White Paper

Evolving strategies for application-specific validation of research use antibodies

Arazo Saadat, Reynaldo Dimagiba, Trinette Chuang, Chun Yang, Robin T Clark, Wayne A Speckmann, Alejandra Solache
EMD Millipore

Introduction

Antibodies are essential for the localization and quantification of a protein's expression with respect to biological state, as well as for protein modification, purification and functional analysis. Antibodies have become indispensable tools for life science researchers and are used in an increasingly diverse number of immunoassay applications, and they have also shown effectiveness in therapeutic applications.

It is known that the performance of an antibody in one application is not necessarily predictive of its performance in a different application, in the context of a different sample source, or with respect to variations in sample preparation protocol[1,2]. On the other hand, biological conclusions drawn from antibody-based analyses are more likely to be replicated when the hypothesis has been tested using multiple techniques and applications.

Determining the suitability of an antibody for a specific application, however, can be time-consuming. As a result, scientists increasingly demand more stringent, application-specific validation and functional characterization of supplier-provided antibodies, enabling researchers to focus on expedient acquisition of results. Despite this, most commercially available antibodies have not been rigorously tested and have only been analyzed via a single method (typically Western blot)[1].

EMD Millipore has developed a high throughput antibody production and validation screening process to ensure that only the highest quality antibodies are offered to our scientific partners. In doing so, we continue in the tradition of Chemicon®, Upstate®, and Calbiochem®, renowned antibody producers that are now part of EMD Millipore. Our proprietary antibody development system uses automation that has been customized to facilitate every feasible step in our production and validation processes. This enables the development of a sizable and rapidly expanding catalog of polyclonal and monoclonal antibodies against a diverse array of targets while still allowing testing and validation of each antibody in a variety of applications. For high value antibody targets, we often strive to develop overlapping antibody content, so that biological behavior can be validated with independently derived antibodies. For example, some targets are offered as both polyclonal and monoclonal derivatives, so that customers can orthogonally validate their results.

The applications we routinely use for antibody validation include Western blot, immunohistochemistry, immunocytochemistry, flow cytometry, immunoprecipitation, bead-based immunoassays and specialized applications, such as chromatin immunoprecipitation and RNA-binding protein immunoprecipitation. For all of our tests, an extensive

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library of relevant sample materials is available. Our rigorous process and attention to detail allow EMD Millipore to consistently produce high quality antibodies that are thoroughly validated, enabling researchers to complete their investigations faster and more cost-effectively.

The first step: screening antibodies by Western blotting

Western blotting (WB) is widely used to assess an antibody's specificity and is an appropriate routine first validation step to determine whether the antibody recognizes the denatured antigen. Observing a single band corresponding to the target's molecular weight is a first indication that the antibody is specific for the selected target. However, the presence of multiple bands or a band exhibiting an unexpected molecular weight cannot always be attributed to failure of the antibody, because these bands may represent the same target with varying post-translational modifications, breakdown products, or splice variants. Thus, an antibody is considered to pass WB specifications if it produces a band (or bands) of the expected molecular weight(s) for the target protein and a reasonable number (i.e. <3) of off-target bands at lower intensity of reactivity.

Typically, WB validation is performed using multiple cell lysates or tissue lysates to explore the range of detectable protein expression in various tissues and species. At EMD Millipore, each antibody we develop is tested using an extensive internal cell bank and lysate library representing diverse growth conditions and treatments. The library of available test samples houses thousands of different cells, tissue lysates and blots, all of which have been tested and QC-controlled. This collection of testing materials provides us with a consistent and reliable source of high-quality testing material for our stringent WB validation studies.

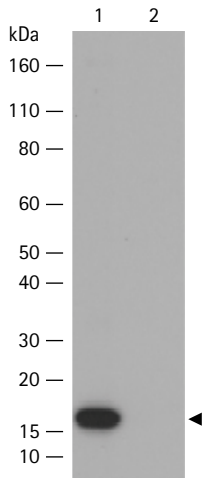


Figure 1.

WB validation of anti-trimethyl Histone H3 (Lys27) antibody. HeLa acid extract (lane 1) and recombinant histone H3 (Cat. No. 14-494, lane 2) were resolved by electrophoresis, transferred to PVDF membranes and probed with 0.1 µg/mL anti-trimethyl Histone H3 (Lys27; Cat. No. ABE44). Proteins were visualized using a donkey anti-rabbit IgG-HRP and a chemiluminescence detection system. Arrow indicates histone H3 band of expected molecular weight (~17 kDa).

