# Unravelling antibody specificity via multiple complementary approaches

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Phosphospecific antibodies are widely used for the analysis of phosphorylation patterns mediated by protein kinases. For this, a large number of commercially available antibodies against phopho-Tyr and phospho-Ser/Thr exist. These analyses largely rely on the availability of probes that specifically recognize a sequence signature, reflecting the respective recognition sequence for the kinase of interest. In general, these antibodies are generated using synthetic (phospho)peptides that bear a canonical consensus sequence for a respective protein kinase. However, on the other hand, the protein kinases of choice may show some promiscuity in regards to the target sequence although the respective antibody may tolerate sequence variations. Here, we analysed substrate peptides of cAMP-dependent protein kinase (PKA) where the canonical recognition site (Arg- Arg -X-pSer) was systematically modified and the interaction of a widely-used PKA-substrate antibody was analysed. We compared three different techniques side-by-side: a Surface Plasmon Resonance (SPR)–based microfluidic assay (Biacore) to generate binding kinetics, a bead-based homogenous assay (Luminex technology) and a solid phase assay (peptide microarrays). All three methods were successfully applied and generated similar results. Besides the stated epitope (RRXpS), additional sequences were found to be tolerated by the antibody. However, not all physiologically relevant PKA target sequences were recognized by the antibody. Our data clearly demonstrate that: (i.) not all phospho-substrates of a given kinase can be identified using a single phospho-specific antibody; (ii.) the antibody has binding preferences for certain epitopes different from the optimal sequence of the kinase; and (iii.) sequence motifs for other protein kinases may additionally be recognized. For binders-based phospho-proteomics approaches, additional experiments may be necessary to overcome the inherent limitations of phospho-specific antibodies.