Detection of phosphorylation using ABfinity antibodies

Phosphorylation is a reversible posttranslational modification regulated by kinases and phosphatases, which can either activate or inactivate a protein. The binding of a highly negative phosphate group (PO_4^{3-}) to a protein alters its charge, thereby significantly changing the conformation of the protein, and ultimately the way the protein functions. Structural changes through phosphorylation of a protein can also facilitate binding to a partner protein. Thus, phosphorylation events regulate protein–protein interactions. Phosphorylation of a protein can also regulate the mechanism of its degradation and removal from the cell by the ubiquitin-proteasome system [1].

Phosphorylation plays an important role in signal transduction pathways. The binding of an external signal, a ligand, to a cell-surface receptor initiates signal transduction. This initial molecular signal is then amplified, leading to activation of multiple downstream target molecules, eventually leading to gene regulation and eliciting a biochemical response within the cell. The relay of signals from one protein to another in the signal transduction pathways is often mediated by phosphorylation events [2].

Phosphorylation of proteins has great impact on cellular events including cell cycle, metabolism, differentiation, and neuronal signal transduction. Hence, detecting phosphorylated targets becomes important in tracking cellular processes, especially in diseased or abnormal phosphorylated states.

Phosphorylation can be detected at the basal level, or it can be increased upon activation of specific signal transduction pathways via stimulation or inhibition. Phosphorylated targets can be enriched by either preserving them at their phosphorylated states or by synchronizing cells to a point in the cell cycle where subpopulations of the phosphorylated targets can be captured (Figure 1).

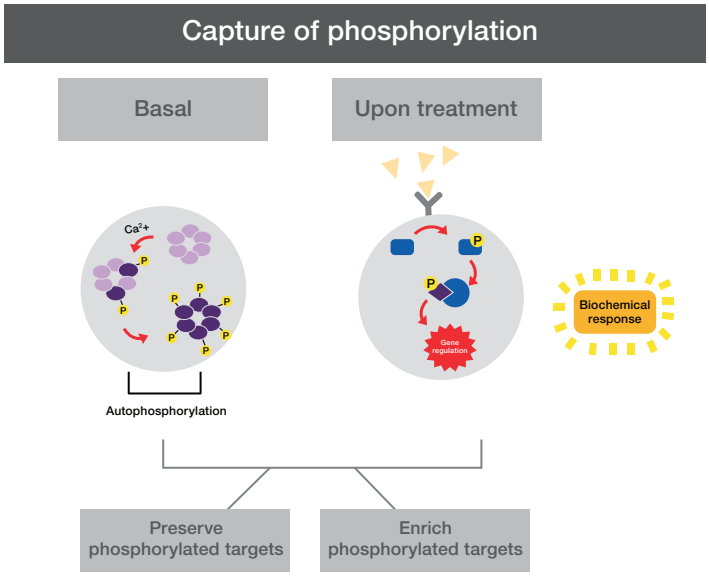

Figure 1. Detection of phosphorylation by preservation or enrichment of subpopulations of phosphorylated proteins in cells.

Unraveling the complexities of cell signaling requires robust reagents (antibodies) that can be used across applications and workflows. This is even more critical when studying transitory forms of proteins with phosphorylation or other posttranslational modifications. There is a growing need for high-quality primary antibodies that not only recognize the protein of interest but can do so each and every time across lots and experiments. Invitrogen™ ABfinity™ antibodies address both issues: specificity of antibody to target proteins and/or posttranslational modifications, and lot-to-lot consistency. ABfinity technology is a recombinant platform that integrates the innate immune response that is available in nature with a recombinant antibody platform where the HC (heavy chain) and LC (light chain) of these antibodies are cloned into mammalian expression systems.

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In this application note we discuss the different types of phosphorylation and their detection using ABfinity antibodies.

Basal phosphorylation

"Basal" is a term used to describe the condition of cells that are not stimulated [3]. This type of phosphorylation need not be transient and can be detected at any time.