Secondary validation: verification of performance in multiple applications

Given the wide range of platforms and techniques used in most antibody-dependent interrogations, an antibody that has been tested in multiple applications is generally more useful to researchers. Accordingly, if our preliminary WB analysis shows a positive result, the antibody is then tested and validated in other applications, such as flow cytometry (Figure 2),

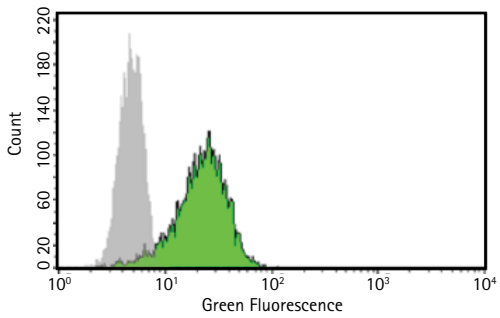


Figure 2.

Example of flow cytometry validation showing specificity of anti-phospho FAK (Tyr397) antibody (Cat. No. ABT135). Jurkat cells that were either untreated (grey histogram) or treated with hydrogen peroxide (green histogram) were analyzed by first staining with anti-phospho FAK (Tyr397) and then donkey anti-rabbit FITC (Cat. No. AP182F). This figure demonstrates the increase in FAK phosphorylation when Jurkat cells are treated with hydrogen peroxide. Phospho-specificity of anti-phospho FAK(Tyr397) was previously determined by Western blot. Flow cytometric analysis was performed on a guava easyCyte™ 8HT flow cytometer.

immunocytochemistry (ICC) or immunofluorescence (IF, Figure 3), immunohistochemistry (IHC, Figure 4), immunoprecipitation (IP) or chromatin immunoprecipitation (ChIP). To pass specifications, the ICC/IF or IHC data should conform to the expected protein expression and subcellular localization pattern of the protein of interest in normal cells or tissues as well as patterns expected for cancers and other forms of disease.

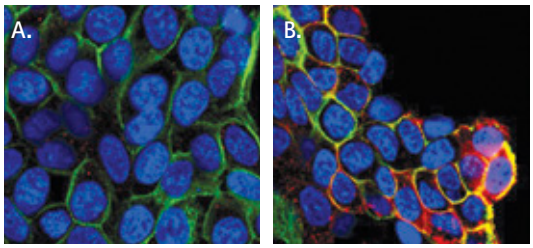


Figure 3.

Immunofluorescence validation showing specificity of anti-phospho EGFR (Tyr1069) antibody (Cat. No. 09-310). Untreated (left) and EGF-treated (right) A431 cells were stained with anti-pEGFR(Tyr1069, red, Cy3), phalloidin (green, Alexa Fluor® 488) and DAPI (blue). EGF treatment is known to increase the phosphorylation of cell membrane receptors, including EGFR, which is detected by the dramatic increase in red signal in the right hand image by the anti-pEGFR antibody. Yellow indicates dual staining of both phalloidin and anti-EGFR antibody.

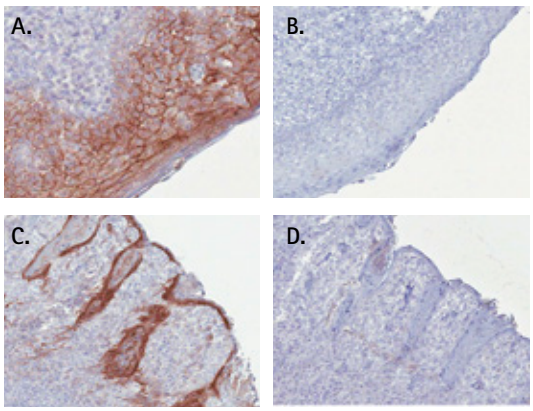


Figure 4.

IHC validation of anti-desmoglein antibody (1:1,000, Cat. No. MABT118) using human tonsil tissue. Human tonsil tissue was analyzed in IHC following antigen retrieval (HRP-DAB detection). As expected, membrane/cell junction immunoreactivity was observed in the stratified squamous epithelium (A) as well as the epithelial cells lining the tonsillar crypts of the human tonsil (C). Treatment of the same tissues with negative control reagent (no primary antibody) resulted in no detectable HRP-DAB signal (B, D). Counterstaining of the tissues with hematoxylin stains the cell nuclei blue.

