Evolving strategies for application-specific validation of research use antibodies

Antibodies have become a vital tool among researchers in the life sciences and are routinely being used in a number of diverse immunoassay applications, including Western blotting (WB), immunoprecipitation (IP), flow cytometry, enzyme-linked immunosorbent assays (ELISA), quantitative immunofluorescence (QIF) and immunohistochemistry (IHC).

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Antibodies can provide a great deal of critical information about a specific protein under both normal and disease conditions, including localisation and quantification of expression with respect to biological state, post-translational modifications and function within a specific cell. User experience has shown that high quality antibodies are most often a combined result of thoughtful, effective strategies for immunogen design, immunisation, screening, purification, validation and finally customer usage and feedback. Testing antibodies in multiple applications reveals much about antibody performance. Therefore, to ensure scientific integrity in biomedical research, it is essential that antibodies developed for research use purposes are rigorously validated, thereby demonstrating specificity, selectivity and reproducibility in the context for which they are to be used. This paper describes the evolving strategies and need for new standards for application-specific validation of research-use antibodies.

Biological conclusions drawn from antibody-based experiments are more likely to be replicated when the hypothesis has been tested using multiple techniques and applications. However, it is well known that the performance of an antibody in one application cannot predict its performance in another application[1,2]. For example, an antibody may recognise a protein that is fully denatured but cannot detect the same protein in its natural conformation; therefore, this antibody would work well for use in Western blotting but not for immunoprecipitation or other applications that require detection of native protein and vice versa. Additionally, it is impossible to predict how an antibody will perform when using different sample sources, or when variations are introduced into sample preparation protocols.

Many commercially available antibodies on the market today have not been rigorously tested and have only been analysed via a single method[1]. Determining the suitability of a specific antibody for a specific application is therefore left in the hands of the researcher. As a result, researchers are

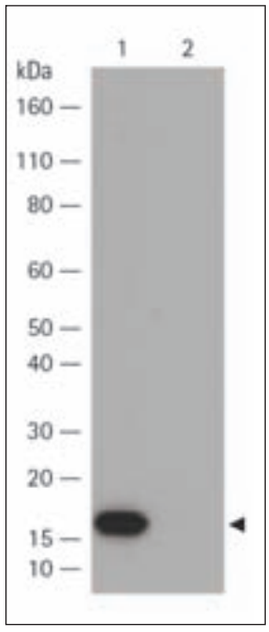

Figure 1
Western blot validation of an anti-trimethyl Histone H3 (Lys27) antibody. Arrow indicates histone H3 band of expected molecular weight (~17 kDa): Lane 1) HeLa acid extract; Lane 2) Recombinant (unmodified) H3 (lane 2)

increasingly demanding more stringent, application-specific validation and functional characterisation of antibodies from suppliers, enabling them to focus on obtaining results more quickly and cost-effectively. Additionally, the impact of antibody-based tests on basic and clinical research has led to several publications that highlight the need for strict validation guidelines for manufacturers[1-3]. Despite these increasing demands, however, there continue to be no universally accepted quality guidelines or standards for the development of antibodies. Therefore, antibody suppliers must evolve and adopt new strategies to validate and develop the highest quality antibodies for use in multiple applications.

The importance of primary and secondary validation

Western blotting is a primary validation step that is routinely used to determine whether a specific antibody recognises the denatured target antigen. The observation of a single band that corresponds to the target's molecular weight indicates specificity for the target antigen (**Figure 1**). However, the presence of multiple bands or a band exhibiting an unexpected molecular weight does not necessarily indicate lack of specificity. These bands may represent the same target antigen with post-translational modifications, breakdown products or splice variants. Therefore, an antibody is considered to pass primary validation specifications if Western blotting produces a band (or bands) of the expected molecular weight(s) for the target antigen and approximately three or fewer off-target bands at lower intensity.