Basal phosphorylation can be in the form of autophosphorylation, as in the case of calmodulin-dependent kinase II (CaMKII). CaMKII is a serine protein kinase that plays a role in controlling cell proliferation and cell cycle progression. It has been widely studied in brain cell models, e.g., neuroblastoma cells like SH-SY5Y [4]. In Figures 2A and 2B, autophosphorylation at the T305/306 site of CaMKII was detected using ABfinity™ Anti-CaMKII [pT305/306] Recombinant Rabbit Monoclonal Antibody. The data illustrate the detection of basal phosphorylation levels without any stimulation or inhibition.

Figure 2B demonstrates specificity of the antibody with a phosphopeptide competition analysis: panel e shows the absence of signal after pre-incubation of the antibody with the phosphopeptide that is used for rabbit immunization, while panel f shows no competition from the nonphosphopeptide.

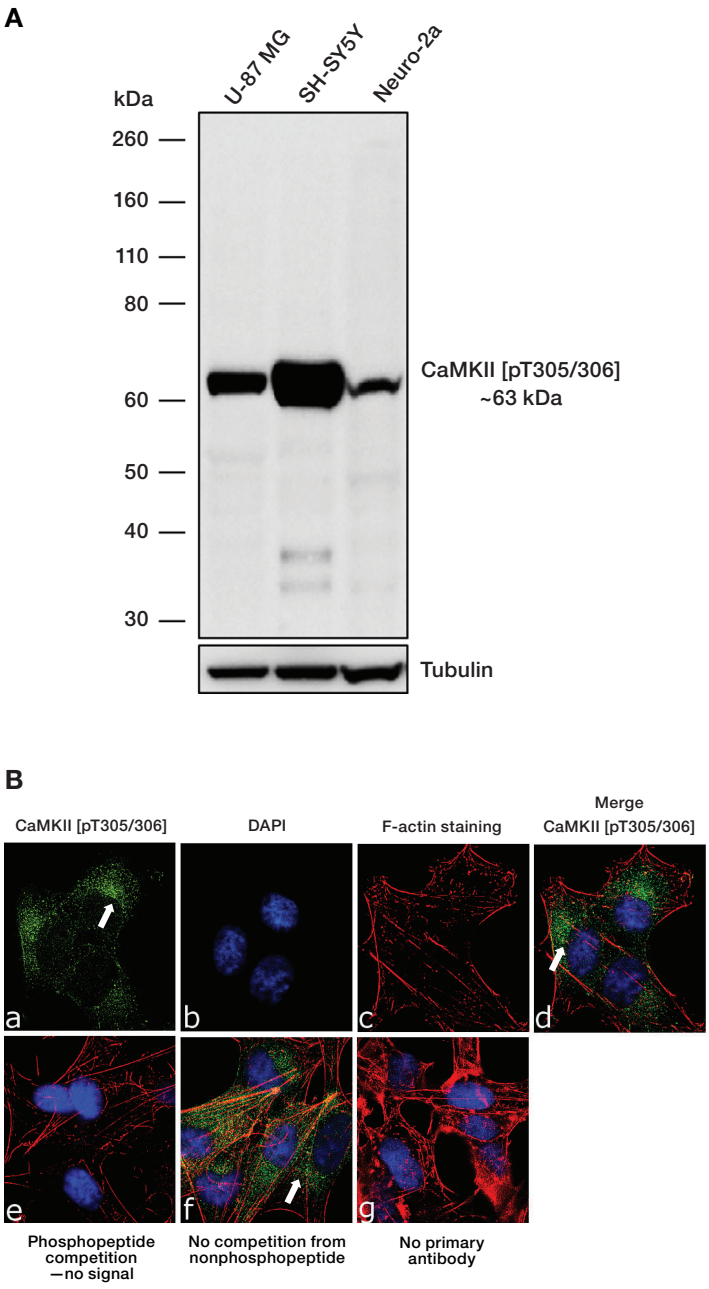


Figure 2. Detection of basal levels of CaMKII [pT305/306] using ABfinity Anti-CaMKII [pT305/306] Recombinant Rabbit Monoclonal Antibody (Cat. No. 702357). (A) Western blotting. **(B)** Immunofluorescence analysis.

