White Paper

Further considerations of antibody validation and usage. How to avoid reviewer challenges.

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The effective use of antibodies in research assays has always depended on multiple factors that determine the accuracy and precision of the component reagents. The confidence in performance borne out of the rigorous testing performed by researchers in developing their own antibodies was generalized when commercial sources became readily available. While high quality testing standards were more the norm for clinical applications of antibodies (Hsi 2001; Bast et al. 2005), fewer concerns were voiced or listened to in the research arena (Saper & Sawchenko 2003). Given that antibody validation can be costly in time and resources, many commercial sources and even research labs have opted to severely limit the degree of testing done on newly created or remade antibodies. Not surprisingly, the research community and even the commercial developers are now re-evaluating their production and validation of antibodies to be more confident in the accuracy and precision of these tools (Couchman 2009; Kalyuzhny 2009; Marx 2013; Voskuil 2014). This growing trend has been accelerated in the past decade by an increase in the retraction of published papers and justifiably, stronger reviewer challenges to interpretations of antibody based data in submitted papers (Saper 2005; McNutt et al. 2014). Antibody validation testing to demonstrate accuracy and precision just makes good science, regardless of who undertakes it.

The interpretation of immunoassay based data is only as strong as its testing and availability of control validation data. In numerous editorials, committee initiatives, and panel discussions, the questions of who is responsible for validation, what constitutes validation, and how often should it be conducted, have been discussed frequently. In a recent discussion lead by the journal Science (McNutt et al. 2014), a panelist described antibody validation as covering four key features that we can identify as components of either precision or accuracy:

- Demonstrating **sensitivity** (precision)
- Demonstrating **reproducibility** (precision)
- Demonstrating **target specificity** (accuracy)
- Demonstrating **application specificity** (accuracy)

Together, these four characteristics define quality antibody development and validation. It also becomes a guide for proper usage and experimental design to minimize reviewer challenges. As a producer and supplier of many cited antibodies and immuno-technologies, scientists at EMD Millipore have worked extensively with researchers who are concerned about their data interpretation and publication. In the sections below we will discuss some important considerations in choosing antibodies and address some of the key reviewer comment trends.

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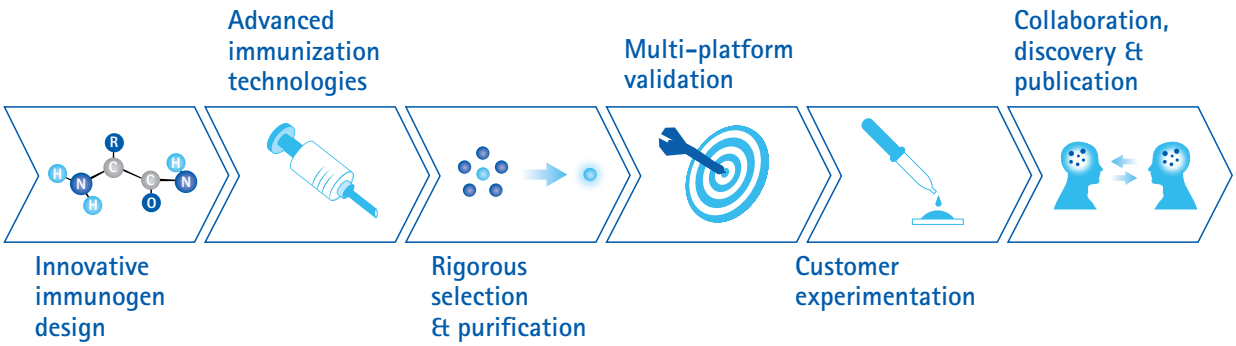
Best practices in selecting an antibody for your immunoassay.

Good forethought in experimental design is fundamental to choosing the components of your immunoassay. The complex nature of antibody development should also be reflected in your planning process. For example, what is the target antigen? Is it internal, external, transmembrane, or secreted? Is the native conformation of the target important for your experiment? Are you trying to detect, measure, localize, isolate, or a combination of any of these?