IHC analysis is used to study protein expression and localization, in the context of subcellular structures, tissues, organs and systems[3]. As part of our IHC validation testing, we always use a negative control to establish that the positive staining is a consequence of the antibody lot being tested, and not an artifact of the staining process (Figure 4).

Strategies for validating antibody specificity

Although several publications have highlighted the importance of stringent specificity validation for antibodies[1-3], there are no set established guidelines or standardization used among the many commercial antibody suppliers. At EMD Millipore, we have developed validation criteria that far exceed the industry standards. We make it our mission to release only those antibodies that meet these criteria, discarding those antibodies that do not meet these stringent specifications.

In addition to the analyses described above, we also use a variety of additional approaches to evaluate antibody specificity, including:

- Peptide inhibition assays
- Peptide microarrays
- Peptide interaction assays using xMAP® beads or ELISA-type assays
- Peptide dot blots

To illustrate the use of some of these methods, we describe below typical validation workflows for some target types for which antibody specificity is particularly important and validation is particularly challenging.

a. Phosphospecific antibody validation

Specificity testing can be especially challenging for antibodies recognizing targets bearing post-translational modifications[4] (PTMs), such as phosphorylation[5], acetylation, methylation, ubiquitination and oxidation. For recognizing specific phosphosites, polyclonal antibodies often display stronger binding, because they can bind to multiple unique epitopes of the phosphopeptide[5].

When producing polyclonal antibodies against PTMs, we increase the chances of specific PTM recognition by meeting high standards of antibody purification. The majority of our polyclonal antibodies are purified using an antigen affinity column. Specificity for modified protein targets is ensured by depletion of the serum with unmodified target protein before affinity purification using the immobilized, modified target protein. Specificity testing is then performed to ensure that the antibody only recognizes the post-translationally modified form of the protein.

The importance of employing diverse strategies to ensure specificity of antibodies against phosphorylated proteins has been documented[4]. Our approaches include:

1. Treatment of cells with appropriate kinase-specific activators and/or inhibitors.
2. Phosphatase treatment to verify phosphospecificity (Figure 5).
3. Comparison of target recognition in cells with or without a knockout point mutation in the gene of interest at the putative site of modification (Figure 6).
4. Blocking with antigen peptide to eliminate all signal: a positive control lysate is preincubated with modified (phosphorylated) and unmodified (nonphosphorylated) antigen-derived peptides, then probed by WB. An antibody passes this test when a single band of the expected molecular weight is absent after incubation with the phosphorylated peptide.
5. Dot blot specificity is confirmed when at least 75% of the total signal is specific to the cognate peptide.
6. IHC or ICC to verify correct subcellular localization or treatment-induced translocation.

If an antibody demonstrates no antigen binding by WB, it may be because the antibody does not recognize denatured antigen. This antibody may still be specific for its intended target in its native conformation, and an IP experiment can be the next step in determining the specificity of the antibody. If the goal is to develop an antibody for use with IHC, IF or native ChIP, then WB screening may not be used as an initial screening method, since detection of denatured protein is not a predictive measure for nondenatured protein detection. As recently reviewed in the histochemistry literature, IHC validation using phosphorylation state-specific antibodies remains challenging[4]. However, technological advances, such as high throughput quantitative image analysis, along with more stringent use of control antibodies to monitor the overall state of cellular phosphorylation (such as the phosphotyrosine antibody family, 4G10®) may unlock the potential power of IHC for illuminating signaling networks.

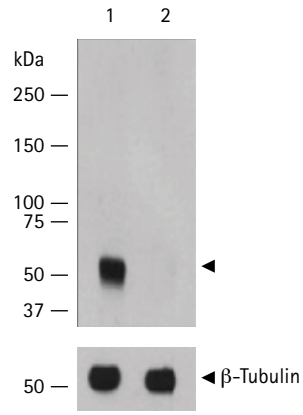


Figure 5.

Specificity testing of phospho-PTEN (Ser380) antibody (Cat. No. 04-1136) using phosphatase treatment. NIH 3T3 cell lysate containing the phosphorylated target, phospho-PTEN (Ser380), was either untreated (lane 1) or treated (lane 2) with alkaline phosphatase, and analyzed by Western blotting with 1:2,000 anti-phospho-PTEN (Ser380). Proteins were visualized using a goat anti-rabbit secondary antibody conjugated to HRP and chemiluminescence detection, and β-Tubulin was detected as a loading control. Phospho-PTEN was detected as expected at ~54 kDa in the untreated, but not the phosphatase-treated lysate, showing that the antibody was specific to the phosphorylation. As a result, this antibody was accepted as a phosphospecific detection reagent.