Validation by Western blotting is best performed using multiple cell or tissue lysates to determine the range of detectable endogenous protein expression in various cells and tissues. Testing against only a recombinant target protein is not considered validation of specificity. Each antibody should be tested against various quality-controlled cell and tissue lysates representing diverse growth conditions and treatments. It is important to note, however, that Western blotting cannot be an absolute standard for antibody specificity for all applications, since many antibodies that work by Western blot may not work for other assays that require the detection of antigen in its native conformation, such as immunohistochemistry or immunoprecipitation.

Given the wide range of antibody-dependent applications used by researchers, a research use antibody that has been validated across multiple applications is generally considered more valuable. Accordingly, if a preliminary Western blot analysis demonstrates a positive result, the antibody should then be tested and validated in other applications, including flow cytometry (**Figure 2**), immunofluorescence (IF; **Figure 3**), immunohistochemistry (IHC; **Figure 4**), immunoprecipitation and/or chromatin immunoprecipitation. IF or IHC data should confirm the expected protein expression and subcellular localisation pattern in normal cells or tissues in addition to patterns expected for cancers and other diseases. As part of any validation testing on cells or tissues, a negative control should also be used to confirm that any positive staining is a consequence of the antibody lot being tested and not an artifact of the staining process (**Figure 4b and 4d**).

If an antibody does not demonstrate target antigen binding by Western blotting, it is possible that the antibody does not recognise the denatured antigen or the protein could be endogenously expressed at very low levels. However, the antibody may still bind to the target antigen in its native conformation. In these cases, an immunoprecipitation assay should be the next step for determining specificity of the antibody. If the goal is to develop an antibody for use with immunohistochemistry, immunofluorescence or chromatin immunoprecipitation, then initial screening by Western blot is not recommended, since detection of denatured protein is not a predictive measure for binding to the non-denatured target.

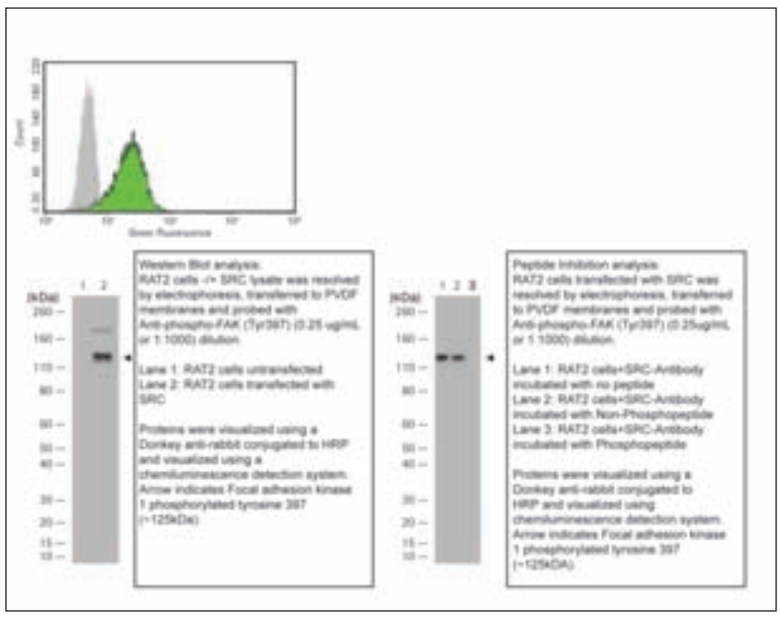

Figure 2: Flow cytometry validation demonstrating specificity of an anti-phospho FAK (Tyr397) antibody. This figure shows an increase in FAK phosphorylation when Jurkat cells are treated with hydrogen peroxide when compared with untreated Jurkat cells

Validating specificity against difficult targets

There are a variety of approaches for evaluating antibody specificity against difficult targets (eg, post-translationally modified proteins). These strategies include the use of peptide inhibition assays, peptide microarrays, peptide interaction assays using beads or ELISA-type assays and peptide dot blots. To illustrate the use of some of these methods, described below are typical validation workflows for targets for which antibody specificity is particularly important in the context of modified or otherwise challenging targets.