Enrichment of basally phosphorylated proteins

Some phosphorylated proteins can be more effectively detected when the phosphorylated species are enriched. Enrichment can be achieved by arresting the cell cycle in a specific phase, by either serum starvation or use of cell cycle–arresting reagents: when cells in culture are arrested, phosphorylated proteins accumulate in the arrested phase.

One such reagent is paclitaxel, which stabilizes microtubule dynamics. When treated with paclitaxel, cells are arrested at G_2/M phase. This leads to accumulation of the phosphorylated form of vimentin (phosphorylated by CDK1 during mitosis) [6], an intermediate filament protein, enabling more efficient detection as shown in Figure 3. Another cell cycle–arresting reagent, nocodazole, leads to the phosphorylation of retinoblastoma protein (Rb), an important tumor suppressor protein, inhibiting microtubule formation and arresting cells in the G_2/M phase. Phospho-Rb is the activated form that is found until the M phase and gets dephosphorylated when cells exit the M phase [7]. In order to not to detect the phosphorylated form of Rb, cells were treated with nocodazole to arrest them in the G_2/M phase, as shown in Figure 4.

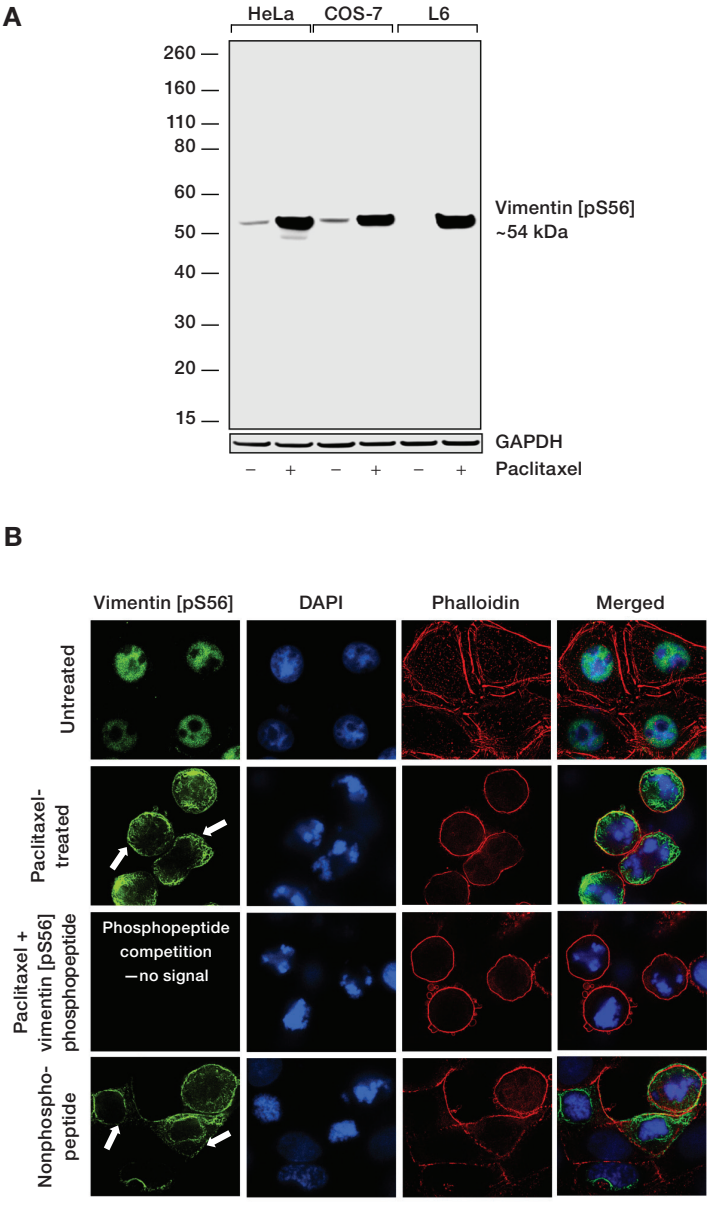


Figure 3. Detection of phospho-vimentin [pS56] upon treatment of cells with the cell cycle–arresting reagent paclitaxel, using ABfinity Anti-Vimentin [pS56] Antibody Rabbit Oligoclonal (7HCLC) (Cat. No. 711212). (A) Western blotting. **(B)** Immunofluorescence analysis.