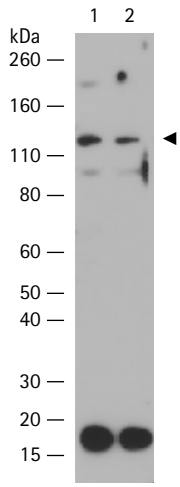


Figure 6.

Example of a non-conforming antibody: specificity testing of a phospho-specific antibody using phospho site-specific mutant cell lysate. Since this target protein is constitutively phosphorylated when overexpressed, antibody specificity was tested by transfecting cells with either a wild type (lane 1) or a phosphosite point mutant (lane 2) gene encoding the target. Although the phosphorylated target is detected as expected at ~120 kDa in the wild type protein-expressing cell lysate, the purified, depleted antibody preparation also detected the protein with the point mutation. This indicated that the protein was not specific to the modification, and was rejected as a specific antibody candidate.

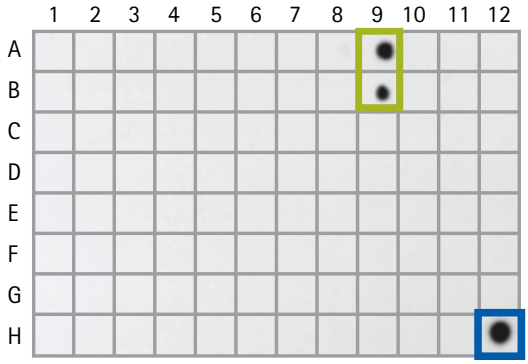
b. Modified histone antibody validation:

Currently, analyses of histone PTMs rely heavily on chromatin immunoprecipitation (ChIP). In ChIP, an antibody against a nuclear protein, such as specific post-translationally modified histone, unmodified histone or a nonhistone target protein, is used to immunoprecipitate associated DNA sequences, which are further analyzed by quantitative PCR (qPCR), microarrays (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-characterized antibodies.

A large percentage of available ChIP antibodies are offered by commercial suppliers, and are often viewed as more fail-safe options. However, unlike EMD Millipore, not all suppliers rigorously or consistently screen their histone antibodies for cross reactivity. We have developed two AbSurance™ Antibody Specificity Histone Peptide Macroarrays for evaluating the specificity of antibodies directed against unmodified histones and key histone modifications (acetyl; phospho; mono-, di-, and trimethyl). The AbSurance™ arrays are comprised of 89 synthetically modified or unmodified peptides based on human histone protein sequences. Using a proprietary process, the peptides are spotted onto a PVDF membrane in 10 and 100 ng quantities, to enable detection of both weak and strong cross-reactivity. These simple and effective peptide membrane arrays can be used to characterize antibodies to key post-translational modifications of histones H2A, H2B, H3, and H4. In sensitive, antibody-dependent analyses, such as ChIP, the AbSurance™ arrays can help to confirm the validity of biological data and have been featured in two recent publications[6,7].

The AbSurance™ H3 array was used to evaluate the specificity of two histone antibodies, and confirmed specific (Figure 7) and non-specific (Figure 8) interactions, similar to results obtained from other labor-intensive screening protocols.

A.



B.