Targeting post-translational modifications

Antibodies that recognise targets with post-translational modifications[4], such as phosphorylation[5], acetylation, methylation, ubiquitination and oxidation, can be especially challenging to validate. In particular the validation of modification-specific antibodies for immunohistochemistry remains particularly challenging[4]. However, technological advances such as high throughput quantitative image analysis, together with more stringent use of control antibodies to monitor the overall state of cellular phosphorylation, for example, may unlock the potential power of immunohistochemistry for illuminating signalling networks.

Polyclonal antibodies that target specific post-translationally modified proteins often display stronger binding than monoclonal antibodies against the same target, because polyclonal antibodies may bind to multiple unique epitopes of the modified peptide[5]. When developing polyclonal antibodies that recognise targets with post-translational modifications, the use of antigen affinity columns during the purification process can significantly improve the specificity of the antibody for the post-translational modification. Depleting unmodified target protein from the serum before affinity purification (using immobilised, modified target protein) increases the specificity for the modified target. Specificity testing can then be performed to confirm that the antibody only recognises the post-translationally modified form of the protein.

It is important that antibody suppliers use diverse strategies to ensure the specificity of antibodies against post-translationally modified proteins[4]. Some of these approaches may include treating cells with appropriate kinase-specific activators and/or inhibitors (**Figure 3 and Figure 5**); comparing target recognition in cells with or without a knockout point mutation in the gene of interest at the site of modification (**Figure 6**); using an antigen peptide to block all signal; confirming

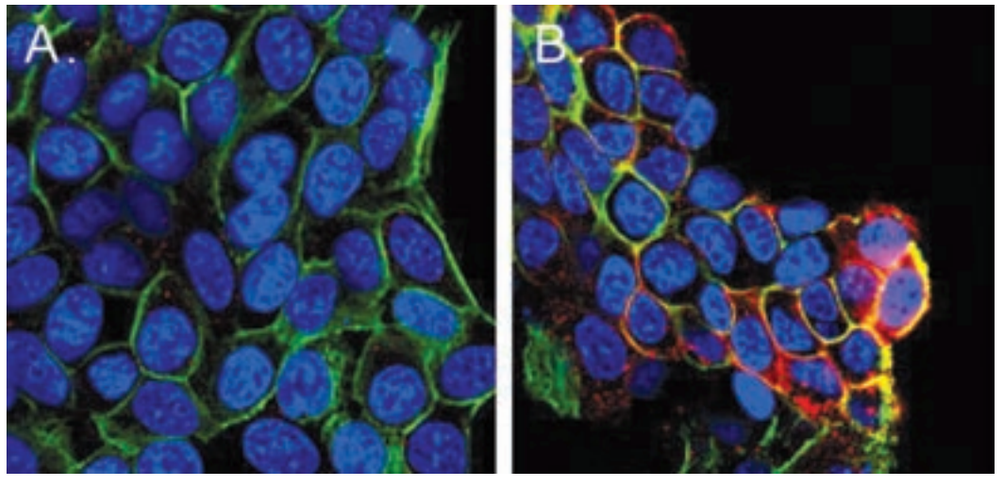

Figure 3
Immunofluorescence validation demonstrating specificity of an anti-phospho Epidermal Growth Factor Receptor (EGFR; Tyr1069) antibody (red). A) untreated A431 cells, B) A431 cells treated with Epidermal Growth Factor

specificity via dot blot (at least 75% of the total signal should be specific to the cognate peptide); and verifying correct subcellular localisation or treatment-induced translocation via immunohistochemistry or immunocytochemistry.

Targeting modified histones

Appropriate analysis of post-translationally modified histones relies heavily on chromatin immunoprecipitation (ChIP). In ChIP, an antibody against a nuclear protein (eg, a specific post-translationally modified histone, unmodified histone or a non-histone target protein) is used to immunoprecipitate associated DNA sequences. These sequences are subsequently analysed by quantitative PCR (qPCR), microarray (ChIP-chip) or next-generation sequencing (ChIP-seq).

