and phosphatases. Anti-phosphotyrosine monoclonal antibodies have been widely used because they react with plethora of proteins containing phosphorylated tyrosine residues. In contrast, the monoclonal antibodies against phosphoserine or threonine residues are unpopular, since their affinity and specificity are less than optimal. To achieve precise characterization of signaling events, it is desirable to raise a good anti-phospho-site-specific antibody to clearly detect phosphorylated species. However, raising this type of antibody is costly and time-consuming, and sometimes results in failure. Use of Phos-tag may provide an alternative method to detect phosphorylated proteins. Phos-tag is a dinuclear metal complex that acts as a novel phosphate-binding tag. Phos-tag molecules preferentially capture phosphomonoester dianions bound to Ser, Thr, and Tyr residues. Phosphorylated proteins can be detected as slower migrating species by electrophoresis and western blotting using PAGE gel containing appropriate amount of Phos-tag acrylamide. Previously Phos-tag acrylamide has been used in 20-100 μM concentrations. We have now found that lower concentrations \(3.5-5 μM) of Phos-tag can dramatically improve the separation between phosphorylated and non-phosphorylated species of a protein with larger molecular mass \(~150 kDa). Here we describe a detailed protocol that includes tips to ensure easier application of this methodology. The procedure should be carried out as in the standard SDS-PAGE unless stated otherwise.

Reagents

Gel solutions Solution A. 30% acrylamide/bis solution \(37.5:1) from BioRad \(Cat# 161-0158). Solution B. Buffer for separation gel \(1.5M Tris/0.4% SDS, pH 8.8). Solution C. Buffer for stacking gel \(0.5M Tris/0.4% SDS, pH 6.8). Solution D. Phos-tag-acrylamide \(commercially available at www.phos-tag.com). 5.0 mM in distilled water \(DW). Keep at 4°C. Solution E. 10 mM $MnCl_2$ solution in DW. Solution F. 10% \(w/v) Ammonium persulfate solution. Transfer buffer \(Tris 25 mM/ Glycine 0.192M/ 20% methanol) Other reagents including sample loading buffer and gel running buffer are the same as the standard SDS-PAGE method.

Procedure

Preparation of SDS-PAGE gel containing Phos-tag 1. Assemble minigel glasses and spacers. We use 100 mm x 100 mm x 1 mm minigel apparatus from Nihon Eido \(Tokyo, Japan). 2. Prepare Phos-tag separation gel solution by mixing solutions A+B+D+E with DW. We normally use 5% gel with 3.5 μM Phos-tag and 7 μM $MnCl_2$ for $_{150\ kDa\ protein.\ Add\ TEMED\ and\ solution\ F.\ Mix\ then\ pour.\ Allow\ to\ polymerize\ for}$ 1 h. 3. Prepare stacking gel solution by mixing solutions A+C with DW. Add TEMED and solution F. Mix then pour. Allow to polymerize for ~ 1 h. **Running the gel** 4. Prepare sample by mixing whole cell lysate \(or

immunoprecipitates) with sample buffer. 5. Load samples onto the gel. Avoid loading dissimilar samples \(i.e. salt concentrations etc.) to two adjoining wells, since this will cause severe distortion of the bands. It is necessary to set a vacant lane between samples and molecular weight markers to avoid such problems. 6. Run the gel at very slow speed with low constant current \(~5 mA). It will require a long time; for our apparatus, it takes about 12 h to finish separation. It seems important to spend a long time for electrophoresis to obtain good separation. It is also important to use the separation gel buffer with appropriate pH \(8.8). We experienced strong effect on gel running speed due to small fluctuations in the buffer pH. **Western transfer** 7. Equilibrate the gel with transfer buffer supplemented with 1 mM EDTA for 10 min. This is required to enhance efficiency of transfer. Then continue equilibration with transfer buffer without EDTA for additional 10 min. 8. Wet PVDF membrane with the transfer buffer. 9. Assemble gel-membrane stack with 3MM filter paper. 10. Transfer in a submarine-type apparatus for 60 min at 100 V constant voltage. Keep the apparatus at low temperature with plenty of ice. **Blocking and detection** 11. This can be done with a standard Western protocol. We found it important not to block too strongly; this may erase faint bands. We normally use 2% BSA/TBS-T overnight at 4°C. Dilution and incubation length should be optimized with each antibody used. Quality of the antibody is particularly important, since non-specific bands may confound interpretation of the data. Thus it is desirable to include appropriate control \(i.e. expression of mutant protein lacking phosphorylation sites).

Figures

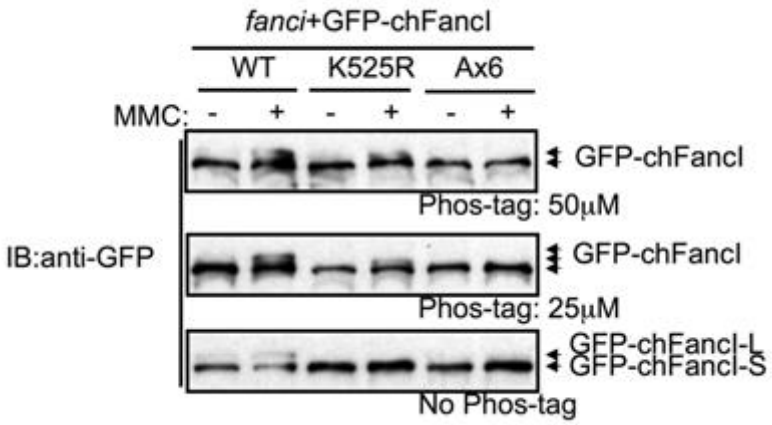

Figure 1

Phosphporylation and monoubiuqitination of chicken FANCI. DT40 cells were treated with MMC 500ng/ml for 6h or left untreated. Whole cell lysate were prepared and blotted with anti-GFP. Wild type DT40 (WT), fanci cells expressing GFP-chicken FANCI lacking monoubiuqitination site (K563R) or carrying alanine substitutions in six putative phosphorylation sites (Ax6) were analyzed. In this condition (Phos-tag 25 μM or 50 μM), it is difficult to clearly separate phosphorylated and non-phosphorylated FANCI.

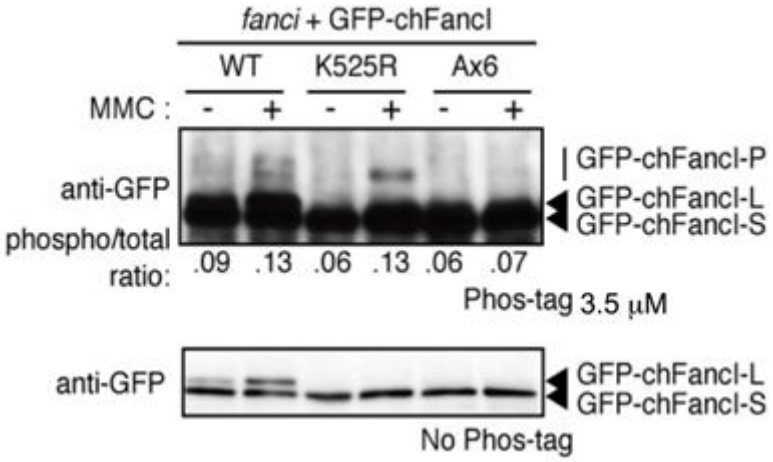

Figure 2

Improved Phos-tag western blotting. Lysates were similarly prepared as in Figure 1. Electrophoresis and western blotting were done as described in this protocol.