Histone H3 Antibody Specificity Array

	1	2	3	4	5	6	7	8	9	10	11	12	
A	H3 1-19 unmod	H3 1-19 R2me1	H3 1-19 R2me2a	H3 1-19 R2me2s	H3 1-19 T3P	H3 1-19 K4ac	H3 1-19 K4me1	H3 1-19 K4me2	H3 1-19 K4me3	H3 1-19 R8me1	H3 1-19 R8me2a	H3 1-19 R8me2s	100 ng
B	H3 1-19 unmod	H3 1-19 R2me1	H3 1-19 R2me2a	H3 1-19 R2me2s	H3 1-19 T3P	H3 1-19 K4ac	H3 1-19 K4me1	H3 1-19 K4me2	H3 1-19 K4me3	H3 1-19 R8me1	H3 1-19 R8me2a	H3 1-19 R8me2s	10 ng
C	H3 1-19 K9ac	H3 1-19 K9me1	H3 1-19 K9me2	H3 1-19 K9me3	H3 1-19 S10P	H3 1-19 T11P	H3 7-26 unmod	H3 7-26 K14ac	H3 7-26 R17me1	H3 7-26 R17me2a	H3 7-26 R17me2s	H3 7-26 K18ac	100 ng
D	H3 1-19 K9ac	H3 1-19 K9me1	H3 1-19 K9me2	H3 1-19 K9me3	H3 1-19 S10P	H3 1-19 T11P	H3 7-26 unmod	H3 7-26 K14ac	H3 7-26 R17me1	H3 7-26 R17me2a	H3 7-26 R17me2s	H3 7-26 K18ac	10 ng
E	H3 16-34 unmod	H3 16-34 K23ac	H3 16-34 K27ac	H3 16-34 K27me1	H3 16-34 K27me2	H3 16-34 K27me3	H3 16-34 R26me1	H3 16-34 R26me2a	H3 16-34 R26me2s	H3 16-34 S28P	H3 26-44 unmod	H3 26-44 K36ac	100 ng
F	H3 16-34 unmod	H3 16-34 K23ac	H3 16-34 K27ac	H3 16-34 K27me1	H3 16-34 K27me2	H3 16-34 K27me3	H3 16-34 R26me1	H3 16-34 R26me2a	H3 16-34 R26me2s	H3 16-34 S28P	H3 26-44 unmod	H3 26-44 K36ac	10 ng
G	H3 26-44 K36me1	H3 26-44 K36me2	H3 26-44 K36me3	H3 26-44 Y41P	H3 47-65 unmod	H3 47-65 K56ac	H3 71-89 unmod	H3 71-89 K79me1	H3 71-89 K79me2	H3 71-89 K79me3	Rat IgG 10 ng	Sheep IgG 10 ng	100 ng
H	H3 26-44 K36me1	H3 26-44 K36me2	H3 26-44 K36me3	H3 26-44 Y41P	H3 47-65 unmod	H3 47-65 K56ac	H3 71-89 unmod	H3 71-89 K79me1	H3 71-89 K79me2	H3 71-89 K79me3	Mouse IgG 10 ng	Rabbit IgG 10 ng	10 ng

Figure 7.

Detection of specific Interactions using anti-trimethyl histone H3 lysine 4 (anti-H3K4me3, Cat. No. 05-745R). (A) Probing an AbSurance™ array with anti-H3K4me3 (1:2,000) shows the expected binding to H3K4me3 peptide (spots 9A and 9B, green box) without cross-reaction to other peptides including H3K4 (spots 6A and 6B), H3K4me (spots 7A and 7B), and H3K4me2 (spots 8A and 8B). Positive control rabbit IgG shown at position 12H (blue box). (B) Map of the AbSurance™ Histone H3 Peptide Macroarray (Cat. No. 16-667) showing the peptide identity and amount spotted in each position.

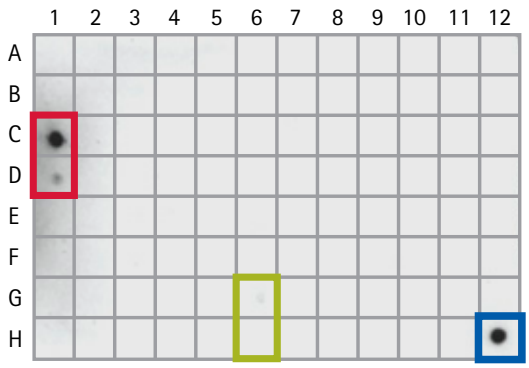


Figure 8.

Detection of non-specific interactions using an antibody to acetyl histone H3 lysine 56 (H3K56ac) obtained from Supplier E. AbSurance™ array data demonstrate weak recognition of the target H3K56ac peptide (spots 6G and 6H, green box) but strong cross-reactivity with H3K9ac (spots 1C and 1D, red box). Primary antibody used at 1:2,000 dilution. Positive control rabbit IgG shown at position 12H (blue box).

Because many users of histone modification antibodies use these antibodies in ChIP, in-house ChIP testing is often used as a validation method. Furthermore, antibody recognition in the context of chromatin can differ from other immunoassays, meaning that validation using a peptide array or Western blot may not be predictive of successful ChIP. EMD Millipore helps researchers avoid ChIP failure due to poor antibody performance by offering ChIPAb+™ antibody/primer sets, which are individually validated and tested in a defined assay, lot by lot (Figure 9). Each set includes a negative control antibody, plus control primers for amplifying a known, enriched locus to help customers perform the same assay with which the antibody was validated as a control for their ChIP protocol and process.