All of these factors should be built into development of quality and should be evaluated prior to selecting an antibody. A complete discussion of these considerations can be found in a recent edition of "An introduction to Antibodies and Their Biological Applications" published by EMD Millipore (2013).

Once you have identified your target antigen and have chosen your detection method, you must then select one or more primary antibodies to detect your target based on the following considerations:

Determine the best application for your research need.

Each application (western blotting, immunohistochemistry, ELISA, flow cytometry, etc.,) has inherent strengths and weaknesses. In addition, it is critical to note that not all antibodies will work with every application. This is chiefly the reason why an antibody cannot be considered 'bad' if it works by Western blotting, but not in immunohistochemistry. The important point is to validate the antibody for your specific application. We recommend that you check the vendor's data sheet or website to see what type of validation data has been provided for the specific application. Any supporting publications would not only strengthen confidence, but also would show how many different protocols could be used with that antibody. A narrow range of usable protocols could be an indicator of high precision, but low tolerance to any assay changes.

Determine the type of sample being tested.

There are several key considerations around the nature of your target protein that may significantly impact antibody performance and subsequently raise reviewer concerns. Not the least of these involves providing sufficient data or published support that the target protein is expressed in the tissue or cell line sample used. Antibody reactivity may also be affected by dynamic expression changes, since cellular proteins can be post-translationally modified, translocated, inserted into membranes, and even degrade. (Table 1)

Table 1. **Target considerations affecting antibody performance**

Target considerations	Good practice
Pre or pro protein	Antibody epitope must be in pre/pro region
Latent or activated protein	Phospho-specific or other PTM specific antibody
Tertiary structure obscures target	Denaturing or degrading protocols needed
Complex Native conformation or multi subunits	Conformation-specific antibody
Intracellular or intramembrane localization	Membrane disruption protocols needed
Live cell	External cell surface epitope needed
Surface moiety	Unfixed/light fixed frozen protocols or antigen retrieval needed

Determine proper sample preparation needs.

One often-overlooked element of developing a good immunoassay is prior planning for the type of sample preparation steps needed. The abundance of the targeted protein, conditions needed to expose the epitope, as well as the sensitivity, selectivity, and precision of the antibody all can greatly affect the accuracy and precision of the immunoassay data. Some of the typical considerations and corresponding sample preparation strategies are shown in Table 2 below. For an extensive discussion on protein sample preparation, see EMD Millipore's 2014 technical guide on protein purification and preparation.

Table 2. **Sample preparation considerations for immunoassays**

Consideration	Sample Preparation Strategy	Relevant Product
The target antigen is in low abundance	Subcellular fractionation and enrichment	ProteoExtract© organelle kits
	Immunoprecipitation	PureProteome™ magnetic beads for affinity purification
Target is a small peptide derived from a larger protein	Size exclusion ultrafiltration	Amicon® Ultra size exclusion spin filters
Target immunogen is obscured in plasma membrane or organelles	Protein extraction	ProteoExtract© Kits
Weak but specific antibody binding	Subcellular fractionation and enrichment	All-in-one purification + concentration system
	Protein concentration and buffer exchange	
Signal from protein of interest is obscured by signal from abundant proteins	Deplete samples of abundant proteins	PureProteome™ kits for Albumin/IgG depletion
Buffer components are interfering with immunodetection	Buffer exchange/dialysis	Amicon® Ultra size exclusion spin filters
		Amicon® Pro all-in-one purification + concentration system
		D-Tube™ dialyzers

Have proper control data.

Validation data is heavily dependent on good controls irrespective of whether the data comes from the antibody manufacturer or the end user. Indeed, recent discussions on the need for better antibody validation suggest that both vendor data and in-experiment user controls should be made available during manuscript review (Couchman 2009; Kalyuzhny 2009; Marx 2013; Voskuil 2014). The following are some considerations in providing appropriate supporting information.

Vendor data.

Search for validation data on a data sheet, certificate of analysis or on the vendor's website and examine the quality of the data. Check to see if only a verification of the presence of antigen is provided (ELISA, Western blotting) or whether there are other in-depth data. Check to see what type of sample was tested (cell lysate, tissue homogenate etc), and under what conditions (antibody concentration, cell stimulation, lysate concentration, etc). Testing only purified recombinant protein may not give the best results when the analysis is performed with real cells or tissue samples.