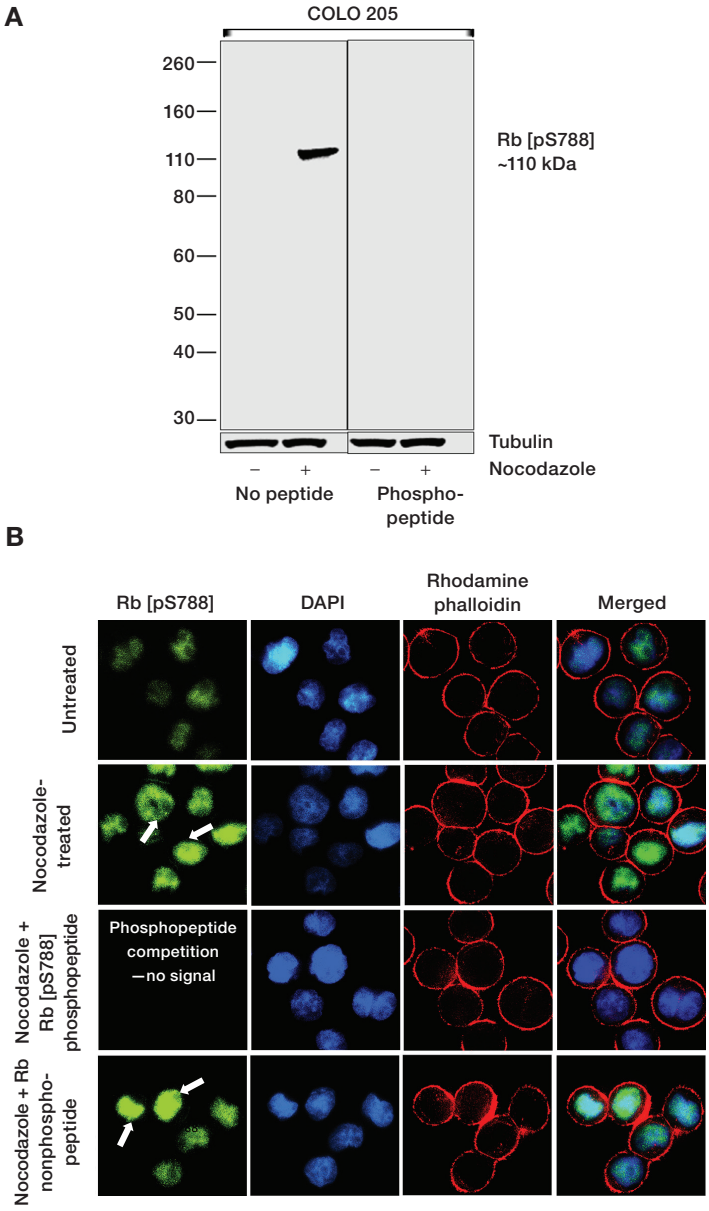


Figure 4. Detection of phospho-Rb [pS788] upon treatment with nocodazole, using ABfinity Anti-Rb [pS788] Recombinant Rabbit Oligoclonal (6HCLC) (Cat. No. 711143). (A) Western blotting. **(B)** Immunofluorescence analysis.

Phosphorylation in response to stimulation

Growth factor pathways are activated upon ligand binding, which initiates signal cascades. For example, the binding of epidermal growth factor (EGF) to the EGF receptor (EGFR) results in a series of downstream phosphorylation events along with the phosphorylation of EGFR itself, as shown in Figure 5. Figure 6 shows that the phosphorylated form of Rac1, a downstream target in the EGFR pathway, was detected via western blotting (Figure 6A) and immunofluorescence analysis (Figure 6B) in a culture treated with EGF [8]. Epidermoid cell lines such as A431, which has high basal levels of EGFR, would make an ideal model to treat with EGF to detect Rac1 phosphorylation [9]. Tyrosine protein kinase Met (c-Met) or hepatocyte growth factor receptor (HGFR) is highly expressed in gastric carcinomas [10]. HGF is the ligand that binds to c-Met, leading to its phosphorylation and activation [11].