ChIP can be a powerful strategy to gain both locus-specific and genome-wide information, but only when using highly specific and well-characterised antibodies. A large number of ChIP antibodies are available through commercial suppliers; however, it is important to note that not all suppliers rigorously or consistently screen their epigenetic antibodies for cross-reactivity.

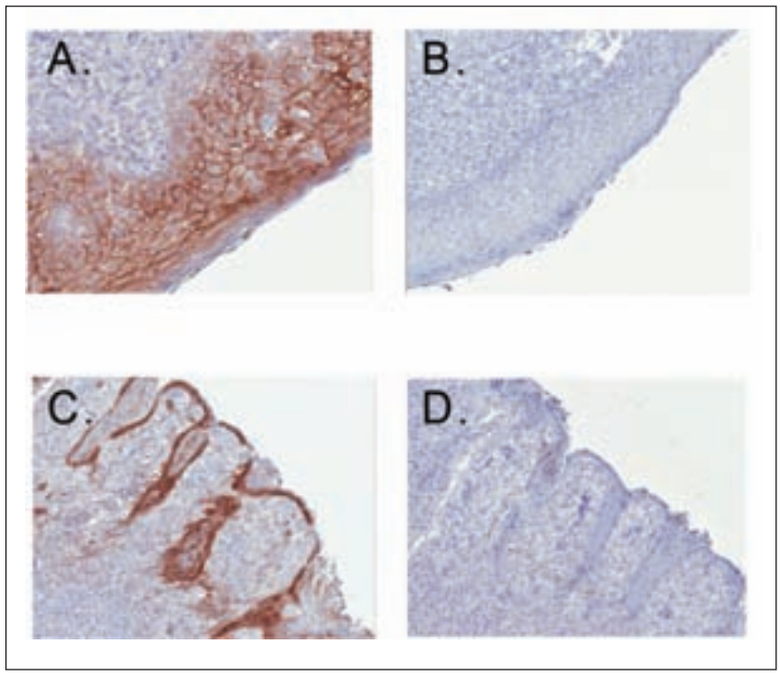

Figure 4
Immunohistochemistry validation of anti-desmoglein antibody using human tonsil tissue. Membrane junction immunoreactivity was observed in the stratified squamous epithelium (A) as well as the epithelial cells lining the tonsilar crypts of the human tonsil (C). Treatment of the same tissues with negative control reagent (B, D) resulted in no detectable signal

Targeting difficult proteins

The current methods used by suppliers to generate antibodies can have significant impact on their success in any of the above applications[8]. In addition to the aforementioned modified proteins, there are some proteins with specific characteristics that make developing antibodies against them particularly difficult. These examples include proteins with sequences that are highly conserved among mammalian species, membrane proteins and small molecules. However, there are multiple technologies and approaches available during antibody development that can address each of these challenging proteins, including novel immunisation strategies, special considerations in antigen design, and automated or high-throughput screening technologies. Each approach is proving to be critical for the successful generation of both polyclonal and monoclonal antibodies for difficult targets.

Novel immunisation protocols designed to produce antibodies with the highest possible affinity and specificity include the use of multiple host animal strains as well as different combinations of protein carriers and dosing strategies. Results show

that these innovations at the point of antigen exposure are able to break immune tolerance for highly conserved antigens and may induce a stronger antibody response, allowing the generation of specific antibodies against many highly conserved proteins.

A genetic immunisation protocol can also be used to address challenges arising from generating antibodies against membrane proteins and small molecules. For example, an immunisation process that targets antigens directly to antigen-presenting cells can induce rapid and effective antibody responses. Additionally, significant effort has been placed on immunogen design, purification, optimisation and screening strategies for antibodies against post-translationally modified proteins, which have resulted in highly specific antibodies generated against modified histones and to many other post-translationally modified targets.