Control data.

Whenever possible, both negative and positive controls should be included in an assay. A positive control sample may be any tissue, cell line, or purified protein that is known to contain the antigen of interest, and has been previously demonstrated to be positive by a reliable method. A negative control sample is one that is known to be devoid of the antigen of interest. In addition to sample controls, one should also use reagent controls including separate controls for primary and secondary antibodies. Appropriate isotype controls should also be used to show that the primary antibody binding is specific and does not result from background signal due to immunoglobulin binding non-specifically.

Lot-to-lot variation.

A key part of demonstrating antibody precision is to measure variability across multiple lots. Because antibodies from different animal bleeds or purification batches may have significantly different titer values, each new batch of antibody must be validated, and conditions optimized before use in an existing assay. For polyclonal antibodies, this is particularly important as new lots may change their performance due to changes in the bulk lot, or the need to remake the antibody using the same antigen. Antibodies remade using the same immunogen sequence may not necessarily react in the same way and should be reevaluated by the manufacturer and the user on the application of interest. This polyclonal antibody variability has led some to suggest that monoclonal antibodies should be the gold standard (Rhodes & Trimmer 2006; Bradbury & Plückthun 2015). There are clear advantages and disadvantages in having a single narrow epitope for a target (Table 3). While monoclonals, by the nature of their design and production, are more consistent across lots, drifts in the clone or variation in purification techniques during new lot production could introduce unexpected variations. Again, vendor data should be available for inspection, but the end user should still validate new lots. (Voskuil 2009). Most savvy antibody users optimize their protocols for a given lot and then order all vials of that lot to limit unnecessary reoptimization.

Table 3: Advantages and Disadvantages of Polyclonal and Monoclonal Antibodies

	Advantages	Disadvantages
Polyclonal Antibodies	Relatively easy to generate and more cost-effective.	Loss of antibody source.
	Multiple epitopes on the same protein can generate many antibodies. Hence, they provide more robust signals.	Different bleeds or lots may give different results.
	Polyclonal antibodies can generate better signals with proteins expressed in low levels.	Immunization of a new animal with the same antigen may lead to different epitopes and different clones may be generated.
	They are compatible with a broader range of applications.	Shared epitopes on different proteins can lead to labeling of proteins other than the antigen protein.
	Polyclonal antibodies provide more flexibility in antigen recognition. For example, they may bind the antigen in spite of polymorphism, heterogeneity of glycosylation etc. Hence, they can identify proteins of high homology or from different species.	Greater batch-to-batch variability is possible.
	Better suited for the detection of denatured proteins.	May produce nonspecific antibodies that can add to background signal.
Monoclonal Antibodies	Different clones of antibodies can be generated to different epitopes on a single antigen.	Production of monoclonal antibodies is more labor-intensive. More work is required, especially in the cloning and selection process.
	Hybridoma cells can serve as an infinite source of the same antibody.	They may be limited in their applications.
	The high specificity of monoclonal antibodies minimizes background and eliminates cross-reactivity.	A vast majority of monoclonal antibodies are produced in mice because of a robust myeloma cell line.
	Their homogeneity is very high and they provide consistent, reproducible results.	High specificity of monoclonal antibodies limits their use in multiple species.
	They bind only to one antigen in a mixture of related proteins.	Monoclonal antibodies are more susceptible to the loss of epitope through chemical treatment of the antigen.
	Batch–to-batch variability is very minimal.	May have lower avidity.

Publishing with Antibodies

Let's return to the basic immunolabeling assumption that regardless of the technique used, a positive signal infers that the specific antibody has bound to the specific antigen. As with any technique, it is good science to verify that your signal is indeed specific and reproducible. Reviewers of publications and grants are becoming increasingly critical of data analysis, and researchers are being challenged to think about the fundamental principles by which laboratory techniques work, and to be more careful about over-interpretation. Even the venerable P value in biostatistics is being attacked and there are more calls for better training of researchers in experimental design and interpretation (Leek & Peng 2015).