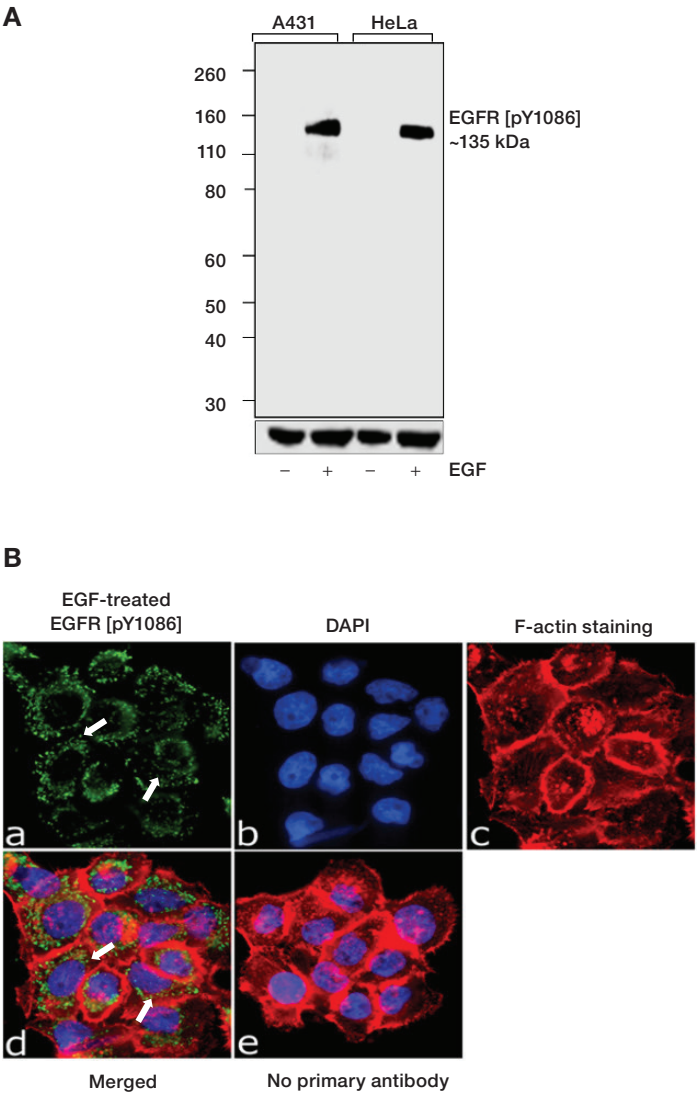


Figure 5. Detection of phospho-EGFR [pY1086] upon treatment of cells with EGF, using ABfinity Phospho-EGFR [pY1086] Recombinant Rabbit Monoclonal Antibody (11H22L16) (Cat. No. 701736). (A) Western blotting. **(B)** Immunofluorescence analysis.