Finally, the efficiency, quality and reproducibility of monoclonal antibody generation can be significantly enhanced through automation and high throughput screening. Monoclonal antibodies are frequently preferred for research use – and overwhelmingly preferred for use in clinical research assays – because of their consistency and specificity[5]. However, the conventional hybridoma technology that is used to generate and screen monoclonal antibodies is both time-consuming and very low throughput. Today, there are novel, semi-automated approaches to producing high quality mouse monoclonal antibodies which combine high-throughput hybridoma production with protein microarray-based screening and selection. These technologies enable standardisation of the monoclonal antibody development process, leading to greater consistency in validation results.

Summary

Antibodies have become a critical research tool within the scientific community and are routinely used in a number of applications. Most commercial antibodies available today have not been rigorously tested, however, and there are no universally accepted validation guidelines or standards to which manufacturers must adhere, leaving suppliers to develop their own internal validation specifications. To ensure scientific integrity, research use antibodies should undergo rigorous validation testing by suppliers that meet or exceed research standards in the context for which they are to be used to ensure that only the highest quality antibodies are offered to customers. It is rigorous and compelling validation criteria, combined with thoughtful effective strategies for immunogen design, immunisation, screening, purification, and finally

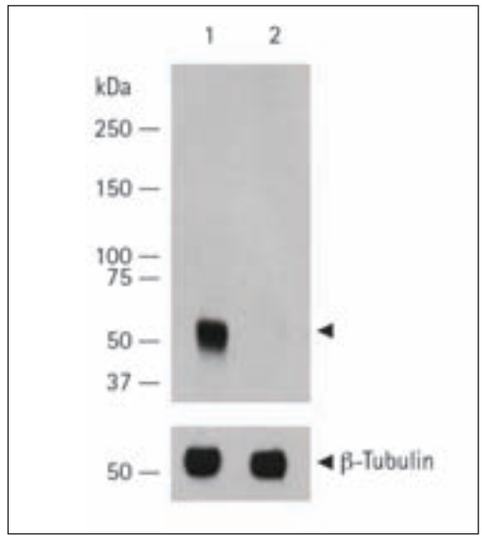

Figure 5: Specificity testing of phospho-PTEN (Ser380) antibody using phosphatase treatment. Phospho-PTEN was detected as expected at ~54kDa in the untreated, but not the phosphatase-treated lysate demonstrating that the antibody specifically binds to the phosphorylated protein: Lane 1) Lysate from untreated NIH 3T3 cells; Lane 2) Lysate from NIH 3T3 cells treated with alkaline phosphatase

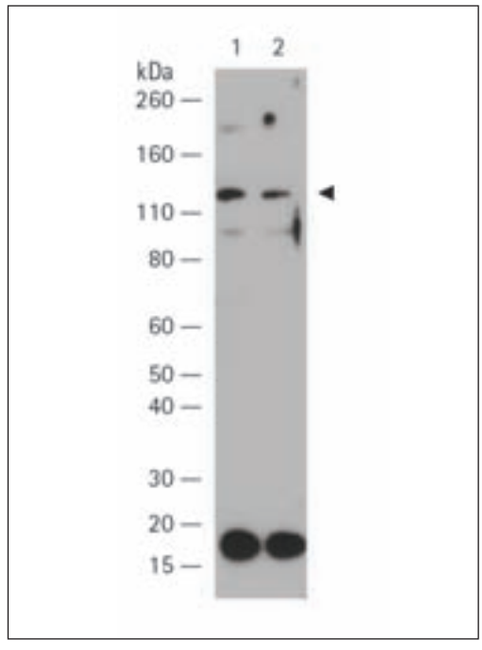

Figure 6: Specificity testing of a phospho-specific antibody using phospho-site-specific mutant cell lysate. Although the phosphorylated target is detected as expected at ~120kDa in the wild type protein-expressing cell lysate (lane 1), the antibody also detected the protein with the point mutation (Lane 2). This indicated that the antibody was not specific to the modified protein and should be rejected as a phosphosite-specific antibody candidate

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customer usage and feedback that distinguish the exemplary providers of reliable, suitable and verifiable antibodies to ensure that only the highest quality antibodies are offered to customers. DDW

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