Most of the reviewer comments redirected to EMD Millipore's Antibody Technical Service Group revolve around challenges to antibody precision or accuracy. This is an important distinction, because often the response to a challenge of antibody specificity is to add more replicate data or add some negative control data. While these are good additions in general, they do not address the fundamental accuracy issue. A better approach would be to use different techniques to confirm the results and use more antibodies to the same protein, but with differing epitopes (mono or polyclonal) to demonstrate specificity. Table 4 reviews some of the common reviewer comments and some best practices in preparing for a response.

Table 4: Common reviewer concerns and how to avoid them.

	Common reviewer comments	Good practice
Accuracy	I am not convinced your antibody is specific	• Use two or more different techniques to verify specificity; for example, WB can corroborate IHC data
		• Use two or more antibodies made against different immunogens or regions of the protein and measure co-localization
		• Test against relevant knockout samples
	I am not convinced your antibody is not cross-reacting with related proteins	• Use two or more antibodies made against unconserved epitopes of the same antigen to confirm results
	Your Western blot shows more than one band or at the wrong size. How can you show specificity?	• Cite published literature on cleavage products or glycosylation patterns
		• Consider running a denatured vs. native gel
		• Reprobe with a different antibody to same protein
	Repeat experiment using antibody with known epitope.	• Many antibody sequences are published by researchers or commercial suppliers and can be requested
		• Sequenced epitopes are not necessary for verifying antibody specificity or experiment reproducibility
		• Publish antibody catalog number and company to aid in peer validation of your data
	Antibody immunogen sequence is not provided so antibody specificity cannot be gauged	• Most reputable vendors will release immunogen sequences if available and not on their website/datasheet
		• For antibodies made against large immunogens like whole proteins, or whole cells, the sequence is less useful. Supporting data using narrow epitope antibodies (polys or monos) and multiple applications is better support
	Antibody staining seems strong and cell specific but is showing up in unexpected location	• Confirm using additional antibodies to different epitopes
		• Confirm subcellular localization using subcellular fractionation and Western or immunoprecipitation
Accuracy / Precision	Antibody failed in Western but seems to work in IHC. This seems low quality.	• It is not uncommon to have antibodies work in one application but not another, especially in monoclonals
		• Check with vendor for specialized protocols
		• The epitope may be blocked in that application. Confirm using additional antibodies to different epitope regions
	Repeat your experiment with monoclonal antibodies for better data interpretation.	• Many monoclonals are available for targets recognized by polyclonals
		• Choose a polyclonal made from a short peptide thus minimizing clonality and epitope
		• Choose a polyclonal antibody validated in multiple applications to demonstrate specificity across sample matrices, epitope treatments and detection environment
Precision	Error bars are disturbingly large on your antibody-based data	• Lock down your antibody protocol and then ensure you have enough antibody from the same lot number, so you don't have to re-optimize each experiment because of lot-to-lot variability
		• Increase replicates
	How much of the signal is actually background?	• Optimize protocol and reduce variability (see above).
		• Perform a peptide inhibition assay
		• Perform experiment without the primary antibody to establish background
		• Co-localize with direct fluorescent labeled primary
		• Use species preabsorbed secondary antibodies
	Antibody signal is weak in the some of the data you provided	• Check protocol for sample handling or penetration issues
		• Adjust primary/secondary antibody concentrations

Conclusions: Furthering the use of validated antibodies

Antibody validation is the key to sound scientific methodology and consequently solid publication. Validation is a process whereby, through the use of specific laboratory procedures, the performance and characteristics of an analytical technique are deemed suitable for the specific intended use. Therefore, good experimental design by both developers and users of antibodies is critical for proper usage and interpretation of antibody-based results. The high accuracy and precision in detecting specific antigens is naturally built into antibody development *in vivo* and most certainly underlies the extensive use and trust in immunotools. As exploiters of this natural immunological development, manufacturers and researchers must be more vigilant in our validation, experimentation, and interpretation so as not to undermine the true value and limitations of antibody based applications.

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