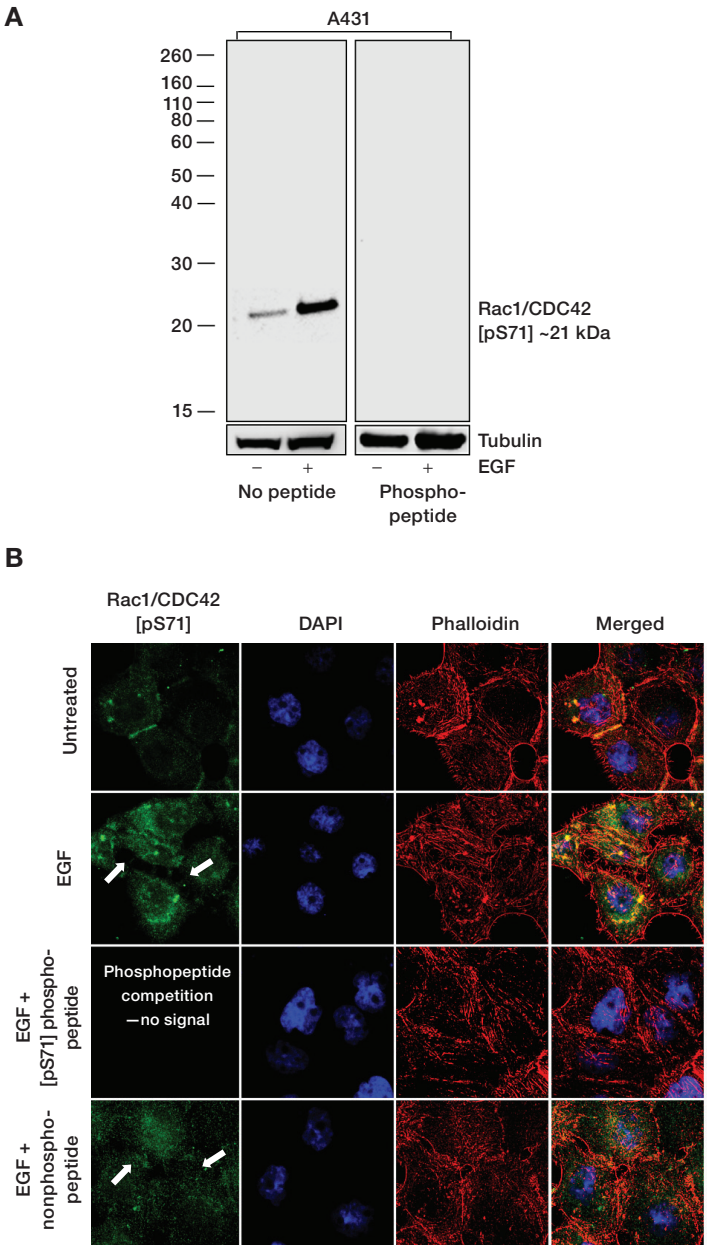


Figure 6. Detection of phospho-Rac1/CDC42 [pS71] upon treatment of cells with EGF, using ABfinity Phospho-Rac1/CDC42 [pSer71] Rabbit Monoclonal Antibody (20H14L7) (Cat. No. 701942). (A) Western blotting. **(B)** Immunofluorescence analysis.

Enrichment of the phosphorylated form of the protein

Efficient detection through enrichment of the phosphorylated form after relevant stimulation can be achieved by blocking the signaling pathway downstream of the phosphorylated target of interest.

Janus kinase 2 protein (JAK2) is phosphorylated at sites Y1007/1008 in the presence of IFN-γ, tagging it for proteasomal degradation. To prevent degradation and to enrich the subpopulations of JAK2 [pY1007/1008], a proteasomal inhibitor like MG132 is added [12] (Figure 7).