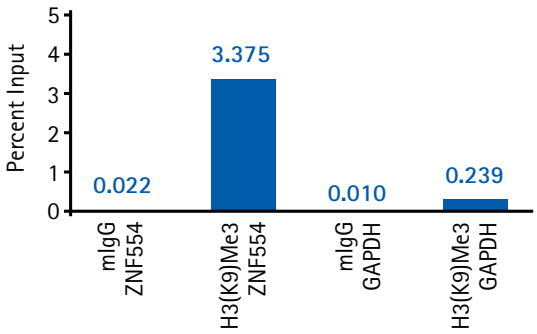


Figure 9.

Chromatin immunoprecipitation validation. ChIP validation of ChIPab+™ 17-10242 Trimethyl Histone H3 (Lys9, Cat. No. 17-10242). Sonicated chromatin prepared from HeLa cells was subjected to chromatin immunoprecipitation using 1 µL of either Normal Mouse IgG, (Cat. No. 12-371B) or 1 µL of Anti-Trimethyl Histone H3 (Lys9) and the Magna ChIP™ G Kit (Cat. No. 17-611). Successful immunoprecipitation of trimethyl Histone H3 (Lys9) -associated DNA fragments was verified by qPCR using ChIP primers specific for the 3' region of the human ZNF554 gene as a positive locus, and human GAPDH promoter primers as a negative locus. Data are presented as percent input of each IP sample relative to input chromatin for each amplicon and ChIP sample as indicated.

New approaches to increasing success in antibody development: making the "impossible" antibody "possible"

The methods employed to generate antibodies can have a profound impact on their success in any of these applications[8]. Furthermore, some target proteins have inherent properties that make them particularly difficult for antibody development; these include proteins that are highly conserved among mammalian species, proteins with post translational modifications (PTMs), membrane proteins, and small molecules. EMD Millipore employs multiple technologies and approaches to address each of those challenging proteins in antibody development. These include novel immunization strategies, special antigen designs and automated or high-throughput screening technologies, which are proving crucial in the successful generation of both polyclonal and monoclonal antibodies to difficult targets.

Our new immunization protocols include multiple strains of mice along with different combinations of protein carriers and dosing strategies to produce antibodies with the highest affinity and specificity possible. Results show that these strategies are able to break immune tolerance for highly conserved antigens in addition to inducing stronger antibody responses, allowing the generation of antibodies to many highly conserved proteins.

In order to address challenges arising from generating antibodies to membrane proteins or small molecules, we have adopted a genetic immunization protocol. This immunization process targets antigens directly to antigen-presenting cells, thus inducing rapid and effective antibody responses. In addition, significant effort has been placed on immunogen design, purification, optimization and screening strategies for antibodies against PTM proteins. These endeavors have resulted in highly specific antibodies against modified histones and to many other PTM targets.

Finally, we have improved the efficiency, quality and reproducibility in monoclonal antibody generation through automation and high throughput screening. Because of their consistency and specificity, monoclonal antibodies are frequently preferred for research, and overwhelmingly preferred for use in clinical research assays5. However, the conventional technology used for hybridoma generation and screening is time-consuming and offers very low throughput. At EMD Millipore, we have implemented a novel, semiautomated approach to producing high quality mouse monoclonal antibodies, combining high throughput hybridoma production with protein microarray-based screening and selection (Figure 10). These technologies have enabled us to standardize our monoclonal development process, leading to consistent results and increased capacity to generate monoclonals—we are now able to generate 300 monoclonal antibodies per year.

Figure 10.
High-throughput Western blotting routinely used in screening and purification of EMD Millipore antibodies.

Summary

EMD Millipore's antibodies undergo rigorous validation testing and must pass stringent guidelines before they are offered to our customers. Our antibodies are among the most thoroughly validated in the industry. We strive to meet the most demanding expectations of our customers and back all of our antibodies with our **"Anti-Fail" guarantee**. If a user is not satisfied with the performance of one of our antibodies in a validated application, we will either provide sufficient technical support to enable success or provide full credit for the purchase of that antibody.

Several new approaches have been implemented into our antibody development process, which have contributed to the increased success in development of difficult-to-produce antibodies. Our exemplary validation criteria, combined with our ability to make the "impossible" antibody "possible," have positioned EMD Millipore as the provider of choice for reliable, suitable and verifiable antibodies.

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