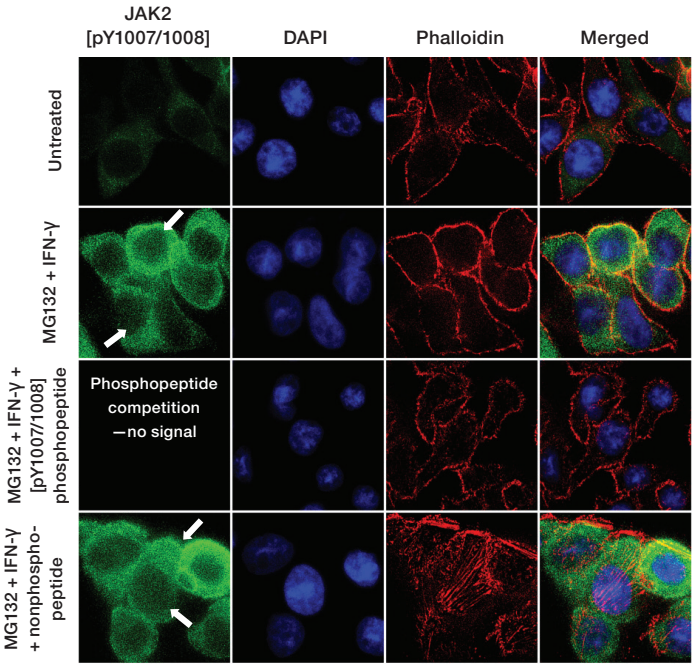
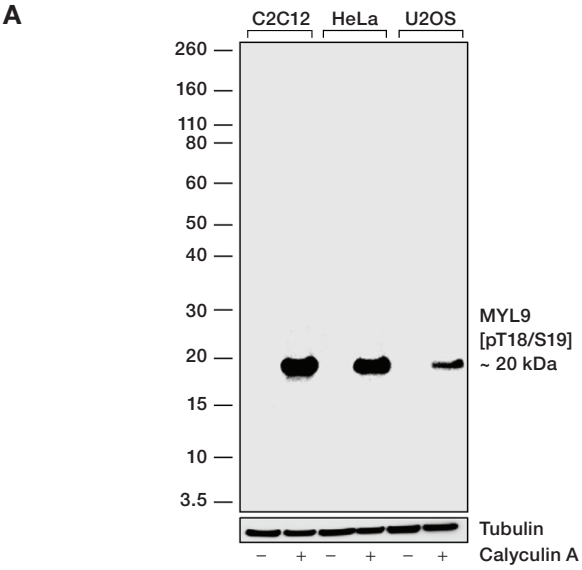


Figure 7. Detection of phospho-JAK2 [pY1007/1008] upon treatment of cells with IFN-γ and MG132, using ABfinity Phospho-JAK2 [pY1007/1008] Antibody Rabbit Oligoclonal (18HCLC) (Cat. No. 710928) in immunofluorescence analysis.

Preserve basal (and transient) phosphorylation

Proteins can be phosphorylated and dephosphorylated rapidly. Phosphorylation takes 1–2 minutes, and it can peak for 10 minutes [13]. The transiently phosphorylated state can be preserved using phosphatase inhibitors to prevent dephosphorylation, thereby allowing the phosphorylated proteins to be captured and detected. Examples of phosphatase inhibitors include okadaic acid and calyculin A. While okadaic acid inhibits only protein phosphatase 2A, calyculin A inhibits both protein phosphatase 1 and 2A. Figure 8A shows transient phosphorylation of myosin regulatory light polypeptide 9 (MYL9) [pT18/S19] that would have otherwise not been captured but is now found with calyculin A treatment. Similarly, protein tyrosine phosphatases can be inhibited by pervanadate, as shown in Figure 9A. Phosphorylated spleen tyrosine kinase, also known as Syk, is shown to be present at basal levels in the monocytic cell line U937 [14], whereas preservation of Syk phosphorylation is required in erythroleukemia cell lines such as HEL 92.1.7, using pervanadate.



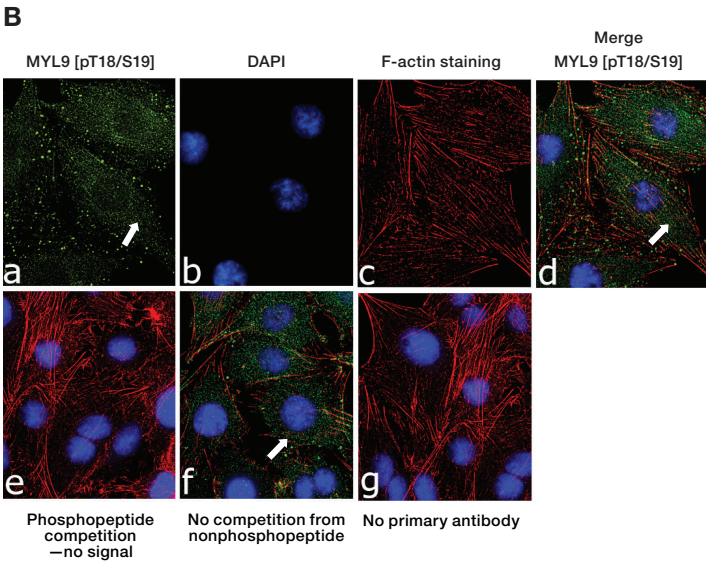
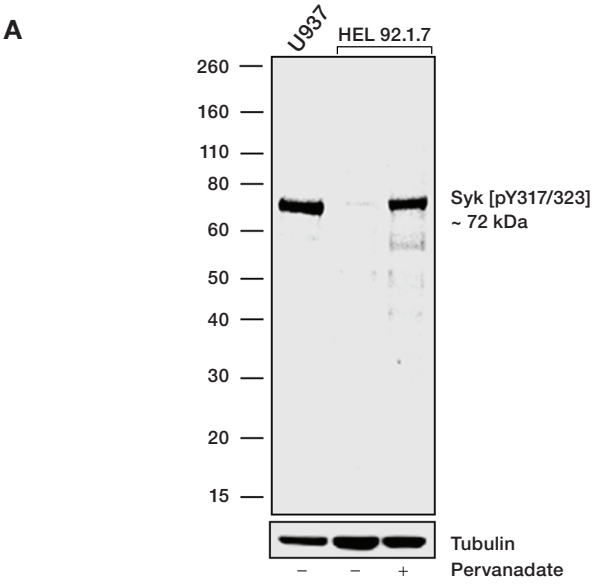


Figure 8. Detection of myosin regulatory light polypeptide 9 (MYL9) [pT18/S19] upon treatment of cells with a phosphatase inhibitor, calyculin A, using ABfinity Myosin Regulatory Light Polypeptide 9 [pT18/pS19] Rabbit Monoclonal Antibody (20H14L7) (Cat. No. 711400). (A) Western blotting. **(B)** Immunofluorescence analysis.



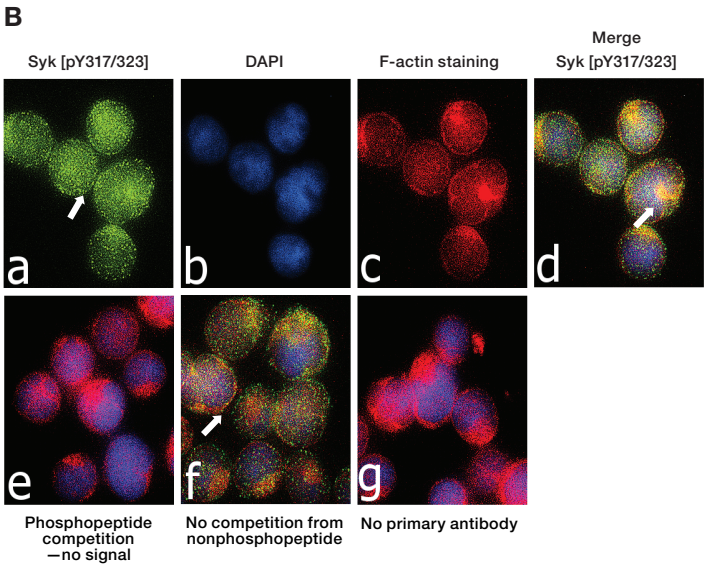


Figure 9. Detection of Syk [pY323] (mouse [pY317]) upon treatment of cells with a phosphatase inhibitor, pervanadate, using ABfinity Syk [pY323] (mouse [pY317]) Rabbit Oligoclonal Antibody (6HCLC) (Cat. No. 711415). (A) Western blotting. **(B)** Immunofluorescence analysis.

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Conclusions

In summary, we have described how we can classify phosphorylation and how the different types of phosphorylation events and/or states can be studied. We have also discussed details of working with highly unstable proteins undergoing phosphorylation, using several examples. All the data shown and described here were generated using ABfinity antibodies, demonstrating that these antibodies are highly efficient in detecting phosphorylation across applications, with lot-to-lot consistency.

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